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EDITORIAL

Role of cholecystokinin and central serotonergic receptors in functional dyspepsia

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Abstract

Symptoms of functional dyspepsia are characterized by upper abdominal discomfort or pain, early satiety, postprandial fullness, bloating, nausea and vomiting. It is a chronic disorder, with symptoms more than 3 mo per year, and no evidence of organic diseases. Dysfunctional motility, altered visceral sensation, and psychosocial factors have all been identified as major pathophysiological mechanisms. It is believed that these pathophysiological mechanisms interact to produce the observed symptoms. Dyspepsia has been categorized into three subgroups based on dominant symptoms. Dysmotility-like dyspepsia describes a subgroup of patients whose symptom complex is usually related to a gastric sensorimotor dysfunction. The brain-gut peptide cholecystokinin (CCK) and serotonin (5-HT) share certain physiological effects. Both have been shown to decrease gastric emptying and affect satiety. Furthermore the CCK induced anorexia depended on serotonergic functions probably acting via central pathways. We believe that abnormalities of central serotonergic receptors functioning together with a hyper responsiveness to CCK or their interactions may be responsible for the genesis of symptoms in functional dyspepsia (FD).

Key words: Functional dyspepsia; Cholecystokinin; Serotonin; Gastric emptying

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INTRODUCTION

The term dyspepsia defies definition, although it is widely used in clinical practice. It comes from the Greek word, dys meaning bad and peptein meaning digestion. It causes much confusion among both patients and clinicians. Dyspepsia itself is not a diagnosis but stands for a constellation of symptoms referable to the upper gastrointestinal tract. A recent working party has recommended that dyspepsia refers to pain or discomfort centered in the upper abdomen (ROME II) [1]. Pain is the unpleasant sensation mainly in or around the midline. Discomfort may be characterized by early satiety, fullness, abdominal bloating, belching, nausea, retching and vomiting. When dyspeptic patients have no underlying identifiable disease process to account for their symptoms, then they are considered to be suffering from functional dyspepsia (FD). FD runs a chronic course and the Rome II criteria states that symptoms have to be present for at least 12 wk, which need not be consecutive, in the preceding 12 mo. The symptoms are persistent or recurrent and not associated with a change in bowel pattern or stool form.

FD is a heterogeneous condition and not all patients present with the same symptoms. The patients may be divided into subgroups based on their symptoms cluster. 3 major subgroups are recognized: Ulcer-like dyspepsia, dysmotility-like dyspepsia and nonspecific dyspepsia. Dysmotility-like dyspepsia describes a subgroup of FD patients whose symptom complex seems to suggest a relation to feeding and involvement of an underlying gastric sensory or motor disorder. The pathogenesis of this common disorder remains unclear but probably involves multiple pathophysiological mechanisms with complex interactions involving the enteric nervous system, the afferent sensory pathways and the brain, the so called brain-gut axis. There appears to be disturbed motor functions with altered visceral sensations and a strong association to psychosocial factors. Present opinion is that FD is a biopsychosocial disorder where dyspeptic symptoms may arise from these interactions[2].

The brain-gut peptide CCK and brain-gut indolamine 5-HT share some physiological effects. 5-HT is involved in gut motility, visceral sensation and other aspects of gut function while CCK is involved in mediation of pain in the gut and nociception in the central nervous system (CNS). CCK and fenfluramine (increases neuronal release of 5-HT) both slow gastric emptying and block stress induced hyperphagia[3-7]. Both neurotransmitters have been

independently implicated as factors affecting food intake [8,9]. Peripheral and central administration of CCK and fenfluramine produces anorexia in both humans and animals. The CCK induced anorexia depended on serotonergic function, probably at central sites[10]. Furthermore intravenous CCK administration stimulates the release of 5-HT and noradrenaline in the paraventricular and supraoptic nuclei, both important in central modulation of feeding and gastrointestinal motility [11].

Stressful events in life are known to alter ingestive behaviors and associated physiological events such as gastric acid secretion and gastrointestinal motility. Evidence also implicated corticotrophin releasing factor (CRF) in the mediation of stress-induced inhibition of upper gastrointestinal (GI) tract and stimulation of lower GI motor function. Endogenous 5-HT, peripherally released in response to stress, seems to be involved in the central CRF-induced effect on the GI tract[12,13]. Acute psychological stress, if produces significant emotional change may lead to an increase in sensitivity to experimental visceral stimuli. Whether chronic stress has the same effects remain to be seen.

The symptom complex described in dysmotility-like FD is usually related to feeding and also suggests an underlying abnormal GI sensory and motor function. There is an indirect correlation between severity of early satiety and gastric emptying rate, as well as an association between bloating and delayed gastric emptying[58]. Severe postprandial fullness and vomiting are independently associated with delayed gastric emptying of solids[19]. Since the neurotransmitter 5-HT and the neuropeptide CCK have been implicated in the regulation of feeding and the control of GI function, they may play an important role in the pathophysiology of functional dyspepsia.

ROLE OF SEROTONIN IN FUNCTIONAL DYSPEPSIA

Serotonin is a monoamine that acts as both a peripheral transmitter in the gut and a neurotransmitter in the brain[14,15]. Within the enteric nervous system (ENS), 5-HT is stored in myenteric neurons and acts as a neurotransmitter[16,17]. It plays an important role in regulating peristalsis and intestinal tone and thought to be one of the most important gut neurotransmitters. Sumatriptan, a 5-HT1 receptor agonist, inhibits antral motor activity, delays gastric emptying and relaxes the gastric fundus[18]. Intravenous injection of 5-HT in dogs have been observed to modulate gastric emptying, and furthermore gastric emptying can be inhibited by injecting fenfluramine into the cerebral ventricles of rats[20,21]. The primary neuronal effect of fenfluramine includes release of 5-HT from nerve terminals and inhibition of reuptake. Some serotonergic drugs induce anorexia through central pathways[22]. Warner suggested that some cases of functional abdominal pain are due to hyperserotoninemia[23]. Furthermore the hyperserotoninaemia of the carcinoid syndrome causes nausea, vomiting, colicky abdominal pain and diarrhea[24]. The majority of patients with FD are however not hyperserotoninemic. Nonetheless, altered sensitivity of 5-HT receptors might have similar consequences as high levels of serotonin. Interestingly, the functional activity of central serotonergic receptors can be studied using a neuroendocrine challenge test.

Neuroendocrine challenge test

The neuroendocrine axis provides an acceptable means of assessing brain 5-HT receptor function. The development of neuroendocrine challenge tests rest on the demonstration that the release of certain anterior pituitary hormones is controlled by brain monoamine pathways. Thus , monoamine function can be assessed by measurement of the hormonal response in plasma which follows stimulation of a particular brain monoamine pathway by a specific drug [25]. The size of the hormonal response is taken as an index of the functional activity of the monoamine synapses with which the drug interacts. It is apparent that for a neuroendocrine challenge test to provide a valid measure of brain 5-HT function, it must demonstrate that the hormone measured is indeed under the control of brain 5-HT pathways and that the drug employed to produce the hormonal response is acting specifically through 5-HT synapses. The release of prolactin (PRL) from the anterior pituitary is under the inhibitory control of dopamine and stimulatory control of 5-HT[25]. When hypothalamic receptors are stimulated by an appropriate 5-HT agonist, an increase in serum PRL takes place, via stimulation of a PRL releasing factor by 5-HT neurons originating in the medial and dorsal raphe nuclei.

Buspirone, an azaspirodecanedione, stimulates central 5-HT1A receptors at the hypothalamic level and brings about PRL release in a dose dependent manner[26,27]. Its effects can be blocked by the antagonist methysergide and pindolol, a specific 5-HT1A receptor blocker. It crosses the blood brain barrier easily and has a rapid onset of action. The extent of prolactin release can thus be used reliably as a measure of central 5-HT1A receptor sensitivity.

The response to the buspirone challenge test in FD patients was compared to normal healthy subjects and patients with peptic ulcer disease[63,64]. Considerable greater prolactin responses were found in FD patients than in healthy controls or PUD patients (Figure 1A). The mean (+/- SEM) increase in plasma PRL after buspirone in male FD patients was 672.2 +/- 65.0 Mu/L, in contrast to male healthy controls with 222.9 +/- 59.9 Mu/L. The corresponding results for female FD and controls are 1428.8 +/- 232.5 Mu/L and 352.5 +/- 33.8 Mu/L respectively. The mean PRL response for the PUD group was 325+/- 91.1 Mu/L. A highly significant difference was obtained when the FD patients was compared to the healthy controls (males; $P<0.05$, females; $P<0.001$). Differences between the responses became apparent around 60 minutes (Figure 1B). There is a significant difference in the prolactin response to Buspirone challenge, between males and females. Why is there such a difference is still not very clear. There is also evidence to suggest that, the prolactin response in females, varies at different times in the female menstrual cycle. All the neuroendocrine challenges were carried out at the same period of the cycle. Interestingly, gastric emptying rates have also being observed to be influenced by the menstrual cycle[74]. This may explain the fact

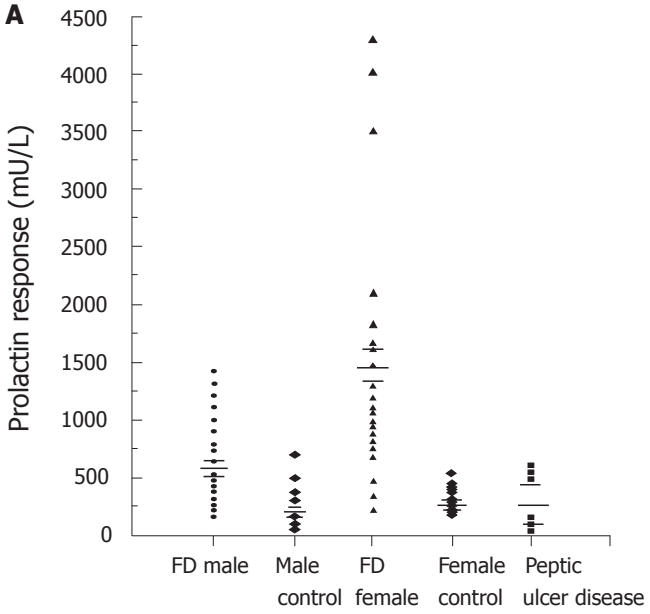

Figure 1A Prolactin response to buspirone challenge in healthy controls and patients with functional dyspepsia and peptic ulcer disease

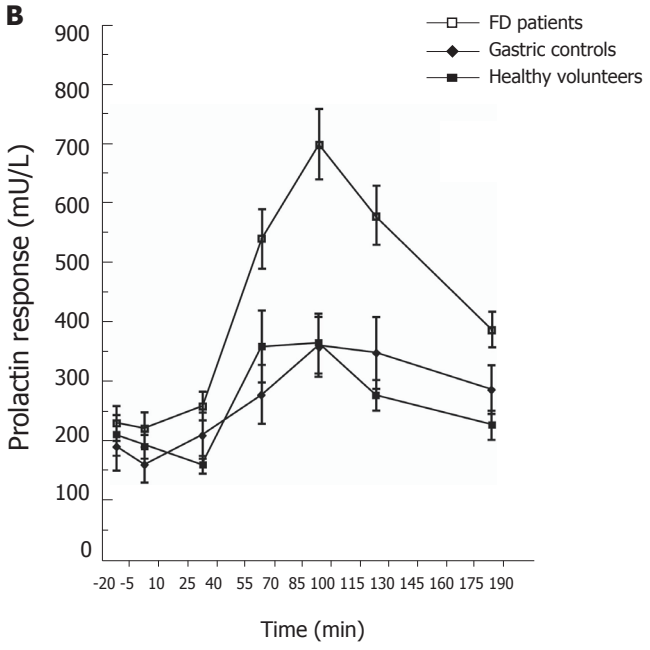

Figure 1B Prolactin response to buspirone 60 mg in male functional dyspeptics, peptic ulcer disease patients and healthy controls.

why FD, is more common in women and generally their symptoms are more severe compared to man. Our results indicated that central serotonergic receptors are considerably more sensitive in FD patients and provide evidence that FD is a disorder characterized by a neurochemical dysfunction in the brain.

Serotonin neuronal system of the brain have been postulated to modulate many basic physiological tasks, including gating of pain perception, control of eating and GI sensorimotor functions. An abnormal central 5-HT receptor function may interfere with any one or all of the above functions. Interference with the pain gating system may affect patients perception to pain or give rise to an abnormal response to nociception from visceral receptors. Fenfluramine, a 5-HT releasing agent and reuptake inhibitor, reduces hunger ratings, delays onset of feeding and provokes termination of a bout of eating. The presence of hypersensitive central serotonergic receptors would function in the same manner as an increase release of 5-HT. Interestingly, FD patients are known to have abnormal feeding behavior with symptoms that are related to meals. Furthermore, gastric motility has been shown to be affected by injection of 5-HT into the cerebral ventricles of animals[4]. Thus a hypersensitive central 5-HT receptor function could easily affect gastric motility.

ROLE OF CHOLECYSTOKININ (CCK) IN FUNCTIONAL DYSPEPSIA

CCK is an established brain-gut peptide that plays an important regulatory role in gastrointestinal function[28]. CCK is involved in the control of food intake and satiety in both man and animals[29-31]. It inhibits gastric motility and emptying via capsaicin-sensitive vagal pathways[32,33]. In humans, CCK regulates gastric emptying under physiological conditions[34,72]. An altered response to CCK may be responsible for the dyspeptic symptoms in FD and may be accountable for the frequently observed abnormal gastric motility. The sensitivity of FD patients to CCK has been tested using a CCK challenge test.

CCK challenge test

Subjects for the provocation test undergo an overnight fast. The test was performed using a synthetic CCK-octapeptide (CCK-8), sincalide. Response to CCK was assessed by an intravenous CCK-8 infusion (6 nanograms/kg/min) over 10 min in a double-blind, cross-over design using normal saline as placebo. A break of 15 min was given between infusion. The CCK test was deemed positive when the infusion reproduced the patients' symptoms (epigastric pain or discomfort, nausea, abdominal distension, bloating, belching and vomiting). Patients' response to the infusion was assessed by a third independent observer and patients scored their response (reproduction of the symptoms) on a visual analogue scale.

When FD patients were subjected to the CCK-8 challenge test, majority (90%) of them responded positively to the challenge test[75]. Commonly reported symptoms included abdominal pain, abdominal bloating and fullness, belching, nausea and occasional vomiting (dysmotility type symptoms). Most of the healthy subjects complained only of very minor symptoms including mild nausea and minor abdominal discomfort (Figure 2A). No subject reported any symptoms on saline (placebo) infusion. Interestingly it was also shown that, intravenous atropine was able to abolish the response to the CCK-8 provocation test in a dose-dependent fashion (Figure 2B). Similarly, oral loxiglumide (CCK-A antagonist) 800 mg, consumed 1 h before the CCK challenge, was successful in controlling the symptoms (Figure 2C). Solid phase gastric emptying were also measured in the FD patients using scintigraphic assessment of a standard breakfast with Tc-99M tin colloid and compared with healthy controls. Solid-phase gastric emptying (analyzed in terms of half-emptying times) dif-

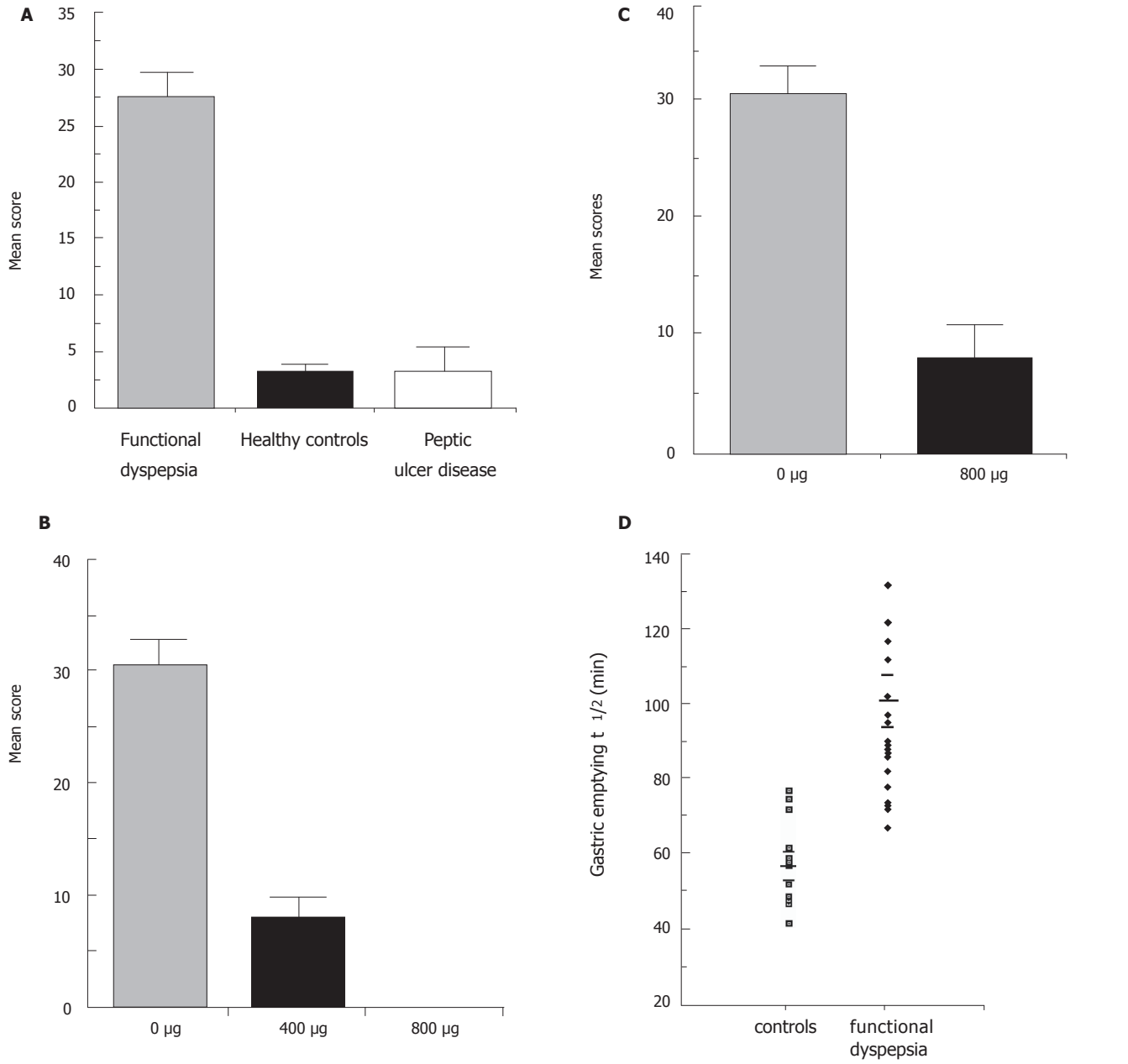

Figure 2 **A**: Response to CCK-8 challenge in healthy controls, patients with functional dyspcpsia and peptic ulcer disease; **B**: Effects of atropine on CCK-8 infusion in functional dyspeptic patients. **C**: Effects of loxiglumide 800mg on CCK-8 infusion in functional dyspeptic patients; **D**: Solid phase emptying rates in functional dyspeptic patients and healthy controls.

fered significantly between the two groups (Figure 2D).

These results indicated that a high proportion of the patients with dysmotility-type FD have an abnormal response to CCK-8 infusion. This abnormal hyperresponsiveness to CCK may account for the genesis of dyspeptic symptoms in FD. It is interesting to note that gastric emptying is also delayed in the FD group. CCK-8 contribution to the slowing of gastric emptying may be due to the stimulation of pyloric contractions and suppression of antral and proximal duodenal motility[34]. The response to CCK-8 challenge may be mediated via a vagovagal reflex arc, resulting in the perturbation of gastric motility. It is possible that CCK-A receptors are involved with the sensory afferent limb, while the cholinergic receptors are responsible for the motor efferent limb of this reflex arc. Ingestion of a meal (especially a fatty meal) leads to the release of CCK that acts locally in a paracrine fashion, and the information generated conveyed to the CNS *via* vagal afferent fibers. This pathway is also responsible for the feedback inhibition of gastric tone and motility. The gastric motor changes can lead to dyspeptic symptoms in FD patients.

CHOLECYCSTOKININ AND SEROTONIN INTERACTIONS IN FUNCTIONAL DYSPEPSIA

Stallone first provided evidence for the interactions between 5-HT and CCK in the control of feeding behavior[10]. It was demonstrated that while the 5-HT receptor antagonist, metergoline, acted to attenuate the CCK-induced anorexia, the peripheral 5-HT antagonist xylamidine, had no

effect. Therefore the inhibitory effect of CCK-8 on food intake depended upon central serotonergic activity[10,35,37]. Studies have shown that dl-fenfluramine can reduce the rate of gastric emptying[21,36]. The reduction in gastric emptying produced by dl-fenfluramine could be blocked by the CCK-A receptor antagonist, devazepide[38]. This suggested that the serotonergic effects on gastric emptying depended upon CCK mechanism. It has also been shown that administration of CCK-8 can excite 5-HT neurons[39], and result in 5-HT being released in the hypothalamic paraventricular nuclei (PVN) and supraoptic nuclei[11,41] both important in central modulation of feeding and gastrointestinal motility. It is likely that endogenous CCK, released post prandially, activates the central 5-HT system, leading to increased release in the PVN. There is evidence that 5-HT acts within the PVN to suppress carbohydrate intake[42]. Activation of central 5-HT mechanisms by CCK-8 may then be involved in the control of satiety. Likewise efferent fibers descending from the CNS to the ENS are important in the control of gastrointestinal functions. Abnormalities involving either the CCK or the 5-HT pathways can result in perturbation of feeding and disruption of gastrointestinal motility. Thus the symptoms encountered in FD may result from a dysfunction of either of the above pathways or interaction between the two.

We examined a group of FD patients (early satiety, upper abdominal pain or discomfort, post-prandial bloating, fullness, nausea and vomiting) who has an abnormal response to the CCK challenge test, a delayed solid-phase gastric emptying and determined their central 5-HT receptor activity using the buspirone stimulation test. Solid phase gastric emptying (half emptying time) were significantly prolonged in the dyspeptic group compared to the controls (mean +/- SE; 90.3 +/- 3.9 min *vs* 54.6 +/- 5.9 min). Dyspeptic patients (mean +/- SE; Females: 1450+/- 132.5 Mu/L, Males: 672.5 +/- 84.9 Mu/L) were found to have a significantly higher ($P < 0.001$) PRL response when compared to the healthy volunteers (mean +/- SE ; Females: 352 +/- 33.5 Mu/L, Males: 187 +/- 44.3 Mu/L).

These results indicated that FD patients with an abnormal response to CCK-8 challenge also have hypersensitive central 5-HT receptor functioning. However, no correlation can be obtained between PRL response and severity of response to CCK challenge (R= 0.24, NS). Patient's response was rated on a Visual Analogue Scale, which is very subjective and patient dependent. A weighted scoring system may be better in assessing patient's response and may thus show a significant correlation then.

DISCUSSION

Dysfunctional motility[19,43,44], visceral hypersensitivity[45,46], impaired accommodation[47,48] and disordered feeding behaviour are pathophysiological abnormalities that have been described in FD. Each of these disorders may result from primary pathologies arising from the ENS, aberrant signal transmission in the afferent and efferent nerves and abnormal integration within the CNS, which can lead to disruption in peripheral GI sensorimotor functions. Psychological factors, either acute or chronic[12,49,50-54] (stress, negative life events, personality traits or illness seeking behaviors) and presence of certain nutrients in the intestine[55-57] (abnormal exaggerated response) can influence pathways of the brain-gut axis resulting in the observed pathophysiological abnormalities and the resultant symptoms.

It has been reported that agents that modulate 5-HT function may be useful in the treatment of visceral hypersensitivity, either directly on perception or through alteration in visceral tone or motility[59-61]. Visceral sensation can be modified at various levels of the brain-gut axis. It is believed that FD patients perceive visceral stimuli in an abnormal manner[62]. It is still uncertain at what level the dysfunction originates. Altered threshold of visceral mechanoreceptor sensitivity, abnormalities in sensory input transmission and a decrease pain threshold at the CNS may all be responsible. Evidence for central dysfunction exists in that central serotonergic receptors have been shown to be hypersensitive in FD patients[63,64]. This was demonstrated by an exaggerated prolactin response to the buspirone challenge test. Furthermore gastric emptying was also delayed in this group of patients. More importantly the gastric emptying rates and the prolactin response were very highly correlated[63]. This observation suggests that in FD, hypersensitive central 5-HT receptors may be involved in mechanisms giving rise to abnormalities of gastric emptying.

Central 5-HT pathways are also implicated in the mechanisms of nociception. Descending 5-HT systems in the supraspinal and spinal pathways are involved in the control of nociception and thus can modify visceral perception and awareness of pain. Pathways arising from the brainstem and projecting to the dorsal horn of the spinal cord can alter the sensitivity of the dorsal horn neurons and thus centrally control the intensity of perception of pain [65,66]. Similar mechanisms have been implicated in the increase perception to visceral sensation[67].

The neuropeptide CCK act on vagal gastric afferents to inhibit gastric emptying and decrease food intake[29,34,70]. CCK receptors have been demonstrated on gastric afferent nerves[71]. Studies have compared various gastrointestinal responses to exogenous and endogenous CCK at plasma levels measured after a meal or intestinal nutrients. Similar plasma CCK levels after endogenous release and exogenous administration, appear to cause equal degrees of gallbladder contraction[73] and inhibition of gastric emptying in man[72,74]. Animal studies suggest that CCK acts via a vagal afferent pathway to decrease gastric motility and this pathway is important in mediating CCK induced delay in gastric emptying[32]. By acting on the vagal afferents, CCK is seen as part of the mechanism by which information from the peripheral GI tract is conveyed centrally to the CNS to modulate feeding behaviour and the autonomic control of the digestive tract. The abnormal response to CCK octapeptide infusion observed in FD patients may be due to sensitized gastric mechanoreceptors or modification in the transmission of sensory impulse. The satiety effect of CCK depends on intact central 5-HT function[10]. It may be possible that hypersensitive central 5-HT receptors may up-regulate the sensory receptors in the gut wall that then respond abnormally to the CCK infusion. Interestingly postprandial CCK levels in a normal healthy person are not significantly different from FD patients. It is conceiv-

able that the gut in a sensitized subject may react more vigorously to external stimuli (stress or presence of nutrients).

SUMMARY

An interaction between the CCK and serotonergic pathways may explain the constellation of symptoms observed in FD. An altered central 5-HT receptors functioning can reset the threshold for sensitivity to CCK (more sensitive to stimuli) in the gut receptors. Consequently, a normal peripheral stimulus (presence of nutrients in the small intestine or food in stomach) will result in an abnormal afferent input that leads to a distortion of GI perception and function[68,69], either through intrinsic ENS reflexes or through the autonomic or central nervous systems. This will also help to explain how stress and psychological factors may precipitate dyspeptic symptoms in FD patients. Depending on the presence and degree of the stressor at the time, different dyspeptic symptoms will predominate.

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REVIEW

Role of cyclooxygenase-2 in the carcinogenesis of gastrointestinal tract cancers: A review and report of personal experience

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Abstract

Selective cyclooxygenase (COX)-2 inhibitors (coxibs) were developed as one of the anti-inflammatory drugs to avoid the various side effects of non-steroidal anti-inflammatory drugs (NSAIDs). However, coxibs also have an ability to inhibit tumor development of various kinds the same way that NSAIDs do. Many experimental studies using cell lines and animal models demonstrated an ability to prevent tumor proliferation of COX-2 inhibitors. After performing a randomized study for polyp chemoprevention study in patients with familial adenomatous polyposis (FAP), which showed that the treatment with celecoxib, one of the coxibs, significantly reduced the number of colorectal polyps in 2000, the U.S. Food and Drug Administration (FDA) immediately approved the clinical use of celecoxib for FAP patients. However, some coxibs were recently reported to increase the risk of serious cardiovascular events including heart attack and stroke. In this article we review a role of COX-2 in carcinogenesis of gastrointestinal tract, such as the esophagus, stomach and colorectum, and also analyze the prospect of coxibs for chemoprevention of gastrointestinal tract tumors.

Key words: Cyclooxygenase-2 (COX-2); Selective COX-2 inhibitors; Esophageal cancer; Gastric cancer; Colorectal cancer

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DEVELOPMENT OF SELECTIVE COX-2 INHIBITORS

The administration of non-steroidal anti-inflammatory drugs (NSAIDs), one of the most prevalent antipyretics and analgesics, is also known to reduce the risk of cancer development in the gastrointestinal tract organs including the esophagus, stomach and colorectum[1, 2]. Vane[3] indicated in 1971 that NSAIDs act upon cyclooxygenase (COX), a rate-limiting enzyme in the arachidonate metabolism. The enzyme catalyzes the biosynthesis of prostaglandin H_2, the precursor of derivatives such as prostaglandins, prostacyclin, and thromboxanes. Up to now there have been at least two isoenzymes of COX reported, COX-1 and COX-2. COX-1 is constitutively expressed in many tissues and it controls homeostasis by maintaining physiological levels of prostaglandins, while COX-2, induced by cytokines, mitogens, and growth factors, is responsible for inflammatory reactions and tumor development. Recently, COX-3, was reported to be related with pain and fever, and identified as an alternative splice of COX-1[4].

COX-2 and PGE2 play an important role in tumorigenesis from the development to invasion and metastasis of carcinoma through various mechanisms. COX-2 expression promotes cell proliferation by the activation of EGFR[5] and inhibit apoptosis by up-regulation of bcl-2[6], and suppresses host immune response[7]. Furthermore, COX-2 induces angiogenesis with VEGF and bFGF expression[8], and facilitates a metastatic potential by up-regulation of uPA and MMP-2[9, 10]. Theoretically, NSAIDs may be a candidate for chemopreventive agents against tumorigenesis by inhibiting COX-2. In fact, two large-scale randomized, double-blind trials demonstrated that aspirin, a representative of NSAIDs, could prevent colorectal adenoma[11, 12].

But the regular use of NSAIDs causes severe adverse effects including gastrointestinal bleeding, a reduction of the renal blood flow, and dysfunction of platelets because they inhibit both COX-1 and COX-2. To avoid these side effects of NSAIDs the development of selective COX-2 inhibitors was gradually aroused after the discovery of

Table 1 Selective COX-2 inhibitors (coxibs) and chemoprevention in gastrointestinal tract tumors

Generic name	Brand name	PhCo[b]	Esophagus				Stomach			Colorectum			
			Cancer cell line	CIA[c]	reflux-induced animal	Human (BE[d])	Cancer cell line	CIA[c]	MIA[e]	Cancer cell line	CIA[c]	MIA[e]	Human (FAP[f])
Tricyclic													
Celecoxib	Celebrex	Pfizer			(23)			(46,47)	(75)	(76,77)	(81,82)	(54)	(56)
MF-tricyclic	EC[a]	Merck			(21)							(53,87)	
Rofecoxib	Vioxx	Merck				(24)						(55)	
Tilmacoxib	Japan Tobacco			(20)						(78)		(88,89)	
Valdecoxib Bextra	Pfizer												
Etoricoxib Arcoxia	Merck												
Methanesulphonamide													
NS-398	EC[a]	Taisho	(18,19,70,71)				(44,45,72)		(49)	(72)	(83)		
Nimesulide	Mesulid	Helsinn					(73)	(48)			(84)	(90)	
Flosulide		Schering	(70)										
Others													
Nabumetone	Relafen	Glaxo Smith Kline									(85)	(91)	
Meloxicam	Mobic	Boehringer Ingelheim								(79,80)	(86)		
Etodolac	Lodine	Wyeth					(74)			(74)			
Lumiracoxib	Prexige	Novartis											

ECa, experimental compound; PhCob, Pharmaceutical company; CIAc, carcinogen-induced animal; BEd, Barrett's esophagus; MIAe, mutation-induced animal; FAPf, familial adenomatous polyposis; Numbers in parentheses show reference numbers

COX-2 in the early 1990s[13]. Some drugs were discovered as a result of a search for selective COX-2 inhibitors, others were revealed as being COX-2 selective after the discovery of COX-2. There are three classes of selective COX-2 inhibitors (Table 1), the first one being 1,2-diarylcyclopentenes (so-called tricyclic compounds), such as celecoxib and rofecoxib; the second one being methanesulphonamide compounds, such as NS-398 and nimesulide; and the third one being NSAIDS-derivates, such as meloxicam and etodolac. Some selective COX-2 inhibitors, which demonstrate chemopreventive effects on gastrointestinal cancers in experiments and human studies, are already commercialized as anti-inflammatory drugs, but no drug except for celecoxib is presently allowed for use in chemoprevention. In this paper we review the role of COX-2 in the carcinogenesis of gastrointestinal tract cancers and also discuss the prospect of selective COX-2 inhibitors for chemoprevention of gastrointestinal tract cancers.

COX-2 IN CARCINOGENESIS OF GASTROINTESTINAL TRACT CANCER

Esophageal cancer

Recently, the incidence of Barrett's esophagus (BE) and esophageal adenocarcinoma (EAC) has been rapidly increasing in individuals of Western countries, particularly, among white males. The sequence of events leading from gastroesophageal reflux disease (GERD) to EAC is thought to involve the development of inflammation-stimulated hyperplasia and metaplasia, followed by multifocal dysplasia and adenocarcinoma. The up-regulation of COX-2 expression in human tissue of esophagitis, BE and EAC has been demonstrated. The incidence of COX-2 protein expression gradually increases with the development of esophageal lesions, from 75% in metaplasia, to 83% in low-grade dysplasia and up to 100% in high-grade dysplasia and EAC[14]. Combined reflux of the duodenal contents with gastric juice contributes to the development of these diseases[15] and BE patients have higher bile acid levels in the stomach than healthy controls and GERD patients without BE[16]. These observations strongly indicate that duodenal juice including bile is associated with the inflammation-metaplasia-adenocarcinoma sequence. In particular, bile acid is likely to play a pivotal role. Zhang *et al*[17] reported that COX-2 was expressed in the esophageal mucosa using a duodenogastroesophageal reflux model and bile acids, not only unconjugated but also conjugated ones, induced COX-2 mRNA, followed by COX-2 protein and PGE2 production.

The suppressive effects of a COX-2 inhibitor, NS398, on the epithelium of BE have been demonstrated in two independent *in vitro* studies[18, 19]. An increase in apoptosis and a suppression of cell proliferation are supposed to be responsible for the inhibition of cancer cells in these articles. Furthermore, some selective COX-2 inhibitors have been reported to prevent the development of esophageal cancer using *in vivo* animal models. N-nitrosomethylbenzylamine-induced esophageal tumorigenesis in rats was prevented by the administration of another selective COX-2 inhibitor, JTE-522[20]. The study was carried out using a carcinogen-induced rodent model, whereas two studies have been reported using an esophageal reflux model. Buttar *et al*[21] showed the preventive effect on EAC of MF-tricyclic in a rat model of BE and EAC induced by duodenogastroesophageal reflux. In their report, MF-tricyclic prevented the development of EAC, but did not suppress the prevalence of BE. On the other hand, celecoxib suppressed not only the development of EAC, but also that of BE in our study.

We have investigated the effect of celecoxib on esophageal adenocarcinogenesis by using duodenoesophageal reflux model, established by Miwa and his colleagues [22, 23]. Male Fisher 344 rats underwent a duodenoesophageal reflux procedure and were divided into two groups. One

Table 2 Incidences of inflammatory changes, Barrett's esophagus, and adenocarcinoma in a rodent duodenoesophageal reflux model

Wk	Group	*n*	RT#	BCH$	Incidence (%) of Barrett's esophagus	Incidence (%) of Adeno-carcinom
10	Control	10	100[c]	100[c]	10	0
	Celecoxib	5	40	40	0	0
20	Control	10	100[c]	100[c]	40	0
	Celecoxib	5	40	40	20	0
30	Control	10	100[c]	100[c]	50	10
	Celecoxib	5	40	40	40	0
40	Control	19	100[c]	100[c]	89[a]	47[b]
	Celecoxib	8	38	38	25	0

RT#, Regenerative thickening; BCH$, Basal cell hyperplasia; [a]$P<0.005$ and [c]$P<0.05$, respectively, control *vs* celecoxib group, Fisher's exact test.

group was given commercial chow (control group), while the other group was given experimental chow containing celecoxib (celecoxib group). The animals were sacrificed sequentially, at 10th, 20th, 30th and finally 40th wk after surgery. In the control group, esophagitis, BE and EAC were first observed at 10th wk, 20th wk and 30th wk, respectively. Their incidences sequentially increased and at the 40th wk reached 100%, 89% and 47%, respectively. In the celecoxib group, the esophagitis was mild and the incidence of BE was significantly lower at each week ($P<0.001$), in comparison with the control group, and EAC was not identified throughout the experiment ($P<0.05$) (Table 2). COX-2 expression was up-regulated at the 10th and 20th wk ($P<0.05$, respectively) (Figure 1). PGE_2 level and proliferative activity were also up-regulated in both groups, but they were lower in the celecoxib group than in the control group ($P<0.05$) (Figures 1 and 2). Apoptosis increased after the celecoxib treatment ($P<0.05$) (Figure 2). Celecoxib thus proved to be effective for preventing reflux esophagitis, BE and EAC by suppressing PGE_2 production in a rodent model.

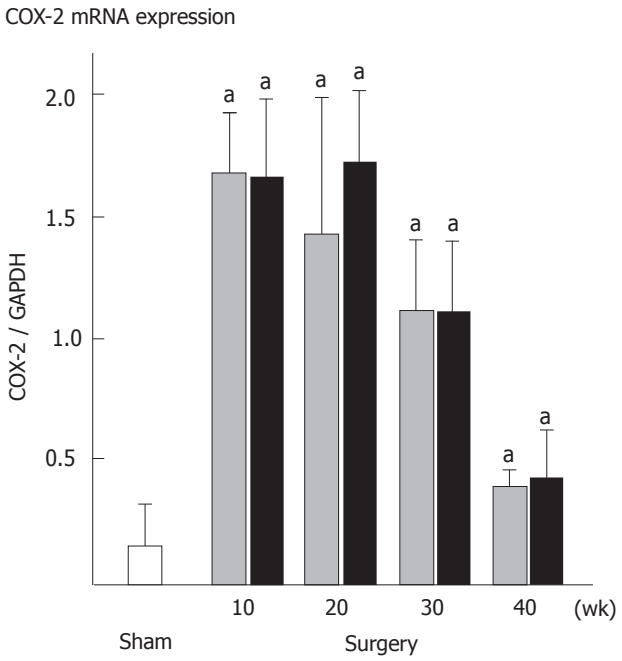
Our results showed surges of COX-2 and PGE_2 between the beginning and the 20th wk in the control group, thus suggesting that the COX-2 expression played an important role in the early phase of the esophageal carcinogenesis in the inflammation-metaplasia-adenocarcinoma sequence. The fact that the suppression of PGE_2 continued throughout the experiment in the celecoxib group may explain that celecoxib suppressed not only the development of EAC, but also that of BE. These data led to perform a clinical chemoprevention study for the patients with BE. Kaur *et al*[24] administered 25-mg/day rofecoxib to twelve patients with BE for 10 days and reported that COX-2 expression, PGE_2 contents and PCNA of epithelium of BE were 3-fold, 2-fold, and 2-fold higher than those of epithelium of normal esophagus, respectively, and all biomarkers decreased after treatment by 77%, 59%, and 62.5%, respectively. Furthermore, a Chemoprevention for Barrett's Esophagus Trial (CBET) was started in 2003 as a phase IIb, multicenter, randomized, double-masked, placebo-controlled study of celecoxib in patients with Barrett's dysplasia[25].

Gastric cancer

Though the incidence of gastric cancer has recently decreased in the United State of America and Western European countries, it is still a major cause of cancer death in many countries, such as Eastern Asia, Eastern Europe, and Latin America. Gastric cancer develops in a multistep process from normal gastric mucosa to chronic active gastritis, to gastric atrophy and intestinal metaplasia, and finally to dysplasia and cancer[26]. According to recent epidemiologic evidence, it is very likely that *Helicobactor pylori* (*H pylori*) plays an important role in this carcinogenic sequence. It is shown that *H pylori* induces COX-2 mRNA/protein levels with the production of PGE_2 in premalignant and malignant lesions[27, 28]. A chronic infection of *H pylori* causes gastritis due to COX-2, iNOS, and other cytokines, but the precise mechanism of *H. pylori* involvement in gastric carcinogenesis remains to be elucidated. Normal gastric mucosa scarcely expresses COX-2, but the expression of COX-2 increases through the multistep process of gastric carcinogenesis. Sun *et al*[29] reported the positive rates of COX-2 by immunohistochemistry in superficial gastritis, gastric atrophy, intestinal metaplasia, dysplasia, and cancer to be 10.0%, 35.7%, 37.8%, 41.7%, and 69.5%, respectively. In addition to these findings several studies have strongly suggested COX-2 expression to be a relatively early event in the sequence of gastric carcinogenesis[30, 31].

Since Ristimaki *et al*[32] first described an elevated expression of COX-2 in gastric carcinoma in 1997, numerous studies have reported the relationship between COX-2 expression and gastric cancer. According to a review article, COX-2 mRNA is up-regulated in 51% to 76% (median 73%) of the tumors by Northern blot or RT-PCR, while COX-2 protein is overexpressed in 67% to 83% (median 73%) by immunoblotting and 43% to 100% (median 62%) by immunohistochemistry[33]. The COX-2 expression is more frequent in intestinal-type than in diffuse-type gastric cancer[34-36], and it also correlates with noncardia cancer[37], tumor size[38], depth of invasion[36, 38, 39], lymph node metastasis[38-42], lymphatic invasion[41, 42], clinical stage[41-42], and angiogenesis[39, 43].

Sawaoka *et al*[44, 45] demonstrated the inhibitory effects of a COX-2 inhibitor, NS-398, on the gastric cell line expressing COX-2 (MKN45) and on its xenograft in nude mice *in vivo*. Hu *et al*[46] examined the chemopreventive effect of indomethacin and celecoxib, using a rat model. They induced gastric cancer by the administration of 100 μg/ml MNNG to Wistar rats for 40 wk and reported the incidence and the tumor multiplicity of gastric cancer of 10 mg celecoxib group to be 18.8% and 0.19, which was significantly lower than 75.0% and 1.0 of the control group, but indomethacin did not show any such preventive effect. Curiously, indomethacin strongly inhibited PGE_2 production in comparison with celecoxib. They supposed that chemopreventive effects of the celecoxib may not be mediated by the inhibition of the COX-2 activity or prostaglandins production alone and thus carried out another experiment to elucidate the cell kinetics[47]. They indicated that both drugs suppressed cell proliferation, but celecoxib



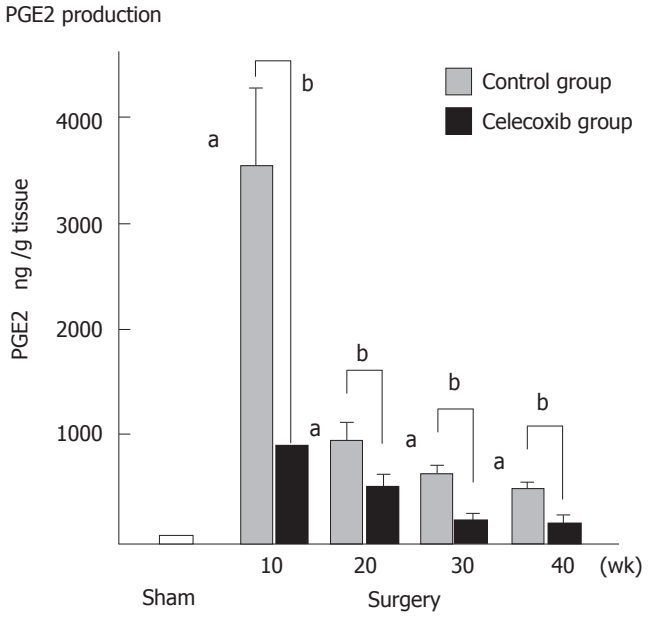
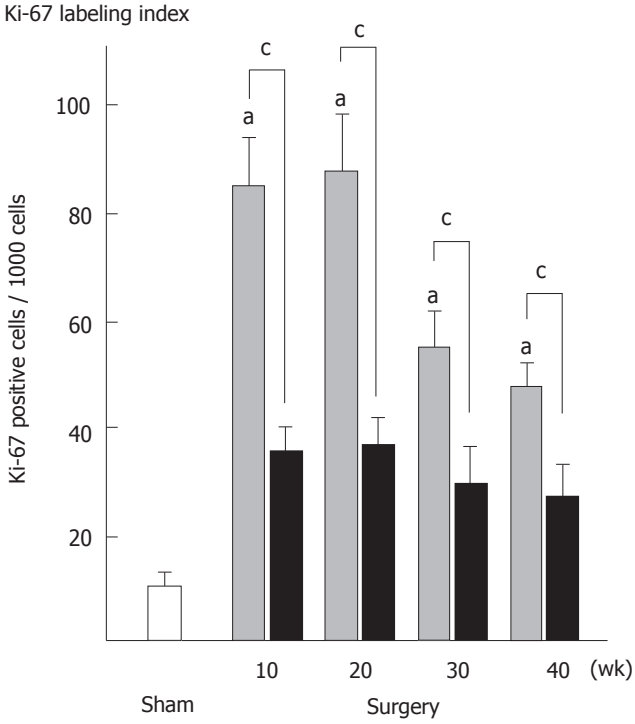


Figure 1 COX-2 mRNA expression and PGE2 production of esophageal mucosa. [a]$P < 0.005$ *vs* sham; [b]$P < 0.01$ between the two groups , Mann-Whitney U test



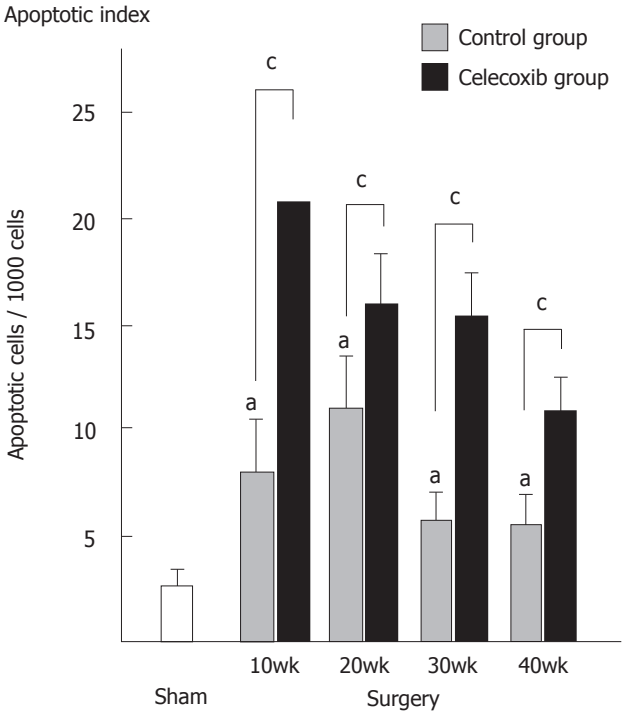


Figure 2 Cell kinetics of esophageal mucosa in a duodenoesophageal reflux model. [a]$P < 0.05$ *vs* sham; [c]$P < 0.05$ between the two groups , Mann-Whitney U test

increased the apoptosis of gastric cell in a dose-dependent manner, whereas indomethacin did not effect apoptosis, thus suggesting that celecoxib inhibits gastric carcinogenesis by the COX-2 independent pathway, such as by the inhibition of the NF-1-κB signaling pathway. Furthermore, Nam *et al*[48] examined the effect of nimesulide on gastric carcinogenesis using an N-methyl-N-nitrosourea (NMU)-induced and an *H pylori*-infected mouse model, demonstrating that gastric tumors developed in 68.8% of mice given both MNU and *H pylori*, whereas the tumor incidence in the mice receiving nimesulide in addition to MNU and *H pylori* was 27.8%.

More recently COX-2 was proven to have a strong relationship with gastric tumorigenesis in a study using transgenic mice[49]. In the transgenic model expressing both COX-2 and microsomal prostaglandin E synthase (mPGES)-1, the animals developed inflammation-associated hyperplastic gastric tumors in the proximal glandular stomach. In addition, NS-398 treatment for four weeks completely suppressed the gastric hypertrophy, thereby reducing the mucosal thickness in the same model. We previously established a rodent duodenogastric reflux model, in which gastric cancer developed for 50 to 60 wk without any chemical carcinogens[50]. We have now started an experiment to prove the chemopreventive effects of

Table 3 Chemopreventive effects of coxibs on intestinal tumors using animal models

Drug				Outcomes		Reference	
Name	Concentration	Term	Animal model	Inhibition rate (%)	*P* value	Reporter (#)	Year
carcinogen-induced rat model							
Celecoxib	1500 ppm	5-16 wk	F344 rat, AOM[a]	40 (ACF)	$P < 0.001$	Reddy *et al* (92)	1996
NS-398	1 mg/kg•bw	5-11 wk	F344 rat, AOM[a]	34 (ACF)	$P < 0.05$	Yoshimi *et al* (83)	1997
	10 mg/kg•bw			47 (ACF)	$P < 0.01$		
Celecoxib	1500 ppm	5-50 wk	F344 rat, AOM[a]	93 (colon tumor)	$P < 0.00001$	Kawamori *et al* (81)	1998
Nimesulide	200 ppm	6-30 wk	ICR mouse, AOM[a]	36 (adenocarcinoma)	NS	Fukutake *et al* (84)	1998
	400 ppm		50 (adenocarcinoma)		$P < 0.05$		
Celecoxib	500 ppm	5-58 wk	F344 rat, AOM[a]	55 (adenocarcinoma)	$P < 0.001$	Reddy *et al* (82)	2000
		1000 ppm	5-58 wk	62 (adenocarcinoma)	$P < 0.001$		
		1500 ppm	5-58 wk	77 (adenocarcinoma)	$P < 0.0001$		
		1500 ppm	22-58 wk	47 (adenocarcinoma)	$P < 0.01$		
Nabumetone	750 ppm	for 18 wk	F344 rat, AOM[a]	15 (ACF)	$P < 0.05$	Roy *et al* (85)	2001
	1500 ppm			37 (ACF)	$P < 0.01$		

to be continued

Table 3 (continued)

Apc gene mutant mouse model							
MF-tricyclic	3,5 mg/kg•d	3-11 wk	ApcΔ716	52 (intestinal polyp)	$P = 0.0037$	Oshima *et al* (53)	1996
	14 mg/kg•d			62 (intestinal polyp)	$P < 0.0001$		
Nimesulide	400 ppm	4-15 wk	ApcΔ850 (Min)	48 (intestinal polyp)	$P < 0.05$	Nakatsugi *et al* (90)	1997
Celecoxib	150 ppm	30-80 d	ApcΔ850 (Min)	29 (intestinal polyp)	$P < 0.01$	Jacoby *et al* (54)	2000
	500 ppm		29 (intestinal polyp)				
	1500 ppm		71 (intestinal polyp)				
JTE-522	0.001 (%)	4-12 wk	ApcΔ474	9 (intestinal polyp)	NS	Sasai *et al* (88)	2000
	0.01 (%)			32 (intestinal polyp)	$P < 0.05$		
Nabumetone	900 ppm	5-15 wk	ApcΔ850 (Min)	50 (small bowel polyp)	$P < 0.05$	Roy *et al* (91)	2001
				65 (large bowel polyp)	$P < 0.05$		
MF-tricyclic	13 mg/kg/d	3-7 wk	ApcΔ850 (Min) + Msh2-/-	48 (intestinal polyp)	$P < 0.001$	Lal *et al* (87)	2001
Rofecoxib	0.0025 (%)	3-11 wk	ApcΔ716	36 (intestinal polyp)		Oshima *et al* (55)	2001
	0.0075 (%)			55 (intestinal polyp)			
JTE-522	0.01 (%)	4-12 wk	ApcΔ474	49 (large adenoma)	$P < 0.01$	Sunayama *et al* (89)	2001
				-28 (small adenoma)	NS		

AOM[a], azoxymethane; Reporter (#), Name of reporter and (#) shows reference number

meloxicam on gastric tumors including gastric adenoma and adenocarcinoma using this model and have preliminarily confirmed a suppressive effect on gastric lesions (data not shown).

Colorectal cancer

Colorectal cancer is one of the most popular cancers and its incidence is increasing with high mortality rates in westernized countries. The relationship between the carcinogenesis and COX-2 is most intensively elucidated in both basic and clinical research about colorectal polyps, adenoma, and cancer. Before the discovery of COX-2, numerous studies about inhibitory effects of NSAIDs on intestinal tumorigenesis were performed using chemical carcinogen-induced animal models and *Apc* gene mutant mouse models[51, 52]. The *Apc* gene plays an important role in colon cancer development. An epoch-making paper was published by Oshima *et al*[53] in 1996 about the contribution of COX-2 to carcinogenic sequence in Wnt/Apc/Tcf pathway. They induced COX-2 mutations in $Apc^{\Delta 1716}$ knockout mice, which led to the development of numerous polyps in the intestine. In COX-2-/- $Apc^{\Delta 1716}$ and COX-2+/- $Apc^{\Delta 1716}$ mice, the number of polyps dramatically decreased by 86% and 66%, respectively, in comparison to that in the littermate COX-2+/+ $Apc^{\Delta 1716}$ mice. They also reported in the same paper that MF-tricyclic suppressed number of polyps in $Apc^{\Delta 1716}$ mice. This is the first report that COX-2 inhibitor reduced the number of intestinal polyps. Following this finding several COX-2 inhibitors have been reported to succeed in polyp reduction in knockout *Apc*

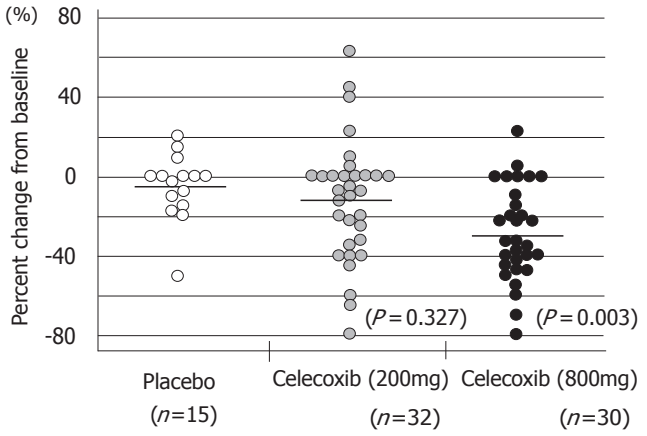


Figure 3 Percentage change from the baseline in the number of colorectal polyps in FAP patients

mice (Table 3).

Both celecoxib and refecoxib, two popular drugs as the first generation of selective COX-2 inhibitors, are now commercially available for orthopedic diseases. Both drugs have been shown to have chemopreventive effects on intestinal polyps in *Apc* mutant mouse models. Jacoby *et al*[54] performed two experiments of adenoma prevention (early phase) and regression (late phase) by celecoxib using the *Min* mice model. They showed that celecoxib decreased not only tumor size and multiplicity in the prevention study, but also caused a decrease in the size of established polyps in the regression study. In the rofecoxib study using $Apc^{\Delta 716}$ mice model, the drug successfully decreased the number and size of polyps in a dose-dependent manner[55].

The *Apc* gene mutation is also responsible for familial adenomatous polyposis (FAP). Some articles have demonstrated the chemopreventive effects of NSAIDs on colorectal polyps of FAP patients[51]. The successful outcomes of selective COX-2 inhibitors in animal models enabled us to start a clinical study of chemoprevention of FAP. Steinbach *et al*[56] of the University of Texas, Anderson Cancer Center, in Houston, reported that treatment with celecoxib significantly reduced the number of colorectal polyps in patients with FAP in 2000. I also joined this trial, which was performed as a double-blind, placebo-controlled study and was supported by a contract with the U.S. National Cancer Institute, and Searle Pharmaceuticals. All patients underwent total colonoscopy at the beginning and end of the study. All polyps observed by endoscopy were photographed and videotaped. Several members in the study group assessed the number and size of the polyps using these records in a completely blind manner. A statistical analysis was independently carried out by a biomathematician. Seventy-seven FAP patients were randomly assigned to treatment with celecoxib (100 or 400 mg twice daily) or a placebo for six months. Twice daily treatment with 400 mg celecoxib brought a 28% reduction in the number of polyps, a 100-mg dose led to an 11.9% reduction. In contrast, the polyp counts in patients who received placebo fell by only 4.5%. (Figure 3). At least a 25% reduction in polyps was experienced by 53% of the patients in the 400-mg treatment group, compared with 31% of the 100-mg group and 7% of the placebo group (Figure 4). The incidence of adverse events was similar among the groups.

Corresponding to these results, the U.S. Food and Drug Administration (FDA) immediately approved the clinical use of celecoxib for FAP patients, since it was considered to be a potentially useful adjunct to current management by suppressing polyp formation in patients with a residual rectum after colectomy and in patients with an intact colon who are awaiting a colectomy. Several years later the preventive effects on duodenal polyps in FAP patients were established by the same group[57]. Thereafter, three large trials of the chemopreventive effect on the recurrence of neoplastic polyps of the large bowel in patients with a history of colorectal adenoma have been initiated. The APPROVe (Adenomatous Polyp Prevention On Vioxx) was designed to examine the effects of treatment with rofecoxib in April 2000. The APC （Adenoma Prevention with Celebrex）cancer trial and the PreSAP（Prevention of Spontaneous Adenomatopus Polyps) cancer trial started using celecoxib in December 1999 and March 2001, respectively. Unexpectedly, all the trials now have been stopped because of an observed increased risk in cardiovascular (CV) events.

HEAD WIND AGAINST COX-2 INHIBITORS

In spite of the advances and successes of COX-2 inhibitors, recently some pharmaceutical companies have abandoned the development or marketing of such inhibitors. The Vioxx Gastrointestinal Outcomes Research Study (VIGOR study) foreshadowed a current tough situation of COX-2 inhibitors. The VIGOR study was originally designed to assess whether rofecoxib is associated with a lower incidence of clinically important upper gastrointestinal (GI) events (gastroduodenal perforation or obstruction, upper GI bleeding, and symptomatic gastroduodenal ulcers) than is naproxen, a nonselective NSAID, among 8 076 patients with rheumatoid arthritis[58]. As expected, 2.1 confirmed the incidence of GI events per 100 patient-years occurred with rofecoxib, in comparison to 4.5 per 100 patient-year with naproxen (relative risk, 0.5; $P<0.001$). However, the VIGOR study also showed the relative risk of developing a confirmed adjudicated thrombotic CV event (myocardial infarction, unstable angina, cardiac thrombus, resuscitated cardiac arrest, sudden or unexplained death, ischemic stroke, and transient ischemic attacks) with rofecoxib treatment in comparison to that with naproxen to be 2.38 ($P=0.002$). On the other hand, another similar study, the Celecoxib Long-term Arthritis Safety Study (CLASS) yielded different results[59]. The CLASS was conducted to determine whether celecoxib is associated with a lower incidence of significant upper GI toxic effects and other adverse effects in comparison with conventional NSAID, ibuprofen or diclofenac. For all 8 059 patients enrolled in the CLASS, the annualized incidence rates of upper GI ulcer complications alone and combined with symptomatic ulcers of celecoxib vs NSAIDs were 0.76% *vs* 1.45% ($P=0.09$) and 2.08% *vs* 3.54% ($P=0.02$), respectively, whereas there was no significant difference in the CV event (myocardial infarction, stroke, and death) rates between celecoxib and NSAIDs. It was later reported

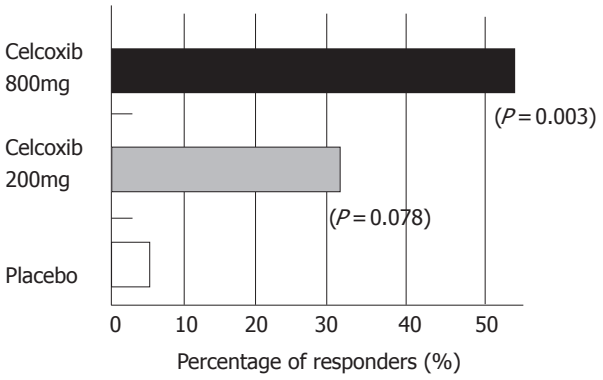


Figure 4 Percentage of responders who showed a 25% or more decrease in the mean number of colorectal polyps

that the adjusted odds ratio for myocardial infarction (MI) among celecoxib users, relative to persons who did not use NSAIDs, was 0.43 in comparison with 1.16 among rofecoxib users, and the use of rofecoxib was associated with a significantly higher odds of MI in comparison wtih the use of celecoxib (adjusted odds ratio for rofecoxib *vs* celecoxib, 2.72, $P = 0.01$) in a study comparing rofecoxib with celecoxib regarding the risk of MI incidence[60].

Merck withdrew rofecoxib from the market in September, 2004 because of an increased risk of serious CV events, including heart attack and stroke, among study patients taking rofecoxib compared to patients receiving placebo (the APPROVe). Japan Tobacco Incorporation has already declined to develop JT-522 for clinical use after phase II study in September, 2003. Regarding celecoxib, in an APC cancer trial, Pfizer demonstrated an increased CV risk over placebo, while the PreSAP cancer trial revealed no greater cardiovascular risk than the placebo. The outcomes of two trials were completely different, but Pfizer nevertheless decided to stop them. The US FDA issued a Public Health Advisory, which stated that the long-term use of NSAIDs and selective COX-2 inhibitors might increase the risk of severe CV events (myocardial infarction, strokes, *etc*) at the beginning of 2005. According to the conclusions of an advisory panel, Pfizer decided to withdraw valdecoxib from the market in April, 2005. Recently, Shaya *et al*[61] performed an observational cohort study to examine the CV risk of COX-2 inhibitors compared with nonspecific NSAIDs except naproxen in Maryland Medicaid enrollees, a high-risk population. But they did not find that COX-2 inhibitors increased CV risk over nonnaproxen NSAIDs. Whether or not selective COX-2 inhibitors really increase the risk of CV events compared with other NSAIDs remains unknown and still controversial.

COX-1 is constitutively expressed in most tissues and cells, such as the kidney, stomach, platelets, and vascular endothelium, while COX-2 expression is induced in fibroblasts, endothelial cells, monocytes, and ovarian follicles[62, 63]. Accordingly, COX-1 alone is expressed in platelets. Ironically, because the selective COX-2 inhibitors hardly suppress COX-1 inducing thromboxane A2, which activates aggregation of platelets, CV risk might be increased among the users of COX-2 inhibitors[64]. In this sense, drugs belonging to the intermediate class of COX-1/COX-2 inhibitors (moderately selective COX-2 inhibitors), such as meloxicam and etodolac, might be reassessed in the near future. But it is very beneficial for most patients that selective COX-2 inhibitors undoubtedly reduce GI disorders about in half compared with NSAIDs[58, 59]. Physicians should select COX-2 inhibitors or NSAIDs, after carefully considering which events are most important for each patient, namely GI or CV events. Recently, COX-2 inhibitors have been found to have new pharmacological advantages. Pyo *et al*[65] reported that NS-398 enhanced the effect of radiation on the COX-2 expressing cells. It was also shown that COX-2 inhibitors had a synergistic antitumor effect in combination with several chemotherapeutic agents, including gemcitabine or 5FU in pancreatic cancer[66], and paclitaxel and carboplatin in non-small-cell lung cancer[67]. Furthermore, the combination of celecoxib and an angiotensin-converting enzyme inhibitor enhanced the antitumor effect through insulin-like growth factor I receptor pathway[68] and low doses of celecoxib was useful for chemoprevention of intestinal polyps in omega-3 polyunsaturated fatty acid-rich diet [69]. These facts are very encouraging to both researchers and clinicians regarding COX-2 inhibitors, thus offering hope for their eventual use in the future.

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REVIEW

Causal role of *Helicobacter pylori* infection in gastric cancer: An Asian enigma

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Abstract

Helicobacter pylori (H pylori) has been etiologically linked to gastric cancer. *H pylori* infection is more frequent in less developed Asian countries like India, Bangladesh, Pakistan, and Thailand and is acquired at early age than in more developed Asian countries like Japan and China. Frequency of gastric cancer, however, is very low in India, Bangladesh, Pakistan and Thailand compared to that in Japan and China. Similar enigma has been reported from Africa as compared to the West. Seroprevalence of *H pylori* infection in adult populations of India, Bangladesh, Pakistan and Thailand varies from 55% to 92%. In contrast, seroprevalence of *H pylori* in Chinese and Japanese adults is 44% and 55%, respectively. Annual incidence rate of gastric cancer in India, Bangladesh, and Thailand is 10.6, 1.3, 7.1 per 100 000 populations, respectively; in contrast, that in China and Japan is 32-59 and 80-115 per 100 000 populations, respectively. Several studies from India failed to show higher frequency of *H pylori* infection in patients with gastric cancer than controls. Available evidences did not support difference in *H pylori* strains as an explanation for this enigma. Despite established etiological role of *H pylori*, situation is somewhat enigmatic in Asian countries because in countries with higher frequency of infection, there is lower rate of gastric cancer. Host's genetic make-up and dietary and environmental factors might explain this enigma. Studies are urgently needed to solve this issue.

Key words: Stomach cancer; *Helicobacter pylori;* Tropical countries; Carcinogenesis; Infectious diseases

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INTRODUCTION

Helicobacter pylori (*H pylori*) is a major cause of gastroduodenal diseases like peptic ulcer and an important risk factor for gastric carcinoma (GC) and primary gastric lymphoma (PGL). Evidences supporting the etiological role of *H pylori* in GC and PGL include higher frequency of isolation of *H pylori* in patients with GC and PGL[1,2], regression or lower rate of occurrence or recurrence of the tumor in patients in whom the infection is eradicated[3-6], occasional reports of recurrence following re-infection[7,8] and development of tumor in patients[5,9] or animals[10,11] infected with the organism. Several meta-analyses also revealed a strong relationship between *H pylori* and GC and PGL[12,13]. Though the evidences available in literature support causal relationship between *H pylori* and GC and PGL, some interesting observations from the Asian countries make such causal relationship somewhat enigmatic[14-15]. Similar enigmatic situation has also been reported from Africa[16]. Here we have reviewed the available evidences on this issue and attempted to explain possible reasons for such an enigma.

FREQUENCY OF *H PYLORI* INFECTION IN DIFFERENT ASIAN COUNTRIES

Figure 1 shows seroprevalence of *H pylori* infection in different Asian countries[4,15,17-29]. Frequency of *H pylori* infection differs markedly in different countries. In the developing countries like India, Bangladesh, Pakistan and Thailand, infection with *H pylori* is more frequent among general population and is acquired at an early age. There are several studies from India that showed that *H pylori* is acquired by most people in early childhood[30]. Gill *et al*[31] from India showed that the prevalence of IgG and IgA antibodies to *H pylori* was 22%, 56% and 87% in 0-4, 5-9 and 10-19 years age groups, respectively. In contrast, in more industrialized and developed regions of Asia like Japan, China and Singapore, frequency of *H pylori* infection has been reported to be somewhat lower[14]. The prevalence of *H pylori* in the United States has decreased to approximately 10% in the white middle and upper class population of 50 years of age or younger[32]. As *H pylori* is transmitted by feco-oral route, overcrowding, poor sanitation, lower socioeconomic status and poor water

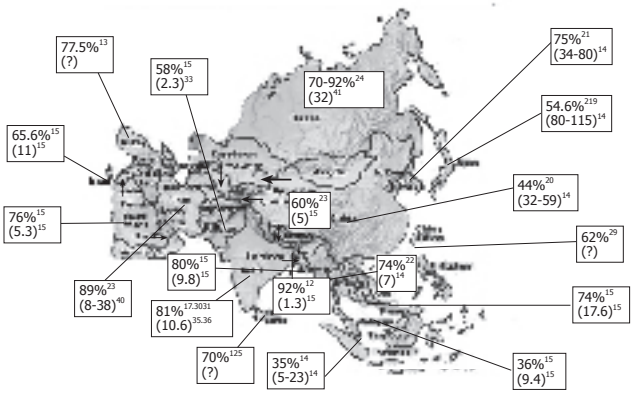

Figure 1 Map of Asia showing frequency of *Helicobacter pylori* infection in each country, crude annual incidence of gastric cancer per 100 000 populations (shown within parenthesis, where data are available). The reference from where data are obtained is shown in superscript. **A:** frequency in the year 1984; **B:** lower annual incidence of gastric cancer for Indians and higher values for Chinese living in Singapore; **C:** studies using PCR among patients with dyspepsia; #: annual-age standardized incidence rates; $: annual age-standardized death certification rate estimated from the graph that appeared in the report (year 1998-99).

supply are some of the major factors that result in higher frequency and lower age of acquisition of *H pylori* in less developed Asian countries[30,33] . Though frequency of *H pylori* as shown in the Figure 1 is not age-standardized, all these data are in adults. As *H pylori* is acquired in early childhood in most developing countries and later in life in most developed countries and as average life expectancy is higher in developed countries as compared to developing countries, age standardization of frequency of infection is unlikely to alter our conclusions.

FREQUENCY OF GASTRIC CANCER IN DIFFERENT ASIAN COUNTRIES

Gastric cancer is the world's second commonest malignancy, having been overtaken only by lung cancer in 1980's[34]. There is a marked international variation in gastric cancer incidence with highest rates reported from Japan. Figure 1 shows annual incidence of gastric cancer per 100 000 populations from Asian countries[14,15,35-41]. It is interesting to note that despite Japan being a developed country with a lower frequency of *H pylori* infection, it has highest frequency of gastric cancer. Similarly, frequency of gastric cancer is quite high in China despite a lower frequency of *H pylori* infection. In contrast, people living in less developed countries of Asia with high frequency of *H pylori* infection[17,18,22,30,31,42-44] that is acquired at an earlier age have the lowest risk of developing gastric cancer[14]. It has also been observed that frequency of gastric cancer differs in different parts within many countries; for example, in Japan[45], variation in gastric cancer risk has been well-documented in different regions and has been presumed to be related to variation in nutrient consumption. In China[46], gastric cancer mortality in Changle county is about 10-fold higher than that in Hong Kong and has been attributed to variation in frequency of *H pylori* infection in the two regions. In India, southern[35] and eastern parts (personal observation) of the country experience somewhat higher frequency of gastric cancer than the northern parts of the country. Interestingly, similar epidemiological observations were made long ago in India in respect of another *H pylori*-related gastroduodenal ailment, i.e., peptic ulcer disease[47].

STUDIES ON ASSOCIATION BETWEEN *H PYLORI* INFECTION AND GASTRIC CANCER IN ASIA

Studies from India failed to show an association between *H pylori* infection and gastric cancer[48-51]. In a study on 50 patients with gastric cancer and 50 controls with non-ulcer dyspepsia, *H pylori* infection was detected less frequently in gastric cancer (38%, 19/50) than those with non-ulcer dyspepsia (68%, 34/50)[48]. An another study demonstrated that 64.7% (33/51) patients with gastric carcinoma and 74.4% (32/43) with non-ulcer dyspepsia had infection with *H pylori* [49]. These studies can be criticized due to small sample size with a consequent type II statistical error. Also, in most of these studies, endoscopy-based tests were used to diagnose *H pylori* infection. Endoscopy-based tests can be false negative in patients with gastric cancer due to gastric atrophy and intestinal metaplasia[52]. However, a recently completed large study from our center in which 279 patients with gastric neoplasms (263 gastric cancer and 16 primary gastric lymphoma) failed to show a higher frequency of *H pylori* infection in patients with gastric neoplasms as compared with the controls (101 non-ulcer dyspepsia and 355 healthy subjects)[53]. In contrast, studies from China and Japan showed association between *H pylori* infection and gastric cancer[54,55].

WHAT IS ASIAN ENIGMA?

Oxford dictionary describes the term "enigma" as a mysterious or puzzling thing. What is puzzling about gastric cancer and *H pylori* infection in Asia? The countries with highest frequency of *H pylori* infection have the lowest risk of gastric cancer in contrast to the countries like Japan and China where gastric cancer risk is highest in the world despite a lower occurrence of *H pylori* infection. This casts major objection to some of the simplified model of gastric carcinogenesis resulting from *H pylori* infection that stated that if the infection is acquired at an early age particularly in presence of malnutrition, it may reduce gastric acid secretion, pangastritis and gastric cancer may be the likely outcome. In contrast, infection acquired later in life and in person with good nutritional status and normal gastric acid secretion would result in hyperchlorhydria and duodenal ulcer disease[56]. It is well documented in the literature that patients with duodenal ulcer infrequently or never develop gastric cancer[5,57]. If this simplified model of gastric carcinogenesis would have been true, India, Bangladesh, Pakistan would have higher frequency of gastric cancer than Japan and China.

WHAT ARE THE POSSIBLE EXPLANATIONS FOR THE ASIAN ENIGMA?

Agent factors

All strains of *H pylori* are not pathogenic. Is it possible

that people living in countries with lower frequency of gastric cancer are infected with non-pathogenic strains of *H pylori* than people living in China or Japan? However, available evidences do not support this hypothesis. Peptic ulcer disease, which is associated with infection by pathogenic strains of *H pylori,* has been reported a common problem in India and Bangladesh[47]. Genotypic analysis of *H pylori* strains from India showed pathogenic strains to be present in more than 80% of adults and children with gastroduodenal diseases as well as in control population[58-59]. Studies that used CagA antibody in patients with non-ulcer dyspepsia have shown that CagA antibody is detected in sera of most patients[60]. From our center, a recently completed large study on 279 patients with gastric neoplasms (263 gastric cancer and 16 primary gastric lymphoma) and controls (101 non-ulcer dyspepsia and 355 healthy subjects) showed that frequency of CagA IgG antibody was similar among the patients with gastric carcinoma and the controls, suggesting that difference in virulence factor of *H pylori*, at least CagA, is unlikely to explain the variation in outcome of *H pylori* infection[53]. In a study from US, Korea and Colombia[61] in which the first-degree relatives of patients with gastric cancer were evaluated to know whether similar strains of *H pylori* or similar environmental factors are responsible for pattern of gastritis. However, this study failed to show any relationship between specific virulence factors or *H pylori* strains and specific histologic pattern or outcome even among those sharing the same environment in childhood[61]. However, several studies from Japan and China[62,63] showed that virulence factors of *H pylori* are strongly associated with gastric carcinoma. Based on the available evidences, one can not conclude that in Asian countries, despite high frequency of *H pylori* infection, low frequency of gastric cancer is related to infection with non-pathogenic strains. Though a study from Africa showed that virulence-associated genes of *H pylori* may partially explain the African enigma[64], the same corollary may not hold well to explain the Asian enigma.

Host's genetic factors

Host's genetic make-up determines in a major way response to any infection, including that to *H pylori*. This is evidenced by the fact that relatives of patients with gastric cancer infected with *H pylori* developed precancerous abnormalities like gastric atrophy and hypochlorhydria more often than those with non-ulcer dyspepsia[65]. Patients with duodenal ulcer, which is also caused by *H pylori,* do not develop gastric cancer in contrast to other conditions associated with *H pylori* infection, such as gastric ulcer, non-ulcer dyspepsia and hyperplastic gastric polyp[5]. These also depict variations in host's response despite infection with the same organism. Japanese immigrants to the United States have higher gastric cancer risk than native-born Americans, though lesser than Japanese living in Japan[66]; this suggests importance of the genetic factors with additive effects of environmental factors.

Difference in carcinogenic risk in people living in different geographical areas might be related to variation in genetic make-up among different races. Specific allelic variation of different genes (polymorphism) present in a proportion of general population may determine variation in carcinogenic potential in different populations in response to environmental carcinogenic exposure, including that to *H pylori* infection[67]. Genetic susceptibility of a person may be important in a number of carcinogenic processes that include: (1) mucosal protection against *H pylori* infection and injury by other carcinogens; (2) mucosal inflammatory response to infection with *H pylori*; (3) degree of apoptotic cell death[68]; (4) carcinogen activation and detoxification by various enzyme systems of the hosts; (5) variability in the repair of mutated DNA; and (6) ability of the cell to proliferate in a controlled manner to repair the damage.

Several studies have been carried out on single nucleotide polymorphism in relation to gastric carcinogenesis[67]. However, many of these studies did not take into account the role of *H pylori* infection and dietary factors in addition to the genetic factors. Therefore, there is need of more data on genetic polymorphism in relation to *H pylori* infection and dietary factors. In fact, genetic studies comparing Asian population with high gastric cancer risk like Japan and China and low cancer risk despite a very high prevalence of *H pylori* infection like India are needed to understand the explanation for the Asian enigma at a molecular level.

Dietary and environmental factors

Diet may play a major role in gastric carcinogenesis. In India, southern[35] and eastern parts (personal observation) of the country experience somewhat higher frequency of gastric cancer than the northern parts of the country. Rice is the staple cereal in eastern India. Non-vegetarian foods, particularly fish, are very common in eastern Indian diet, which is also spicy with more salts. Diet in southern India is somewhat similar to that in eastern India with rice, fish, excess spice and salt being commonly eaten. In contrast, northern Indian diet is mainly wheat-based and a greater proportion of people are vegetarian. Tobacco smoking, high-temperature food intake, spicy food and rice eating have been shown to be risk factors for gastric cancer in India[69,70]. In another study, consumption of dry fish has been shown to be a risk factor for gastric cancer in India[71]. Diet has been considered to be a major factor for increased frequency of gastric and esophageal cancer in Kashmir province of India[72]. Similar observations have also been made in several countries, including Japan where northern districts have reported a higher frequency of gastric cancer than southern district and this has been related to increased dietary intake of salts in northern districts[14]. Tobacco use and alcohol consumption are the other factors that may influence the international variation in frequency of gastric cancer[15]. Possible explanation of Asian enigma might be related, at least in part, to difference in diet between different countries.

CONCLUSIONS AND FUTURE DIRECTIONS

The available evidences clearly show that *H pylori* alone is not the only independent factor in gastric carcinogenesis. Host's genetic make-up and dietary factors play a major role in determining whether or not a person infected with *H pylori* will develop gastric atrophy, intestinal

metaplasia and gastric cancer. This has major importance in preventive strategies of gastric cancer. Despite *H pylori* being an important agent for causing gastric cancer, a recent randomized controlled trial from high risk region of gastric cancer in China failed to show benefit of eradicating *H pylori* in preventing gastric cancer[73]. This might be related to the fact that only 1-2% people infected with *H pylori* develop atrophic gastritis per year, which is a precancerous lesion[74]. Racial and genetic factors are also important as evidenced by difference in gastric cancer risk in different populations, and a recent study, though not from Asia, showed differences in IgG subclass responses between subjects from Gambia and United Kingdom[75]. Unless randomized controlled trials of eradication of *H pylori* among people who are not only infected with *H pylori* but also carry multiple genetic factors which increase their predisposition to developing gastric cancer are undertaken, it is difficult to obtain meaningful conclusions about how useful *H pylori* eradication would be to prevent gastric carcinoma. In fact, in foreseeable future, a day may come when an individual infected with *H pylori* may be able to know, using mathematical modeling and his genetic make-up, as to what would be his risk of developing gastric cancer, and based on that his physician may advise him whether he should undergo *H pylori* eradication treatment and/or modification of his diet to reduce risk of gastric cancer. Since gastric cancer is likely to be a multifactorial disease, which include genetic, dietary and environmental factors and not *H pylori* alone, all these factors need to be considered while constructing the model.

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ESOPHAGEAL CANCER

Evidence of human papilloma virus infection and its epidemiology in esophageal squamous cell carcinoma

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Abstract

AIM: To look for the evidence of human papilloma virus (HPV) infection in esophageal squamous cell carcinomas (ESCC) and to investigate the potential role and epidemiology of HPV infection in the pathogenesis of esophageal carcinomas in Henan emigrants.

METHODS: Papilloma virus（PV）and HPV were determined by Ultrasensive™ S-P immunohistochemistry (IHC) and *in situ* hybridization (ISH) in esophageal carcinoma tissues (82 cases) and the normal mucosa (40 cases).

RESULTS: IHC revealed that the positive rate of PV was 75.0%, 68.18% and 72.5% respectively while the HPV (16/18-E6) positive rate was 45.0%, 36.36%, 37.5%, respectively in esophageal carcinoma tissue specimens from Henan emigrants,the local citizens and patients in Hubei Cancer Hospital. The PV and HPV (16/18-E6) were negative in all normal esophageal mucosa specimens. No correlation was found between HPV in esophageal squamous cell carcinoma tissues and in grade 1-3 esophageal squamous cell carcinoma cells. *In situ* hybridization showed that the HPV (16/18) DNA positive rate was 30.0%, 31.8%, 25.0%, respectively in the 3 groups of samples. No positive hybridization signal was found in 40 normal esophageal mucosa specimens. The positive rate of HPV (16/18) DNA in the esophageal carcinoma specimens was significantly higher than that in normal mucosa specimens ($P < 0.05$). The positive rate was not different among the 3 groups of esophageal carcinoma tissue specimens ($P > 0.05$).

CONCLUSION: HPV infection is high in esophageal carcinoma of Henan emigrants, local residents and patients in Hubei Cancer Hospital. HPV is closely related with esophageal squamous cell carcinoma. HPV infection may play an important role in esophageal squamous cell carcinoma.

Key words: Human papillomavirus; Esophageal squamous cell carcinoma; Immunohistochemistry; *in situ* hybridization

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INTRODUCTION

Chaihu area in Hubei Province, China is a high incidence area of esophageal carcinoma because almost 40 000 residents were emigrated from Henan, the highest incidence province of esophageal carcinoma incidence area. The incidence and mortality of esophageal cancer in Hubei have been increased since 1970s ($\geqslant 131/10^5$)[1]. Studies have shown that HPV plays a key role in the development of squamous cell carcinoma at various body sites, including cervix, anogenital region and oral cavity[2-4]. Esophageal squamous epithelium is one of the susceptible sites to HPV. So far, there is no report on the correlation of HPV infection and high incidence of esophageal carcinomas in Henan emigrants and its epidemiology has not been fully studied. In this study, we determined the incidence of the high-risk HPV types 16 and 18 in patients with esophageal cancer using ultrasensitive™ S-P immunohistochemistry (IHC) and in situ hybridization (ISH) and investigated the potential role and epidemiology of HPV infection in the pathogenesis of esophageal carcinoma.

MATERIALS AND METHODS

Tissue collection

Twenty specimens of esophageal carcinoma were collected from Zhongxiang Chaihu Hospital in the high-

incidence area. The average age of the patients was 58 years (range 41-58 years). Twenty patients were all Henan emigrants. Twenty-two specimens were collected from Macheng district. The mean age of patients was 55.7 years (range 33-67 years). Forty specimens were collected from Department of Pathology, Hubei Cancer Hospital. The mean age of patients was 56.9 years (range 37-75 years). The patients were all natives. All the patients were histologically diagnosed as esophageal squamous cell carcinoma.

All the samples were cut into 5-μm thick sections using Leica RM2135 microtome. H-E staining was performed before IHC and ISH. At the same time, all the samples were confirmed by pathologists and 40 normal esophageal mucosa tissue specimens were used as controls. PV and HPV (16/18-E6) antibody and Ultrasensive™ S-P immunohistochemistry kit were purchased from Maixin Biotechnology Company (Fuzhou, China). Biotinylated HPV (16/18) DNA probes and *in situ* hybridization detection systems were purchased from DAKO Company.

Immunohistochemistry

Immunohistochemistry (Ultrasensive™ S-P method) for detection of PV and HPV (16/18-E6) was performed following the manufacturer's instructions. Briefly, paraffin-embedded sections were dewaxed, antigen retrieval was performed by heating the sections in 10mM of citrate buffer (pH 6.0) for 90 s. The tissue sections were treated with 3% hydrogen peroxide in PBS containing 0.01 mol/L sodium phosphate (pH 7.2), then with 0.15 mol/L NaCl to block endogenous peroxidase and normal rabbit serum to block non-specific binding sites. Mouse monoclonal anti- HPV (16/18-E6) antibody was used as the primary antibody at a dilution of 1:100. Rabbit polyclonal anti-PV antibody was used as the primary antibody at dilution of 1:50 and 1:100, respectively. Peroxidase activity was measured with 3, 3'-diaminobenzidine. The primary antibody was absent in negative controls. Sections were counterstained with hematoxylin. The positive control sections were supplied by Maixin Bio Company.

In situ hybridization

All in situ hybridizations were performed using in situ hybridization detection systems and biotinylated HPV (16/18) DNA probes from DAKO Company. Briefly, paraffin-embedded sections were dewaxed and then digested by immersion in 0.8% pepsin solution in 0.2N HCl at 37℃ for 10 min. Following digestion, the sections were rinsed 4 times in deionized water, then immersed in 0.3% H_2O_2 for 20 min, and rinsed 5 times in deionized water. The slides were dried in air for 15 min, one drop (approximately 20 μL) of probe was applied to the sections, and covered with coverslips. The probe and HPV target DNA were denatured by placing the slides on a PCR cycler at 90℃ for 5 min. Following denaturation, slides were transferred to a pre-warmed humid chamber for hybridization at 37℃ for 60 min. Following hybridization, coverslips were removed by immersing the slides in 1×TBST at room temperature. The slides were transferred to fresh TBST bath before strigent washing at 58℃ for 30 min followed by rinsing the slides 3 times in 1×TBST, one min each. The slides were placed on a level surface and enough streptavidin-AP reagent was applied to each section to cover the tissue, incubated for 20 min at room temperature and then enough BCIP/NBT substrate solution was applied to each section to cover the tissue. The slides were incubated at room temperature for 60 min, counterstained in nuclear fast red and cover slips were mounted. Purple-blue ISH signals were observed under microscope and photos were taken for analysis. Slides with PV and HPV positive esophageal cancer tissues were used as positive controls. The hybridization solution without probe or streptavidin-AP reagent was used as a negative control.

Evaluated standard of results and statistical analysis

Without any knowledge of any patient's clinical and pathological data, all slides were evaluated independently by two pathologists. An evaluated standard of results was established corresponding to the staining intensity of positive cells: - , negative; +, 5-25% positive cells; ++, 26-50% positive cells; +++～+++++, positive cells > 50%. Statistical analyses were performed with SPSS 10.0 software and Fisher's exact probability test was used to analyze the correlation between HPV expression and clinicopathologic features of ESCC. $P<0.05$ was considered statistically significant.

RESULTS

Immunohistochemical data of PV and HPV (16/18-E6)

IHC revealed that the positive rate of PV was 75.0% (15/20), 68.18% (15/22) and 72.5% (29/40) respectively and the HPV positive rate was 45.0% (9/20), 36.36% (8/22), 37.5% (15/40) respectively in esophageal carcinoma tissues from Henan emigrants, the local citizens, and patients in Hubei Cancer Hospital. No PV and HPV were detectable in all normal esophageal mucosa tissues. Only few samples showed weak staining. No correlation was found between HPV infection in esophageal carcinoma tissues and grades of esophageal carcinoma cells. The positive rate of HPV in three groups of esophageal cancer samples was significantly higher than that in normal mucosa samples ($P < 0.01$). The positive rate was not obviously different among the three groups of esophageal carcinoma tissue samples. PV concentration at 1:50 and 1:100 showed the same positive rate (Table 1, Figures 1 A-1C).

In situ hybridization data of HPV (16/18) DNA

In situ hybridization showed that the HPV (16/18) DNA positive rate was 30.0% (6/20), 31.8% (7/22), 25.0% (10/40) respectively in esophageal carcinoma tissues from Henan emigrants, the local citizens, and patients in Hubei Cancer Hospital. Forty normal mucosa tissue specimens showed negative results (Table 2). Among the three kinds of esophageal carcinoma tissue samples, no statistically significant difference was found ($P>0.05$), while significant difference was found between normal mucosa tissues and the three kinds of esophageal carcinoma tissue samples ($P<0.05$). Positive signals of HPV (16/18) DNA were located in nuclei. Positive cells located in the center of carcinoma were in the shape of small shuttle (Figure 2A). The negative control is shown in Figure 2B.

Table 1 Expression of PV and HPV (16/18-E6) in esophageal carcinoma and normal mucosa

Origin of ESCC samples	Patients (*n*)	Positive rates of PV (%)	Positive rates of HPV (%)
Henan emigrants	20	(15/20) 75.00	(9/20) 45.00
Local residents	22	(15/22) 68.18	(8/22) 36.36
Hubei Cancer Hospital	40	(29/40) 72.50	(15/40) 37.50
Normal mucosa	40	(0/40) 0.00	(0/40) 0.00

Table 2 Expression of HPV (16/18) DNA in esophageal squamous cell carcinoma and normal mucosa tissues

Origin of ESCC samples	Patients (*n*)	-	+	+ + ~ + + + +	Positive rate (%)
Henan emigrants	20	14	3	3	30.0
Local residents	22	15	5	2	31.8
Hubei Cancer Hospital	40	30	6	4	25.0
Normal mucosa	40	40	0	0	0.00

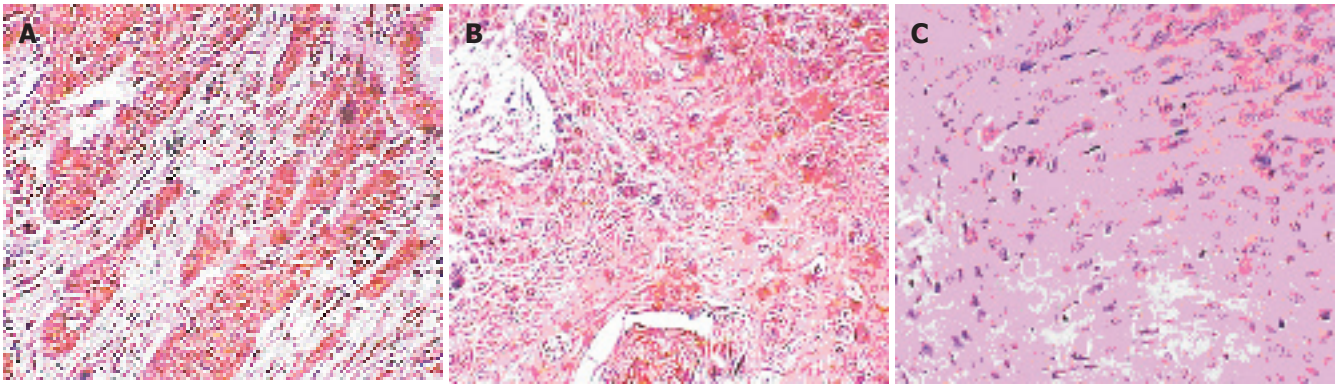

Figure 1 Positive expression of PV (**A**) and HPV(16/18-E6) (**B**) and negative expression of HPV (16/18-E6) (**C**) in esophageal carcinoma (IHC×100)

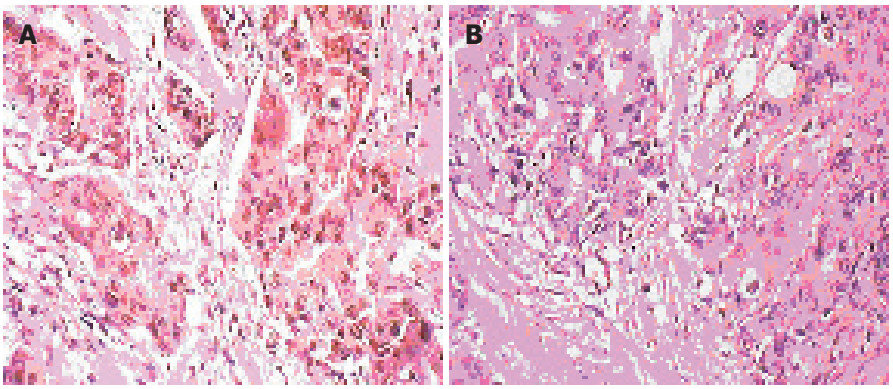

Figure 2 Positive (**A**) and negative (**B**) expression of HPV(16/18) DNA in esophageal squamous cell carcinomas (ISH×100)

DISCUSSION

Esophageal carcinoma is one of the major cancers in China. It may be closely related with heredity, environment, diet and infection with some microorganisms. One of the characteristic features of esophageal carcinoma is its variation in both geographic location and way of life. At present, it is generally believed that esophageal carcinoma is a kind of disease involving many factors. Esophageal carcinoma usually shows a typical course of carcinogenesis. Progression to malignancy during HPV-associated carcinogenesis is related to gene amplification and activation as well as high expression of many cancer genes because of mutation and deletion of cancer suppressor genes[7]. However, its mechanism has not been fully elucidated.

In 2004, approximate 1 000 questionnaires of epidemiology to Henan emigrants showed that the high incidence and mortality of esophageal carcinoma in Henan emigrants are related with environment, diet and genetic susceptibility.

Why HPV infection is related with etiology of esophageal cancer remains unclear[8]. HPV infection is first suggested as a contributory factor for the development of

esophageal cancer in 1982 by Syrjanen *et al*[9]. The presence of HPV antigen has been demonstrated by immunohistochenmical techniques[10]. Subsequently, many studies on HPV infection in esophageal cancer have been reported[11-13, 21]. However, the involvement of HPV remains controversial. Up to now, no report is available on esophageal carcinoma of Henan emigrants. In our study, the PV and HPV positive expression in esophageal carcinoma tissues was determined by Ultrasensive^{TM} S-P immunohistochemistry and *in situ* hybridization. The results showed that the positive rate in three groups of samples was high. HPV positive cells were found in the central region of tissue sections, suggesting that the positive signals (i.e. HPV DNA in the section area) are free of contamination which might come from the experiment. In immunohistochemistry, the PV and HPV positive rates were 75%, 68.18%, 72.5% and 45%, 36.36%, 37.5% respectively, while the expression rate of HPV was 30.0%, 31.8%, 25.0% respectively *in situ* hybridization in the 3 groups of samples, suggesting that HPV infection may be an integral part of a multistep process leading to esophageal cancer in high risk area. The results are consistent with other reports[14,15,20]. The positive rate of HPV detected by immunohistochemistry was higher than that by *in situ* hybridization in our study. These differences probably result from variations in the specificity and sensitivity of the analytical techniques used. *In situ* hybridization is more sensitive and specific than immunohistochemical method. Studies have generated contradictory data possibly due to the geographical location with respect to either low or high incidence areas[16,17]. In addition, variations in infection rate of HPV from the same geographical areas have been confirmed[18,19].

In conclusion, HPV infection is high in esophageal carcinoma of Henan emigrants. HPV is closely related with esophageal squamous cell carcinoma.

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GASTRIC CANCER

Apoptosis induced by preoperative oral 5′-DFUR administration in gastric adenocarcinoma and its mechanism of action

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Abstract

AIM: To study the apoptosis induced by preoperative oral 5′-DFUR administration in gastric adenocarcinoma and its mechanism of action.

METHODS: Sixty gastric cancer patients were divided randomly into three groups (20 each group) before operation: group one: 5′-DFUR oral administration at the dose of 800-1200mg/d for 3 - 5 d, group two: 500mg 5-FU + 200 mg/d CF by venous drip for 3 - 5 d, group three (control group). One or two days after chemotherapy, the patients were operated. Fas/FasL, PD-ECGF and PCNA were examined by immunohistochemistry and apoptotic tumor cells were detected by *in situ* TUNEL method. Fifty-four patients received gastrectomy, including 12 palliative resections and 42 radical resections. Six patients were excluded. Finally 18 cases in 5′-DFUR group, 16 cases in CF + 5-FU group, and 20 cases in control group were analyzed.

RESULTS: There was no significant difference in patient mean age, gender, white blood cell count, haematoglobin (HB), thromboplastin, perioperative complication incidence, radical or palliation resection, invasion depth (T), lymphonode involvement (N), metastasis (M) and TNM staging among the three groups. However, the PCNA index (PI) in 5′-DFUR group (40.51 ± 12.62) and 5-FU + CF group (41.12 ± 15.26) was significantly lower than that in control group (58.33 ± 15.69) ($F = 9.083$, $P = 0.000$). The apoptotic index (AI) in 5′-DFUR group (14.39 ± 9.49) and 5-FU + CF group (14.11±9.68) was significantly higher than that in control group (6.88 ± 7.37) ($F = 4.409$, $P = 0.017$). The expression rates of Fas and FasL in group one and group three were 66.7% (12/18) and 50% (9/18), 43.8% (7/16) and 81.3% (13/16), 45.0% (9/20) and 85% (17/20), respectively. The expression rate of FasL in 5′-DFUR group was significantly lower than that in the other two groups (χ^2=6.708, $P = 0.035$). Meanwhile, the expression rate of PD-ECGF was significantly lower in 5′-DFUR group (4/18，28.6%) than in CF + 5-FU group（9/16，56.3%）and control group (13/20，65.0%) ($\chi^2 = 7.542$, $P = 0.023$). The frequency of Fas expression was significantly correlated with palliative or radical resection ($\chi^2 = 7.651$, $P = 0.006$), invasion depth ($\chi^2 = 8.927$, $P = 0.003$), lymphatic spread ($\chi^2 = 4.488$, $P = 0.034$) and UICC stages ($\chi^2 = 8.063$, $P = 0.045$) respectively. By the end of March 2005, 45 patients were followed up. The 0.5-, 1-, 2-, 3-year survival rates were 96%，73%，60%，48%, respectively, which were related with T, N, M and Fas expression, but not with PD-ECGF and FasL expression.

CONCLUSION: Preoperative oral 5′-DFUR administration may induce apoptosis of gastric carcinoma cells and decrease tumor cell proliferation index, but cannot improve the prognosis of patients with gastric cancer. Down-regulation of FasL and PD-ECGF expression mediated by 5′-DFUR may be one of its anti-cancer mechanisms. Fas expression correlates with the progression of gastric carcinoma and may be an effective prognostic factor.

Key words: Apoptosis; Preoperative chemotherapy; 5′-DFUR; Gastric carcinoma

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INTRODUCTION

Cell apoptosis and proliferation regulate homeostasis.

Disordered balance results in tumorigenesis. Cell apoptosis can hinder tumor growth[1-3]. Therefore, induction of tumor apoptosis is a good anti-cancer therapy. At present, the relationship between chemotherapy and cancer cell apoptosis has drawn more and more attention. However, studies on induction of apoptosis in gastric carcinoma are performed *in vitro*. Inducers reported include γ-ray, beta-ionone, biological response modifiers, chemotherapeutics, *etc*[4-8]. Chemotherapeutics-induced apoptosis is one of its anti-cancer mechanisms. Arsenic trioxide[9], hydroxycamptothecin[10], cisplatin[11] , paclitaxel[12], fluorouracil and its derivant [13,14] , oxaliplatin[15] can induce apoptosis in human gastric carcinoma cells. 5'-deoxy-5-flurouridine (doxifluridine or 5'-DFUR (known as Furtulon) is a selective anticancer medicine, which can be converted into 5-FU by thymidine phosphorylase (the same substance as traversing platelet-derived endothelial cell growth factor, PD-ECGF)[14,19,20]. Accordingly, the concentration of 5-FU is high in tumor tissue[19, 21]. The therapeutic index of doxifluridine is ten times that of 5-FU[19]. Doxifluridine has been widely used in treatment of breast cancer, colorectal carcinoma, ovarian adenocarcinoma, bladder cancer and gastric carcinoma[22-26].

In this study, we used doxifluridine as an apoptosis inducer to study the change of apoptosis and expression of proliferative cell nuclear antigen (PCNA), Fas and Fas ligand (FasL), and PD-ECGF in gastric adenocarcinoma and its mechanism of action.

MATERIALS AND METHODS

Patients

Patients who were diagnosed as malignant gastric neoplasm (age ⩽ 70 years, Karnofsky's scale >90) and could endure chemotherapy and operation, were enrolled in this study. The patients were divided into three groups (20 each): group 1: 800 mg - 1 200 mg/d 5'-DFUR for 3-5 days, group 2: 500 mg 5-FU + 200 mg/d CF by venous drip for 3-5 d and group 3 (control group). One or two days after chemotherapy, the patients underwent surgery. From Oct. 2001 to Oct. 2003, 60 gastric cancer patients (20 in each group) were enrolled (37 males and 17 female, mean age 57.5 years, range 32-70 years) .

Groups

Of the 60 patients, 54 underwent gastrectomy (including 12 palliative resections and 42 radical resections, and 6 were excluded. No perioperative mortality occurred during the first 30 days after surgery. Complications were recorded in four of 54 patients resected, one gastric perforation on the fourth day after 5'-DFUR oral chemotherapy, one preoperative gastrorrhagia and one postoperative cerebral infarction in CF+5-FU group, one venous thrombosis of lower extremities in control group complicated by cerebral hemorrhage during thrombolysis treatment. The patients with gastric perforation and gastrorrhagia required emergency laparotomy.

Immunohistochemistry

PCNA, Fas/FasL and PD-ECGF expression was determined by En Vision immunohistochemistry[27] .The kit was purchased from DAKO Co., USA. Apoptotic cells were detected by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) method. The kit was purchased from Bochringer Mannheim Co., Germany. The proliferation index (PI) and apoptotic index (AI) of gastric carcinoma cells were evaluated by PCNA immunohistochemical staining and *in situ* TUNEL.

Result assessment

The stained cells had Fas/FasL or PD-ECGF positive expression. Brown-stained nuclei were considered as positive cells of PCNA and apoptosis, brown cellular membrane and cytoplasm were considered as Fas or FasL positive cells (Figures 1A and 1B), brown or yellow staining in cytoplasm and/or cell nuclei was considered as PD-ECGF positive cells (Figure 1C). PCNA index (PI) and apoptotic index (AI) of positive cells in 1000 tumor cells were calculated under high power field (*400) of microscope.

AI = TUNEL mark cells/tumor cells (more than one thousand)*100%

PI = PCNA mark cells/tumor cells (more than one thousand)*100%

Statistical analysis

All data were analyzed by ANOVA and chi square test. $P < 0.05$ was considered statistically significant.

RESULTS

Patients and groups

Fifty-four patients underwent surgical resection, including 18 cases in 5'-DFUR group, 16 cases in CF+5-FU group, and 20 cases in control group. There was no significant differences in patient mean age, gender, white blood cell count, haematoglobin (HB), thromboplastin, perioperative complication incidence, radical or palliation resection, invasion depth (T) , lymphonode involvement (N) , metastasis (M) and TNM staging among the three groups.

AI and expression of PCNA, Fas/ FasS-L, PD-ECGF in gastric carcinoma

The expression of PI, AI, FasL and PD-ECGF was not statistically significant as compared to the operation procedure (radical or palliation resection), early or advanced tumor, T, N, M and UICC stages. In contrast, positive staining of Fas was closely related to radical resection but not with serosal invasion, lymphnode metastasis and early UICC stages (Table 1, Figure 1).

Influence of preoperative chemotherapy on expression of AI, PI, Fas/FasL and PD-ECGF

Either 5'-DFUR or CF+5-FU preoperative chemotherapy could significantly inhibit cell proliferation and induce apoptosis as compared to control group. The frequency of Fas expression had no significant difference among three groups. However, the frequency of FasL expression was significantly lower in 5'-DFUR group (50%) than those in CF+5-FU group (81.3%) and control group (85%) . There was no significant difference between groups two and

Table 1 Expression of PI, AI, Fas/ FasL compared to operation procedure, early or advanced tumor, T, N, M and UICC stages

	PI (%)	AI (%)	Fas +	Fas -	FasL +	FasL -	PD-ECGF +	PD-ECGF -
Way of resection								
radical	46.31±15.30	12.13±9.65	26	16	29	13	19	23
Palliation	50.74±21.27	9.40±8.35	2	10	10	2	7	5
P/χ^2 Value	0.654	0.791	7.651		0.949		0.641	
P Value	0.422	0.378	0.006		0.33		0.423	
Early cancer								
Yes	45.29±12.34	14.54±12.38	7	2	6	3	3	6
No	47.70±17.51	10.92±8.70	21	24	33	12	23	22
F/χ^2 Value	0.154	1.123	2.908		0.166		0.949	
P Value	0.697	0.294	0.088		0.684		0.33	
Invasion depth								
T1-2	46.65±15.76	11.96±9.81	20	8	19	9	12	16
T3-4	47.99±17.92	11.06±9.04	8	18	20	6	14	12
F/χ^2 Value	0.086	0.121	8.927		0.552		0.652	
P Value	0.770	0.729	0.003		0.457		0.419	
Lymphonode								
N0	50.37±16.34	10.51±9.89	13	5	14	4	9	9
N1-4	45.76±16.87	12.03±9.20	15	21	25	11	17	19
F/χ^2 value	0.917	0.312	4.488		0.415		0.037	
P value	0.343	0.579	0.034		0.519		0.847	
Metastasis								
M0	46.73±15.79	11.51±9.10	26	19	33	12	22	23
M1	50.10±21.5	11.60±11.24	7	2	6	3	4	5
F/χ^2 value	0.301	0.001	3.798		0.166		0.059	
P value	0.585	0.980	0.051		0.684		0.808	
UICC stage								
Ⅰ	50.42±16.84	10.98±9.99	13	4	13	4	9	8
Ⅱ	42.69±13.24	11.78±10.65	5	4	6	3	2	7
Ⅲ	46.03±16.2	12.73±10.10	7	9	11	5	7	9
Ⅳ	48.01±20.18	10.52±7.25	3	9	9	3	8	4
F/χ^2 value	0.452	0.148	8.063		0.434		4.352	
P value	0.717	0.931	0.045		0.933		0.226	

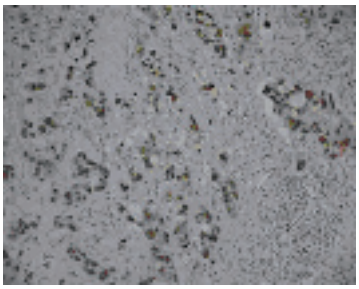

Figure 1 The expression of PCNA (*200).

three. The PD-ECGF expression was detected in 28.6% of 5'-DFUR group, 56.3% of CF+5-FU group and 65.0% of control group (Table 2).

Prognosis

By the end of March 2005, 45 patients were followed up. During the follow-up, 22 cases died, 23 remained alive (including one patient with cerebral hemorrhage after operation). The 0.5-, 1-, 2-, 3-year survival rates were 96%, 73%, 60%, 48% respectively, which were related with T (χ^2=30.32, *P*=0.0000), N (χ^2=22.10, *P*=0.0000), M (χ^2=17.04, *P*=0.0000) and Fas expression (χ^2=12.24, *P*=0.0005, Figure 2), but not with PD-ECGF (χ^2=0.78, *P*=0.3775) and FasL expression (χ^2=0.7, *P*=0.7967).

DISCUSSION

Gastric carcinoma is one of the most common malignant tumors and remains a leading cause of cancer-related death worldwide [15, 16, 28-30]. In China, it still ranks the first[31, 32]. Operative resection is the most effective treatment nowadays. However, the 5-year survival rate was 25%~60% even after radical resection[33-36], micrometastasis is the major reason for recurrence[29,37,38]. Preoperation chemotherapy can inhibit micrometastasis, but strong chemotherapy may potentially increase operation complications in resectable patients[30,35,36]. It was reported that surgical resection may serve as a stimulus for the growth of residual tumor[30]. Inada *et al*[3] have reported the effectiveness of preoperative 5-Fu venous chemotherapy, which can induce cancer cell apoptosis *in vivo*. Our study showed that preoperative venous CF+5-Fu chemotherapy and 5'-DFUR oral administration could induce gastric carcinoma cell apoptosis and inhibit cancer cell proliferation. Wang *et al*[22] reported that preoperative 5'-DFUR chemotherapy is able to partially inhibit the expression of extracellular signal-regulated kinase (ERK) which is closely related with cell proliferation in breast cancer. PCNA index decrease in our studies might be related with inhibition of ERK-1 and ERK-2 expression. Liang *et al*[39] studied apoptosis in ovarian cancer and found that apoptosis induced by che-

Table 2 Influence of preoperative chemotherapy on expression of Fas/FasL, PD-ECGF and AI, PI

Group	*N*	Fas		FasL		PI	AI	PD-ECGF	
		+	-	+	-	(%)	(%)	+	-
5'-DFUR	18	12	6	9	9	40.51 ± 12.62	14.39 ± 9.49	4	14
CF+5-FU	16	7	9	13	3	41.12 ± 15.26	14.11 ± 9.68	9	7
Control	20	9	11	17	3	58.33 ± 15.69	6.88 ± 7.37	13	7
X2/F value		2.379		6.708		9.083	4.409	7.542	
P value		0.304		0.035		0	0.017	0.023	

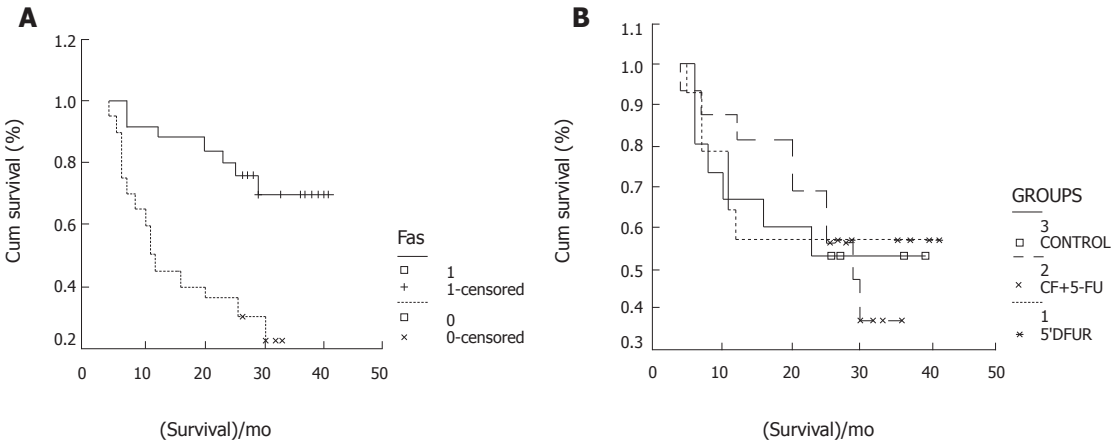

Figure 2 Fas expression (**A**) and survival rate (**B**) in three groups.

motherapy is decreased, suggesting that apoptosis induced by chemotherapy has a time limit. Therefore, preoperative chemotherapy-induced tumor cell apoptosis can inhibit malignant behavior in some degree.

Fas(CD95/APO-1)/Fas ligand (FasL) system is one of the major apoptotic pathways and plays an important role in maintenance of cell colony, elimination of malignant transformation cells and regulation of immune system[1-3, 40]. In general, Fas/FasL system plays an important role in prognosis and immune escape. Fas of activated T lymphocytes and tumor-infiltrating lymphocytes(TIL) can increase apoptosis[1-3,40-45]. Tumor cells combined with activated T lymphocytes and TIL can kill surrounding normal infiltrating lymphocytes, escape immune system[1,2,22,23,43,44]. Our investigation showed that preoperative 5'-DFUR oral administration chemotherapy could down regulate FasL expression, which may be one of its mechanisms underlying tumor cell apoptosis. But CF+5-FU venous chemotherapy had no influence on Fas/FasL expression, suggesting that 5'-DFUR and 5-FU have a different mechanism of action. Induction of 5-FU is relevant with activation of apoptosis gene bax[45], and expression of bcl-2 oncogene[13].

PD-ECGF, one of the angiogenesis factors, extracted from fresh thrombocytolysis matters is the same substance as thymidine phosphorylase(TP), PyNPase[20,46-49]. It can promote angiogenesis, cell proliferation and inhibit apoptosis[46-49]. Konno *et al*[47] reported that expression of PD-ECGF in gastric carcinoma has a positive correlation with PCNA, and PD-ECGF can also promote tumor growth. Osaki *et al*[20] found that increased PD-ECGF expression is closely related with decreased apoptosis in gastric carcimoma. The mean apoptotic index in early and advanced gastric cancer is significantly lower in positive PD-ECGF than in negative PD-ECGF. Koizumi *et al*[21] determined PD-ECGF expression in gastric carcinoma and found that the responsive rate to chemotherapy is 56.8% in positive PD-ECGF group and 0% in negative group, and 82.4% in PD-ECGF over expression group, indicating that chemosensitivity is closely related with PD-ECGF expression. PD-ECGF can increase the activity of 5-FU and other anti-cancer medicines[50]. Cytokine such as interferon, can up-regulate PD-ECGF and cytokine levels, thus detecting PD-ECGF level in tumor tissue can predict chemotherapy sensitivity and its efficacy[18,26,51]. PD-ECGF inhibitors can decrease tumor angiogenesis, prevent infiltration and metastasis[52]. PD-ECGF is the key enzyme for the metabolism of Fortulon. Satoh B *et al*[14] reported that by oral administration of 5'-DFUR, 1 200 mg per day for seven days significantly decreases PD-ECGF activity and acidic protein. Our results showed that preoperative oral administration of Fortulon for 3 - 5 d can down-regulate PD-ECGF expression and induce cancer cell apoptosis in gastric carcinoma. Over expression of PD-ECGF is an important factor of tumor metastasis [53]. Our results also showed that patients with PD-ECGF overexpression had a higher tendency toward liver metastasis. For this reason, down regulation of PD-ECGF expression by preoperative 5'-DFUR chemotherapy plays a certain role in preventing postoperative recurrence and metastasis of gastric carcinoma. It was reported that PD-ECGF level is closely related with tumor metastasis[24,25].

Kabayashi and kimur[8] performed a multicenter clinical trial of preoperative chemotherapy for gastric cancer and

found that oral administration of 5'-DFUR may induce apoptosis of gastric carcinoma and decrease proliferation index in old patients with advanced gastric cancer, but cannot improve their prognosis. However, Fas expression is often presented in early stage of tumors and shows a better prognosis. Thus, Fas expression in gastric carcinoma may be an effective prognostic factor for survival.

In conclusion, 5'-DFUR or CF+5-FU chemotherapy can induce apoptosis and inhibit proliferation of tumor cells. Down regulation of FasL and PD-ECGF induced by 5'-DFUR may be one of its anti-cancer mechanisms. Fas expression is correlated with progression of gastric carcinoma and may be an effective prognostic factor.

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VIRAL HEPATITIS

Budesonide induces complete remission in autoimmune hepatitis

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Abstract

AIM: Prednisone and azathioprine represent the standard treatment for autoimmune hepatitis (AIH). However, only 65% of the patients enter complete histological remission. Recently, budesonide (BUD) was reported to be a promising alternative. In this study we assessed the efficacy and safety of BUD in AIH.

METHODS: Eighteen patients (12 women, 6 men; mean age 45.4 ± 21 years) with AIH were treated with BUD (Budenofalk®) 3 mg thrice daily and followed up for at least 24 wk. Seven patients also had features of primary biliary cirrhosis ($n = 5$) or primary sclerosing cholangitis ($n = 2$). Advanced liver fibrosis or cirrhosis was present in 6 patients.

RESULTS: Fifteen (83%) patients had a complete clinical and biochemical remission. Ten patients, including five with acute hepatitis, were given BUD as first-line therapy, of which seven enter remission. Three patients, two with liver cirrhosis, did not improve. All patients with second-line therapy experienced long-term remission. A histological remission was also seen in three patients. Clinically relevant BUD-induced side effects were recorded only in patients with liver cirrhosis ($n = 4$).

CONCLUSION: BUD is effective in remission induction in the majority of our patients with AIH. Side effects and treatment failure was mainly observed in patients with liver cirrhosis.

Key words: Budesonide; Autoimmune hepatitis; Complete remission; Adverse events

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INTRODUCTION

Autoimmune hepatitis (AIH) is a chronic inflammatory liver disease characterized by a female preponderance, hypergammaglobulinemia and circulating non-tissue specific autoantibodies. AIH usually responds to immunosuppressive therapy; and prednisone (PRD) with or without azathioprine (AZA) is the treatment of choice. This results in histological remission in approximately 65% of the cases. Several patients become intolerant to this regimen and in some it is even contraindicated[1]. Thus, new first-line and salvage therapies are needed, and several drugs are currently under investigation[2].

Budesonide (BUD), a nonhalogenated glucocorticoid, is of particular interest for the treatment of autoimmune liver diseases: it has a 15-fold greater receptor binding capacity than PRD, and a high hepatic first-pass clearance, exceeding 90% of the orally administered dose. Hence it was reported to be free of systemic effect in patients without advanced liver fibrosis[3,4]. However, as yet, data of BUD for the treatment of AIH are very limited and controversial.

In our present study we evaluated the efficacy and safety of BUD as first- and second-line therapy for AIH.

Patients

Eighteen patients (12 women and 6 men; mean age 45.4 ± 21 years, range 21 and 68 years) were selected from a collective of fifty patients with the initial diagnosis of AIH and treated with BUD between January 2002 and July 2004. Selection was based on the following criteria:

Criteria for AIH were: (1) serum alanine aminotransferase (ALT) level at least three times of the upper limit of normal (ULN); (2) serum immunoglobulin G (IgG) level at least 1.5 times of ULN; (3) positive test result(s) for non-tissue or organ-specific autoantibodies (titer ⩾ 80); (4) liver histology consistent with the diagnosis of AIH. Definite diagnosis of AIH required the presence of at least three of the four criteria or 16 points or more on aggregate score proposed by the International Autoimmune Hepatitis Group[5]. An "overlap" syndrome of primary biliary cirrhosis (PBC) and AIH was defined by the simultaneous

Table 1 Patients' characteristics

Patient (Gender/Age)	Diagnosis	IAHG Score	Autoantibodies (titer) ANA	SMA	ANCA	AMA	ALT (<0.85μmol/s.L) pre-Budesonide	post-Budesonide	ALP (<2.15μmol/s.L) pre-Budesonide	post-Budesonide	HAI pre-Budesonide	post-Budesonide	Fibrosis (Ref. 6)	Therapy (wk)	Response
First-line therapy															
B.M-L. (F/65)	AIH-PBC	19	1280	-	-	-	15.18	0.22	7.12	1.44	5	3	3	24[1]	CR
D.B. (M/26)	AIH	14	320	-	-	-	16.4	0.78	3.19	0.95	7	nd	4	36	CR
D.C. (F/49)	AIH	22	640	-	-	-	1.91	0.89	3.89	3.53	4	nd	5	24	NR/AE
H-S.M. (F/31)	AIH	17	160	80	-	-	4.43	0.6	1.62	0.9	7	nd	2	50	CR
K.M. (F/62)	AIH	14	-	-	-	-	3.45	3.59	7.86	6.29	5	nd	4	12[2]	NR
L.M. (M/60)	AIH	16	640	640	-	-	4.1	0.35	nd	nd	1	nd	0	82	CR
N.G. (F/64)	AIH-PBC	12	2560	-	-	640	10.73	0.38	7.53	1.63	11	1	0	82	CR
S.T. (M/40)	AIH	12	-	-	>80	-	8.39	0.57	7.84	1.36	1	nd	0	24	CR
T.H. (F/50)	AIH	19	640	160	-	-	3.38	6.77	1.54	1.74	9	nd	3	4[2]	NR
T.E. (F/49)	AIH-PBC	15	-	640	-	1280	2.27	0.58	4.21	1.61	5	2	5	112	CR
Second-line therapy															
B.R. (M/39)	AIH	14	160	-	-	-	4.42	0.6	2.16	0.63	6	2	1	100	CR
B.S. (F/25)	AIH	17	160	-	-	-	16.48	0.41	4.89	1.38	6	1	3	116	CR
D.H. (F/61)	AIH-PBC	14	>2560	-	-	-[3]	2.37	0.45	11.91	1.24	5	3	4	32	CR
F.C. (F/41)	AIH	17	2560	2560	-	320	10.1	0.55	4.6	0.88	6	nd	2	72	CR
G.W. (M/68)	AIH-PBC	6	-	-	-	-	28.65	0.18	4.1	0.77	3	nd	4	110	CR
K.C. (F/40)	AIH	16	640	160	-	-	10.1	0.68	4.6	1.77	4	nd	1	50	CR
S.H-D. (M/64)	AIH-PSC	11	2560	-	-	-	2.01	0.6	30.13	2.3	4	1	1	80	CR
S.U. (F/41)	AIH-PSC	14	1280	-	-	-	20	0.3	5.1	1.65	6	nd	1	124	CR

CR = complete response; NR = no response; ANA = antinuclear antibodies; SMA = antibodies to smooth muscle; AMA = antimitochondrial antibodies; ANCA = antineutrophil cytoplasmic antibodies; 3 = anti-Sp100 positive; PSC = primary sclerosing cholangitis; PBC = primary biliary cirrhosis; ALT = alanine aminotransferase; ALP = alkaline phosphatase; AE=adverse event; HAI = Hepatitis Activity Index; 1 = the therapy was continued with azathioprine; 2=discontinuation of the therapy because of no response;

association of these disorders.

Diagnostic criteria of *PBC* used in this study were: (1) a positive test for antimitochondrial antibodies (AMA) in serum (titer≥ 80); (2) serum alkaline phosphatase (ALP) level at least 2 times of ULN or gamma glutamyl transferase (GGT) activity at least 5 times of ULN; (3) a diagnostic or compatible liver histology. Two of these criteria were required for the diagnosis of PBC.

Eleven of our patients were categorized as AIH alone, and seven satisfied the revised international criteria for definite diagnosis of AIH (≥ 16 points) and five the probable diagnosis of AIH [5]. The median score of patients with AIH was 16 points (range, 11 to 22 points). Five patients presented also with features of PBC. Primary sclerosing cholangitis (PSC) was diagnosed in two patients who had elevated cholestatic and aminotransferase enzyme activities, concentric periductal sclerosis and interface hepatitis on liver biopsy, serum antinuclear antibodies and elevated IgG level (Table 1). The median score of patients with overlap syndrome was 13.6 points (range, 6 to 17 points).

Absence of biliary obstruction was assessed by ultrasound. None of the patients had a history of excessive alcohol consumption (>30 g/d), and there was no evidence of exposure to hepato- or cholangio-toxic drugs. Serological tests for hepatitis B and C virus infection were negative. Metabolic liver disease including hereditary hemochromatosis, Wilson`s disease and α1-antitrypsin deficiency was excluded by appropriate biochemical tests and histologically.

Of the eleven patients with AIH alone, ten patients were designated as type 1 AIH. Antinuclear antibodies (ANA) alone were found in four patients, ANA and anti-smooth muscle antibodies (SMA) in six. Antibodies against anti-neutrophil granulocyte were present in a single patient. One patient remained autoantibody-negative. One patient with AIH had high-titer AMA without any clinical or histological evidence of PBC. Whereas all patients with AIH-PSC overlap syndrome were ANA-positive, of the patients diagnosed as having an overlap of AIH and PBC two presented AMA in serum, and a further one had PBC-specific ANA recognizing Sp100 protein (Table 1).

Clinical presentation of our patients included acute hepatitis ($n=10$), with jaundice in two cases, and chronically elevated serum liver function test(s) ($n=8$). Advanced liver fibrosis which was defined according to Ishak *et al*[6] as stages ranging from stage 4 to stage 6 was diagnosed in four patients with AIH and in two cases with overlap syndrome. The diagnosis of liver cirrhosis was made in seven patients, three with AIH and four with an overlap syndrome - [clinical stage Child A ($n=5$) and Child B ($n=2$)]. Four patients had concurrent immunologic diseases, including one patient each with Sharp`s syndrome, multiple sclerosis, Sjögren`s syndrome, and panniculitis.

Ten patients received BUD as first-line therapy (Table 1). Five of them had presented with acute hepatitis mimicking viral hepatitis. The median of ALT activities was 13.61 μmol/s.L (range, 10.73-38.89 μmol/s.L; reference interval 0.17-0.85 μmol/s.L). Eight patients were treated with BUD as second-line therapy (Table 1). Four of them experienced an acute exacerbation of autoimmune liver disease in spite of an immunosuppressive therapy. Exacerbation of the disease was defined as > 2x elevation of ALT activity above the ULN. Five patients treated in the second-line group were intolerant to PRD or AZA or proved to be resistant to PRD (Table 2).

Liver biopsy

Liver biopsies were obtained from all patients to assess

Table 2 Patients receiving budesonide as second-line therapy

	Pre-budesonide therapy PRD (mg/d)	AZA (mg/d)	URSO (mg/d)	Diagnosis	Side effects of PRD or AZA
Acute exacerbation of the disease					
B.S.	0	100	0	AIH	no
D.H.	0	100	0	AIH-PBC	no
F.C.	0	100	0	AIH	no
G.W.	10	0	0	AIH-PBC	no
No response to PRD					
S.H-D.	40	0	1000	AIH-PSC	Headache, hypertension
Side effects of the drugs					
K.C.	10	100	0	AIH	Weight gain of 20 kg
B.R.	5	0	0	AIH	Osteoporosis, myopathy
S.U.	10	50	750	AIH-PSC	Pancreatitis

inflammatory activity and stage of fibrosis before the initiation of therapy. All biopsy specimens were fixed immediately in 10% neutralized formalin and subsequently embedded in paraffin. Deparaffinized serial sections were stained using a hematoxylin and eosin stain (H&E), periodic-acid Schiff reagent with and without diastase pretreatment, Masson`s trichrome stain, reticulin stain, and iron stain. At least eight sections (three sections stained with H&E and one section for each special stain) were evaluated per biopsy specimen. Grading and staging of hepatitis was performed according to the modified Histological Activity Index[6]. Follow-up tissue examinations were done only in patients who had a complete clinical and laboratory resolution and consented for a follow-up liver biopsy. Our protocol was approved by the local Ethics Committee of the Otto-von-Guericke University of Magdeburg. Informed consent was obtained from all patients.

Treatment

BUD (Budenofalk®), 3 mg thrice daily, was administered to each participant with an intention to treat for at least six months. Patients who met the criteria for a complete biochemical response and were free of BUD-related side effects were treated for an indefinite period of time. Drug intolerance, exacerbation of disease or treatment failure justified discontinuation of the medication any time. Therapy was discontinued beyond 6 mo in three patients (wk 4, wk 12 and wk 24) because of treatment failure or deterioration of disease. Patients who experienced a complete biochemical response and had also side effects of BUD or were suspected to be at risk for drug-induced side effects were given AZA in a dose of 1-1.5 mg/kg body weight. Significant cholestasis or pruritus was treated with Ursodeoxycholic Acid (Ursofalk®) in a dose of 10-15 mg/kg body weight in three divided doses.

Patients were monitored at 1-mo intervals in the first three mo of therapy and at 3-mo intervals afterwards by determining serum ALT, ALP, bilirubin, and IgG levels. Treatment outcomes included clinical and biochemical remission, treatment failure, and drug toxicity or side effects. Remission was defined as absence of clinical symptoms, normal serum ALT, ALP, and IgG levels. In patients with an overlap syndrome, remission included also the near normalisation of GGT ($\leqslant$2x ULN). Treatment failure connoted clinical and biochemical deterioration. The development of intolerable cosmetic, biochemical, and/or somatic changes during treatment indicated drug toxicity (= intolerable side effects).

Statistical analysis

Descriptive analyses were used to characterize the study population.

RESULTS

The serum ALT level improved significantly, and completely normalised in fifteen patients (overall response rate 83%). Before initiation of the therapy the median ALT level was 12.44 μmol/s.L (range; 1.48-28.65 μmol/s.L, reference interval 0.17-0.85). At the end of our evaluation, the median of ALT activity was 0.77 μmol/s.L (range 0.18-5.79 μmol/s.L). The median time to complete normalisation of transaminase or cholestatic liver enzyme activities was about three months. The deterioration of ALT activity was noted in a woman (TH) with advanced liver fibrosis and acute hepatitis who did not respond to BUD and had a corticosteroid-dependent AIH. Under PRD (40 mg/d) she entered a complete biochemical remission, but after reducing the doses of PRD under 20 mg she experienced again an acute exacerbation of the disease. She is now in remission receiving 150 mg AZA and 5 mg PRD. BUD therapy was ineffective in two women with liver cirrhosis after 12 and 24 wk. One of them (KM) did respond to PRD and was put on AZA after six months therapy. The other one (DC) did not respond to corticosteroid and is awaiting liver transplantation. A young man (ST) with a history of pathological elevated ALT activities, fatigue and malaise lasting for five years whose liver biopsy revealed no hepatitic changes responded rapidly to BUD leading to complete normalisation of serum liver tests and disappearance of clinical symptoms.

Levels of serum bilirubin with 5.95 mg/dL (reference interval 0.1-1.1 mg/dL), ALP with 6.62 μmol/s.L (reference interval 0.8-2.15 μmol/s.L) and IgG with 2068 mg/dL (reference interval 900-1600 mg/dL) also improved in all patients with initially pathological serum levels. The median of all these parameters (serum total bilirubin: 0.81 mg/dL, range 0.33 to 2.47; ALP: 1.41 μmol/s.L, range 0.63-2.40; IgG: 1385 mg/dL, range 928-3140) was normal at the end

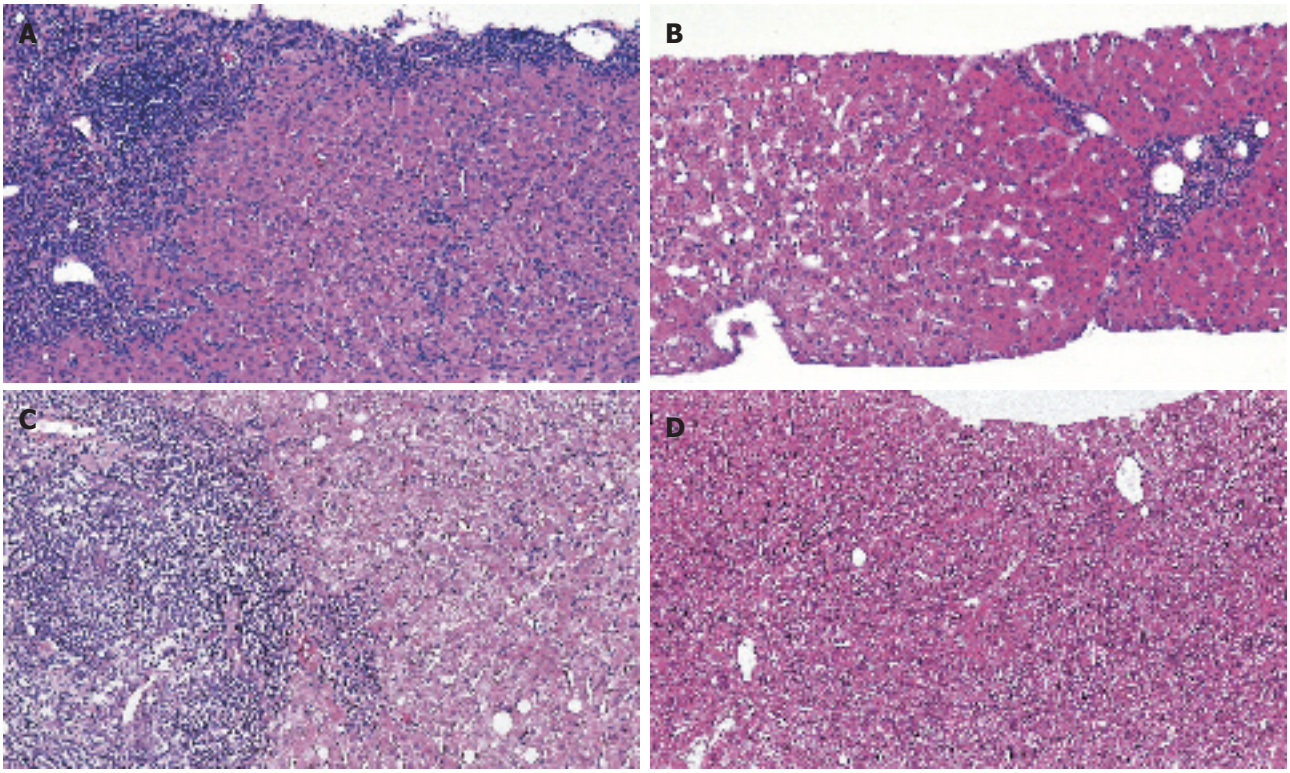

Figure 1 Histology of biopsy specimens obtained from a patient with AIH (**A**, **B**) and from a patient with AIH and PBC (overlap; **C**, **D**). Before treatment was started (**A**, **C**), liver biopsies showed a severe hepatitis with interface and lobular activity. Following administration of budesonide, follow-up biopsies were obtained 10 mo later from each patient and showed a significant improvement of disease activity (**B**, **D**).

of our evaluation.

Side effects of BUD therapy were noted in six patients (33%). However, clinically relevant side effects including abdominal pain ($n = 1$), weight gain of > 3 kg ($n = 3$), acne ($n = 2$) and hair lost ($n = 1$) and cushingoid appearance ($n = 3$) were reported only in patients with liver cirrhosis. Discontinuation of the therapy was warranted only in one woman (DC) who did not respond to BUD and developed intolerable gastrointestinal symptoms. No one of the patients developed impaired glucose tolerance or overt diabetes mellitus. A man with insulin-dependent diabetes mellitus showed no deterioration of his diabetes under BUD therapy. Changes in bone mineral density were not determined during the follow-up.

Histology

Twenty-six liver biopsies were obtained from 18 patients, of which eight were follow-up biopsies from seven patients. Histopathological changes compatible with AIH were found in eleven patients (Figure 1). Biopsy specimens from five patients showed histological signs of an overlap with either PBC or PSC (Figure 1). In a single biopsy from one patient the histological changes were very mild, with few portal tracts showing only a scattered inflammatory infiltrate, which were then categorized as unspecific changes, inconclusive for AIH. Follow-up biopsies obtained from seven patients showed a remarkable improvement of disease activity in all patients. Using the modified Ishak`s scoring system[6], disease activity was assessed with regard to periportal or periseptal interface hepatitis (maximum possible score 4), confluent necrosis (maximum possible score 6), lobular inflammation (maximum possible score 4), and portal inflammation (maximum possible score 4). By adding up the individual categories, a mean total score of 5.17 (range, 1-11) was found in the pretreatment biopsies and of 2.33 (range, 1-5) in the post-treatment biopsies (Figure 1). Staging of AIH (maximum possible score 6 - cirrhosis, probable or definite) ranged between 0 and 5. The absence of portal fibrosis (stage 0) was found in three patients, and advanced fibrosis (stage 5) was found in two. The mean values for staging showed no difference between specimens obtained before (mean value 2.39; range, 0 - 5) or after (mean value 2.10; range, 0 - 4) treatment. A mild steatosis of 20% or less was found only in four biopsy specimens of four patients. Steatosis was not found in any follow-up biopsy specimen.

DISCUSSION

BUD induced clinical and biochemical remission in the majority of our study patients with AIH including those with an overlapping PBC or PSC. Complete remission or significant improvement of liver histology was confirmed in the majority of our patients with a follow-up liver biopsy. In contrast, only three patients, two with liver cirrhosis and one with severe, corticosteroid-dependent disease, did not benefit from the therapy with BUD. 33% of patients experienced side effect(s), but only patients with advanced liver fibrosis or cirrhosis had severe adverse events as reported previously in patients with PBC because of the reduced hepatic metabolism of the drug[7]. Discontinuation of the drug was warranted only in one

patient with liver cirrhosis because of drug intolerance and non-response. These findings support the value of BUD in the management of treatment-naive patients with AIH and also as salvage therapy in individuals who are intolerant to PRD or AZA or experience an acute exacerbation of the disease in spite of an immunosuppressive therapy (Table 2).

Published data with BUD in patients with AIH are limited and controversial. Currently, two papers presenting a total of 23 patients with AIH have been published in the English literature[8,9]. The first report on BUD in AIH came from Sweden. This study included 13 patients with AIH who were treated with BUD as second-line therapy, initially with a daily dose of 6 to 8 mg, after they had experienced a relapse after discontinuation of the first-line therapy with PRD and AZA. Two patients were shown to have liver cirrhosis at presentation. Using BUD, ALT level normalized within 12 wk of treatment in the majority of the study patients[8]. Further small studies including a total of 36 patients with treatment-naive AIH published as abstract from Europe suggested that BUD may induce complete biochemical remission in about two-thirds of patients after one year of therapy who received the drug as first-line therapy. The frequency of the reported drug-related side effects ranged from 29% to 55%.

In contrast, in the second published report, experiences on 10 patients receiving BUD in AIH from a single center in the U.S., suggested that BUD as second-line therapy is able to induce remission only in a minority of patients with severe steroid-dependent AIH and is inferior to PRD[9]. In this report, all patients receiving BUD experienced at least one side effect. These results may be influenced by patient selection, as the presence of fibrosis increases the risk of side effects and advanced fibrosis and steroid-dependent disease are frequently associated with treatment failure and adverse events to drugs used in AIH.

Our data indicate that BUD is able to induce not only biochemical but also histological remission in patients with treatment-naive AIH. Furthermore, BUD can also be used as salvage therapy in those cases where PRD and AZA fail or patients become intolerant to these drugs. Patients presenting with severe acute hepatitis or having an overlap syndrome with PBC or PSC experience the same frequency of remission. As advanced liver fibrosis or cirrhosis is frequently associated with side effects and treatment failure, initiation of this therapy in histologically proven liver cirrhosis may deserve further considerations.

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BASIC RESEARCH

Altered blood-brain barrier permeability in rats with prehepatic portal hypertension turns to normal when portal pressure is lowered

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Abstract

AIM: To study the blood-brain barrier integrity in prehepatic portal hypertensive rats induced by partial portal vein ligation, at 14 and 40 d after ligation when portal pressure is spontaneously normalized.

METHODS: Adult male Wistar rats were divided into four groups: Group I: $Sham_{14d}$, sham operated; Group II: PH_{14d}, portal vein stenosis; (both groups were used 14 days after surgery); Group III: $Sham_{40d}$, Sham operated and Group IV: PH_{40d} Portal vein stenosis (Groups II and IV used 40 d after surgery). Plasma ammonia, plasma and cerebrospinal fluid protein and liver enzymes concentrations were determined. Trypan and Evans blue dyes, systemically injected, were investigated in hippocampus to study blood-brain barrier integrity. Portal pressure was periodically recorded.

RESULTS: Forty days after stricture, portal pressure was normalized, plasma ammonia was moderately high, and both dyes were absent in central nervous system parenchyma. All other parameters were reestablished. When portal pressure was normalized and ammonia level was lowered, but not normal, the altered integrity of blood-brain barrier becomes reestablished.

CONCLUSION: The impairment of blood-brain barrier and subsequent normalization could be a mechanism involved in hepatic encephalopathy reversibility. Hemodynamic changes and ammonia could trigger blood-brain barrier alterations and its reestablishment.

Key words: Blood-brain barrier; Rats; Prehepatic portal hypertension

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INTRODUCTION

Portal hypertension (PH) and hepatic encephalopathy are major complications in human cirrhosis or portal vein thrombosis. Hyperdynamic splanchnic circulation and hyperaemia associated to systemic circulatory alterations are usually present[1,2].

Our laboratory found in the last years, the presence of derangement in the CNS in a prehepatic portal hypertensive rat model. Increases in the uptake and release of norepinephrine in diencephalic and telencephalic discrete brain regions and significant increments in tyrosine hydroxylase activity in these nuclei were described in previous publications[3-5].

Finally, morphologic alterations in astrocytes (AS) and endothelial cells (EC) located in the hippocampal region (CA1 and CA4) were also found in this experimental model[6].

The morphological cell components of BBB are basically AS, EC and pericytes. Two of this, AS processes and EC, were found altered in this model and in acute acetaminophen intoxication fourteen days after portal vein ligation[7,16,17].

Beside this, forty days after portal vein stricture, portal pressure spontaneously turns to normality. The aim of the present experiment was to investigate if normalization of PP was accompanied by the reestablishment of BBB properties and relate these findings with plasma ammonia concentration.

MATERIALS AND METHODS

Animals and surgical procedures

Male Wistar rats, with an average weight of 240 g were utilized and animal welfare was in accordance with the guidelines of the Faculty of Pharmacy and Biochemistry and approved by the Ethical Committee of the Faculty accordingly with Helsinki's Declaration. The animals were placed in individual cages, with free access to food (standard laboratory rat chow) and water, and 12 h light cycle: 8 a.m. - 8 P.M. Special care for perfect air renewal was taken.

Four groups of rats were used: $Sham_{14d}$- sham operated rats; PH_{14d} - Portal vein stenosis; both groups were used 14 d post-surgery. $Sham_{40d}$- sham operated; PH_{40d}- Portal vein stenosis; used 40 d after surgery.

Each group had separated subgroups for portal pressure determination, serum and CSF fluid determinations, Trypan blue and Evans blue injection respectively.

Portal hypertension was obtained by calibrated stenosis of the portal vein (PH) according to Chojkier *et al*[1]. Rats were lightly anesthetized with ether and then a midline abdominal incision was made. The portal vein was located and isolated from the surrounding tissues. A ligature of 3.0 silk sutures was placed around the vein, and snugly tied to a 20-gauge blunt-end needle placed along side the portal vein. The needle was subsequently removed to yield a calibrated stenosis of the portal vein. Operations were performed at 2 PM to obey circadian rhythm. Fourteen days after portal vein ligation, animals exhibit an increase in portal pressure. After 20 d portal pressure begins to fall down to normal values approximately after 30 d.

Sham operated rats underwent the same experimental procedure except that the portal vein was isolated but not stenosed. Animals were placed in individual cages and allowed to recover from surgery. Portal pressure was measured the 14^{th} d and the 40^{th} d after surgery in the corresponding group, by puncture of the splenic pulp. Animals were sacrificed by decapitation between 2 and 4 PM to avoid circadian variations.

Experimental procedures

Portal pressure measurement Fourteen and forty days after the corresponding operation, the rats were anaesthetized with sodium pentobarbital (40 mg/kg), intraperitoneally (ip). Portal pressure was measured through a needle placed in the splenic pulp, and maintained in place by cyanoacrylate gel. The needle was cannulated to a polyethylene catheter (50) filled with a heparinized saline solution (25 U/mL) and connected to a Statham Gould P23ID pressure transducer (Statham, Hato Rey, Puerto Rico) coupled to a Grass 79D polygraph (Grass Instruments, Quincy, MA).

Biochemical determinations Plasma was obtained from blood drained from aorta artery puncture. Under anesthesia, samples of cerebrospinal fluid (CSF) were obtained by cisternal puncture for pro-tein determination according to Bradford[8]. Plasma ammonia concentrations were determined using Ammoniac Enzyimatic UV Kits (Biomerieux, France). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined in plasma using a commercial standardized and optimized Boehringer-Mannheim (Germany) kits.

Brain water content Cortical brain zones were utilized for the determination of water content in order to quantify possible brain edema. Gravimetric method was employed according to Marmorou *et al*[9].

Trypan blue transcardial perfusion Rats were perfused with Trypan blue (TB, Sigma Chemical Co. St. Louis MO. USA.) solution and then fixed with paraformaldehyde. TB solution (0.5%) was prepared by dissolving 1 g of TB in 200 mL of PBS, with gentle heat. The solution was allowed to cool at room temperature, added to the filtrate and placed on ice for immediate use. The temperature of TB solution was 10-12 ° C at the time of perfusion. Rats were anaesthetized with ethyl urethane (1mg/kg) and perfused transcardially with 200 ml of TB solution; followed by 300 mL of ice-cooled paraformaldehyde (2% in PBS). The flow rate of perfusate was maintained at 25 mL/min. Brains were dissected and post-fixed overnight in 30% sucrose for 2 d. Subsequently, the brains were placed in powdered dry ice and stored at -80 °C until processed for microscopic studies. Slices of brain tissue were obtained with cryostat in section of 300 microns according to Paxinos and Watson[10]. Hippocampal slices were evaluated under light microscope and expressed as positive (+) or negative (-) for TB staining. Medial eminence and choroids plexus staining were used as control of TB adequate perfusion. This method was adapted from Ikeda *et al*[11].

Evans blue test Evans blue (EB, Sigma Chemical Co. St. Louis MO. USA.) dye (25% in 0.9% NaCl solution) was intravenously injected at dose of 25 mg/kg in rats under ether anesthesia. One hour after the injection, animals were sacrificed by decapitation. Brains were weighed, clipped and individually placed within formamide p.a. (2mL/brain). These tubes were kept at 37°C for 48 h. The content of dye extracted from each brain was determined by spectrophotometer (Photometer 4010, Boehringer) at 620 nm and compared to standard graph created through the recording of optical densities from serial dilutions of EB in 0.9% NaCl solution[11, 12].

Statistical analysis

Results were expressed as mean ± SE. For multiple comparisons ANOVA, followed by Newman-Keuls or Student Newman Keuls tests were used. A $P<0.05$ was considered significant.

RESULTS

Portal pressure

The portal pressure (Figure 1) in Group Sh_{14d} was 7.6 ± 1.90 ($n=6$), in Group PH_{14d} 14±1.80 ($n=6$), in Group PH_{40d} was 2.52 ± 1.35 ($n=3$), Sh_{40d} 7.7 ± 1.98 ($n=5$). Differences between the following groups were significant: Sh_{14d} *vs* PH_{14d} ($P<0.001$); PH_{14d} vs. PH_{40d}, ($P<0.001$); Sh14d vs PH_{40d} ($P<0.01$). When sham groups (Sh_{14d} *vs* Sh_{40d}) were compared differences were not significant.

Plasma protein concentration.

Group PH_{14d} presented 87.40 ± 8.00 *mg/mL* ($n=4$); Group PH_{40d}, 107.60 ± 2.10 *mg/mL* ($n=5$); Group Sh_{14d}, 108.80±7.60

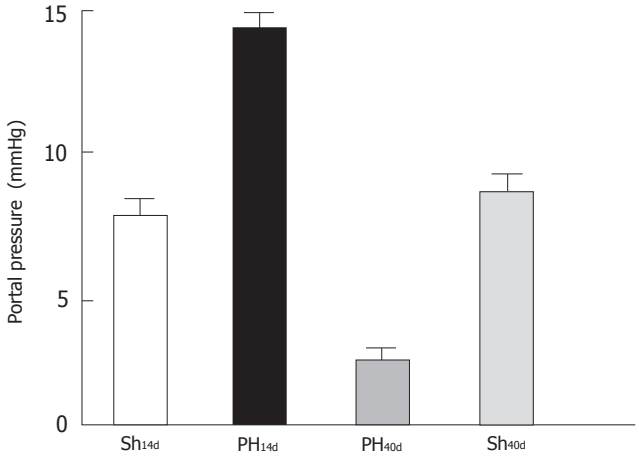

Figure 1 Portal pressure (mmHg). Group Sh14d *vs* Group PH_{14d} ($P<0.001$). Group PH14d *vs* Group PH_{40d} ($P<0.001$). Group Sh14d vs PH40d $P<0.01$. Sh14d *vs* Sh40d (n.s).

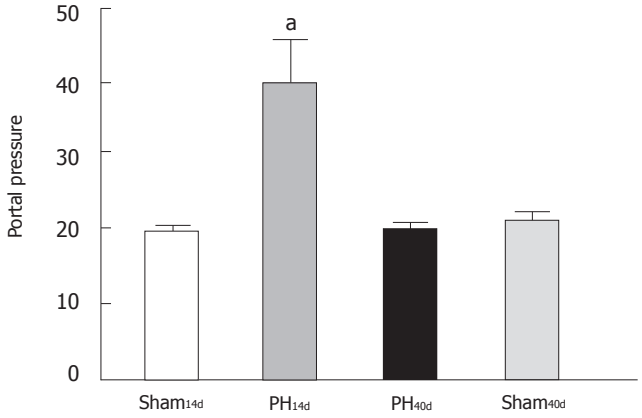

Figure 2 Protein content in cerebrospinal fluid. Hatched column corresponds to PH_{14d} ham shows an increased protein filtration through the BBB. This increment is not present, in black column,corresponding to PH_{40d} Results are expressed as mean ± SE.

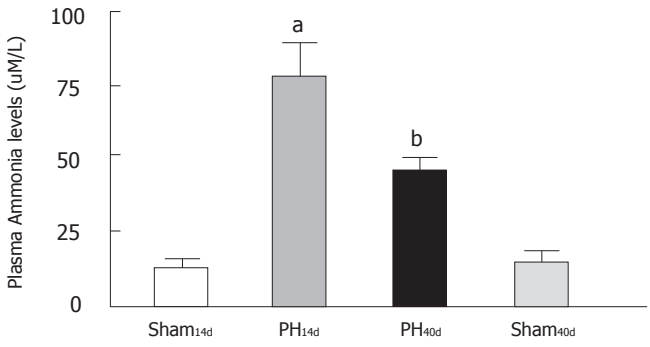

Figure 3 Plasma ammonia levels.significant elevation of plasma ammonia content in PH14d rats correspond to hatchet column. After 40 days of portal vein ligation, the portal pressure normalized, but plasma ammonia is still high (black column). $^{b}P<0.001$ and $^{a}P<0.05$, compared to Sham column.

mg/mL ($n=4$) and Group Sh_{40d}, 113.00 ± 2.10 *mg/mL* ($n=4$) (PH_{14d} *vs.* $Sham_{40d}$, $P<0.05$). Results showed a plasma protein concentration decrease tendency in portal hypertensive animals of day 14, which was only significant when compared with sham operated group after 40 d. This tendency was opposite to that observed in CSF.

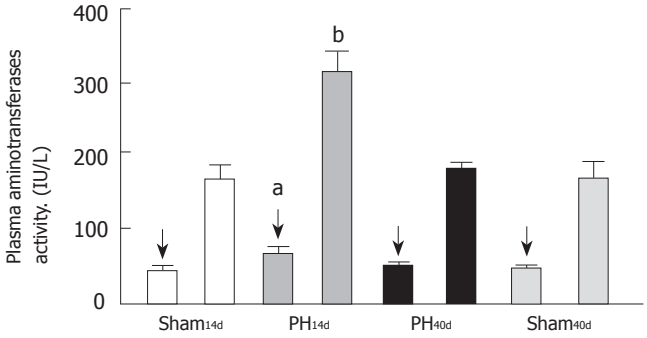

Figure 4 Plasma aminotransferases. The first column of each group corresponds to ALT activity (arrows) and the 2nd to AST activity. Both enzymes activities appear elevated in PH_{14d} (hatched columns) when compared to $Sham_{14d}$ ($^{b}P<0.001$; $^{a}P<0.05$).

Protein content in cerebrospinal fluid

Protein content in cerebrospinal fluid was 40.60±6.80 *μg/mL* in Group PH_{14d} ($n=4$), 20.62±0.69 *μg/mL* ($n=5$) in Group PH_{40d}, 19.52±0.80 *μg/mL* ($n=4$) in Group Sh_{14d}, and 21.70 ±2.27 *μg/mL* ($n=4$) in Group Sh_{40d}. Statistical analysis indicated that there were significant differences between $Sham_{14d}$ and Group PH_{14d} ($P<0.05$) and between Group PH_{14d} and Group Sh_{40d} ($P<0.01$) (Figure 1). According to these results PH_{14d} rats had increased protein permeability through the BBB. This increment was reverted when CSF protein was measured 40 days after portal vein stricture (Figure 2). These changes were not related to the above-cited variations in plasma protein values.

Ammonia plasma levels (Figure 3)

Portal hypertensive rats showed that ammonia plasma level was 79.00 ± 15.00 *μm/L* in Group PH_{14d}, ($n=8$), 44.76± 4.51*μm/L* in Group PH_{40d} ($n=5$), 12.99 ± 3.94 *μm/L* in Group Sh_{14d} ($n=11$) and 11.00 ± 4.20*μm/L* in Group Sh_{40d} ($n=9$) (Figure 2). This graphic shows a clear increase of plasma ammonia in PH_{14}, and a decrease in plasma ammonia levels when portal pressure is normalized. Levels in Group PH_{40d} are still higher than in Group Sh_{14d}, with statistical significance between groups: Sh_{14} vs. PH_{14} ($P<0.001$); Sh_{40} *vs* PH_{40} ($P<0.01$) and Sh_{14} *vs* Sh_{40} (NS). Group I_{14d} and Group IV_{40d} showed a very similar and low plasma concentration. When comparing groups PH_{14} with PH_{40}, P value is <0.01, thus indicating a better ammonia metabolism.

Plasma transferases activity (Figure 4)

Plasma ALT activity was 63.00 ± 9.00 *IU/L* in Group PH_{14d} ($n=6$), 44.00±4.50 *IU/L* in Group PH_{40d} ($n=7$), 39.00±4.00 *IU/L* in Group Sh_{14d} ($n=6$) and 39.00±3.50 *IU/L* in Group Sh_{40d} ($n=6$). Statistical analysis shows an increase of PH_{14d} levels when compared to groups Sh_{14d} and Sh_{40d} ($P<0.05$).

The activity of plasma AST was 316.0 ± 23.0 *IU/L* in Group PH_{14d} ($n=6$), 167.0±14.0 *IU/L* in Group PH_{40d} ($n=7$), 155.0 ± 25.0 *IU/L* in Group Sh_{14d} ($n=6$) and 155.0 ± 32.0 *IU/L* in Group Sh_{40d} ($n=6$). Statistical calculations outline

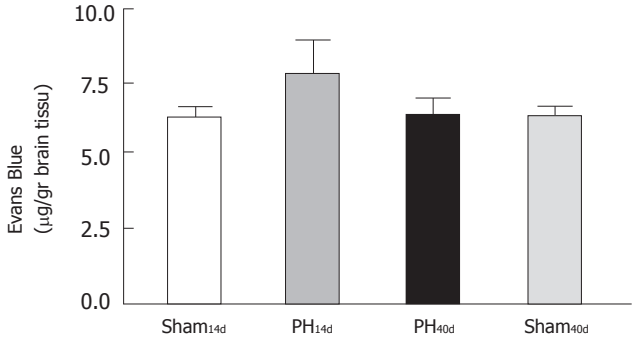

Figure 5 Evans blue test.The systemic injection of Evans blue showed a statistically significant increase of the dye presence in the hippocampal region in PH_{14d} presence in the indicate $p < 0.01$ when compared with the other 3 groups.

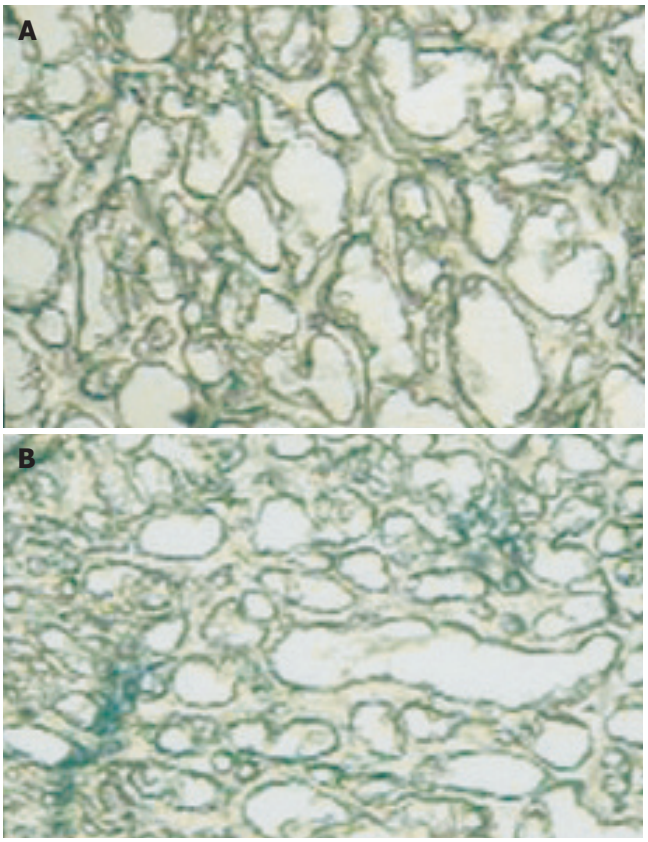

Figure 6 **A:** In the rest of the groups, including that were portal pressure normalized 40 days after portal vein stricture and Sham groups, the image is negative for Trypan blue dye (100 x); **B**: Light microscopy showed a marked Trypan blue dye diffusion in brain hippocampal of PH rats of 14 d (100 x).

an increment of the enzyme in PH_{14d} group, when compared with groups of Sh_{14d} ($P < 0.001$), PH_{40d} ($P < 0.01$), and Sh_{40d} ($P < 0.001$). The elevation of their activity usually indicates some level of rat liver alteration, which is observed in this model of prehepatic portal hypertension.

Water content in cortical brain areas

Water content in cortical brain areas (H_2O/gr %) was 79.21 ±0.17 in Group PH_{14d} ($n = 6$), 79.21 ± 0.17 in Group PH_{40d} ($n = 7$), 78.95 ± 0.18 in Group Sh_{14d} ($n = 6$) and 77.26 ± 0.29 in Group Sh_{40d} ($n = 6$). No significant differences were found between the groups.

Trypan blue and Evans blue tests

Trypan blue test showed the presence of the dye limited to the vascular area, not involving other areas in Groups Sh_{14d} and Sh_{40d}. Meanwhile, EB appeared positively in vascular area and diffusely in perivascular area in Group PH_{14d}. In Group PH_{40d} both were detected in vascular area and very slightly in perivascular areas. These findings demonstrate the existence of an increased BBB permeability to the dye in PH_{14d} rats, which turns to normal when pressure levels are normalized 40 d later (Figures 6 A and B).

The systemic injection of EB confirmed quantitatively the results found with TB. The results, expressed in μg/g of brain tissue, were 6.170±0.380 in Group Sh_{14d} ($n = 12$), 8.680 ±0.700 in Group PH_{14d} ($n = 9$), 6.300 ± 0.590 in Group PH_{40d} ($n = 6$) and 6.250 ± 0.450 in Group Sh_{40d} ($n = 6$). Statistical comparison showed that there was significant difference between Group PH_{14d} and Group PH_{40d} ($P < 0.01$) and between Group Sh_{14d} and Group PH_{14d} ($P < 0.01$). There was no significant difference between Group Sh_{14d} and Group PH_{40d}; Group Sh_{40d} and Group Sh_{14d} shared almost identical values (Figure 5).

DISCUSSION

The BBB is formed by complex tight junctions of the brain capillary endothelial cells in close relation with the astrocyte processes. These morphological and functional structures make possible a selective transport across BBB and a non-selective brain distribution of drugs[13]. Three cellular components characterize BBB: Endothelial cells, astrocyte end feet and pericyte[14]. Tight junctions present between cerebral endothelial cells form a diffusion barrier that restricts the influx of most blood-borne substances from entering the central nervous system (CNS). Dysfunction of BBB complicates a number of neurological diseases[15].

Alterations in astrocytes architecture as well as changes in its metabolism due to ammonia detoxification, osmotic balance and cell homeostasis are a well-known feature of hepatic or porto-systemic encephalopathy[16,17].

In previous publications, we described the alteration of BBB in this model of prehepatic portal hypertension, with or without acute acetaminophen intoxication[18,19]. In this paper, the addition of Evans blue technique confirms previous results and adds a quantitative measurement to morphologic findings.

No difference in brain water content was found in this model of partial portal vein ligation, opposed to changes in brain water content found in rats with acetaminophen intoxication as previously described[19]. The presence, however, of EEG modifications[18] and rota rod test modifications[19], led us to classify it as a probable sub-clinic encephalopathy. The lack of a rise in brain water content observed in this model is probably due to the fact that the chronic nature of the portal pressure rise and porto-systemic circulation probably allows some kind of compensation mechanism[16].

Alterations of BBB permeability have been studied in different models of cirrhosis and porto-caval shunting, observing different kind of BBB changes like for some aminoacids but not others[20-22], Evans blue[23] , but not for 14C mannitol or 3H-glutamate[22].

Our results show an altered BBB permeability, qualitative and quantitative, in portal vein ligated rats 14 d after surgery (PH_{14d}) and posterior normalization after 40 d of portal vein stricture (PH_{40d}). This alteration is shown by significant differences observed in protein concentration in CSF and concentration of Evans blue dye in CNS and confirmed by microscopic observation of Trypan blue diffusion. It is interesting to stress out that in PH_{14d} group, protein concentration in CSF is elevated despite a tendency to fall in plasma protein when compared to control (Sh_{14d}). There were no differences in plasma or CSF protein content between Sh_{14d} and PH_{40d} or Sh_{40d}, nor in the same groups when comparing Evans Blue concentration.

In this study, a decrease in portal pressure accompanies normalization of BBB permeability expressed as CSF protein content and diffusion of Evans blue and Trypan blue dyes. Despite these results, ammonia remains moderately elevated after 40 d, indicating the presence of some amount of portal systemic shunting and that some levels of increased ammonia may not interfere with BBB normal function. This presence of portal systemic shunting could also explain the fact that portal pressure is lower in PH_{40d}, when compared to the sham operated group. In spleen-portographies done in the past, we observed in rats of more than 30 d of portal ligation, some cases of porto-portal collateral circulation shunted the silk ligature (personal observation, unpublished).

The fact that BBB altered permeability is resolved but plasma ammonia concentration is not completely normal remains unexplained. Further studies should be conducted to clarify the responsibility of vasoactive substances released in portal hypertension in BBB alterations in this rat model.

The elevated AST and ALT may indicate some kind of liver alteration present in this model with an otherwise almost normal liver function. Astrocyte changes and ammonia detoxification in the brain are probably some of the mechanisms implicated in BBB alteration and posterior normalization. Other factors have been implicated like cytokines and carbon monoxide[23-25].

The normalization of BBB permeability could be a necessary part of hepatic encephalopathy reversibility. This could be a simple model for further studies in BBB function and reversibility of permeability mechanisms useful for a faster recovery of hepatic encephalopathy in patients.

We confirm the spontaneous tendency of this model to normalize portal pressure. No changes in cortical brain water content were found. Following the spontaneous decrease of portal pressure at 40 d, the BBB altered permeability reestablished its properties. Despite BBB permeability normalization, ammonia concentration remained moderately high, but significantly lower than the portal hypertension group 14 d after surgery. This could be a useful model to study BBB alterations and posterior normalization in portal hypertension with a low grade of encephalopathy.

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BASIC RESEARCH

Alanyl-glutamine dipeptide inhibits hepatic ischemia-reperfusion injury in rats

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Abstract

AIM: To investigate the protective effect and mechanism of alanyl-glutamine dipeptide (Ala-Gln) against hepatic ischemia-reperfusion injury in rats.

METHODS: Rats were divided into group C as normal control Group (n=16) and group G as alanyl-glutamine pretreatment (n=16). Rats were intravenously infused with 0.9% saline solution in group C and Ala-Gln -enriched (2% glutamine) 0.9% saline solution in group G via central venous catheter for three days. Then all rats underwent hepatic warm ischemia for 30 min followed by different periods of reperfusion. Changes in biochemical parameters, the content of glutathione (GSH) and the activity of superoxide dismutase (SOD) in liver tissue, Bcl-2 and Bax protein expression and morphological changes of liver tissue were compared between both groups.

RESULTS: One hour after reperfusion, the levels of liver enzymes in group G were significantly lower than those in group C (P<0.05). Twenty-four hours after reperfusion, the levels of liver enzymes in both groups were markedly recovered and the levels of liver enzyme in group G were also significantly lower than those in group C (P<0.01). One and 24 h after reperfusion, GSH content in group G was significantly higher than that in group C (P <0.05). There was no statistical difference in activities of SOD between the two groups. One and 24 h after reperfusion, the positive expression rate of Bcl-2 protein was higher in group G than in group C (P<0.05) and the positive expression rate of Bax protein was lower in group G than in group C (P < 0.05). Histological and ultrastructural changes of liver tissue were inhibited in group C compared to group G.

CONCLUSION: Our results suggest that Ala-Gln pretreatment provides the rat liver with significant tolerance to warm ischemia-reperfusion injury, which may be mediated partially by enhancing GSH content and regulating the expression of Bcl-2 and Bax proteins in the liver tissue.

Key words: Alanyl-glutamine dipeptide; Hepatic ischemia-reperfusion injury; Glutathione; Bcl-2; Bax

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INTRODUCTION

Temporary clamping of the portal triad, ie, inflow occlusion by the Pringle's maneuver, is a common strategy to minimize bleeding during hepatic resection. Unfortunately, this method resulted in hepatic ischemia-reperfusion (I/R) injury and may cause postoperative functional disorder of the liver. Development of new pharmaceutical strategies to attenuate hepatic I/R injury is important for achieving a better clinical outcome. Hepatic I/R injury factors usually include oxygen radical species, intracellular calcium overloading, inflammatory cytokines and infiltration of neutrophils[1]. However, its mechanism has not been fully elucidated.

Glutamine (Gln) is a conditionally essential nutrient during serious injury or illness, and plays a vital role in the metabolism of tissue[2-4]. Recently, Gln has been demonstrated to protect against I/R injury of gut, heart and skeletal muscle and its possible mechanism is partially related to preservation of the content of glutathione (GSH)[5-7].

Recently, apoptosis has been indicated as an important mode of cell death during hepatic I/R injury[8]. Apoptosis is governed by a number of regulating genes, such as *bcl-2* and *bax* and the ratio of *bcl-2* to *bax* determines survival

or death following an apoptotic stimulus[9]. Animal studies demonstrate that GSH depletion is associated with decreased *bcl-2* gene expression and increased apoptosis in cholangiocytes[10]. Overexpression of *bcl-2* gene by transferring this gene into hepatocytes with adenovirus is resistant to hepatic I/R injury[11].

However, there are few studies dealing with the effect of Gln on hepatic I/R injury. Consequently, we designed this experiment to study the effect of Gln on hepatic I/R injury in rats and its possible mechanisms. Dipeptide L-alanyl-L-glutamine is soluble in water and remains stable after heat sterilization[12]. Peptidase activity in all body compartments shows rapid hydrolysis and release of appropriate amino acids after intravenous infusion. Therefore, alanyl-glutamine dipeptide (Ala-Gln) was used in this experiment.

MATERIALS AND METHODS

Experimental animals

Male Wistar rats weighing 250-300 g (supplied by the Experimental Animal Center of Shengjing Hospital) were housed in a standard animal laboratory with free activity and access to water and chow. They were kept under constant environment conditions in a 12 h light-dark cycle. All operations were performed under clean conditions.

Intravenous drugs and other relevant chemicals

Dipeptiven (Ala-Gln solution) was from Sino-Sweden and Fresenius Pharmaceutical Corp. LTD. GSH and superoxide dismutase (SOD) detection kits were purchased from Nanjing Jiancheng Bioengineering Institute, China. Mouse anti-rat Bcl-2 and Bax monoclonal antibodies were provided by Beijing Zhongshan Biotechnology Co.Ltd, China.

Animal model and grouping

Under urethane anesthesia (intraperitoneal injection, 10 mg/kg), a silastic catheter was inserted through the right external jugular vein into the superior vena cava, tunneled subcutaneously and brought out through the skin of mid-scapular region. A flexible spring guarded the catheter, and then hooked up to an infusion pump. All rats were maintained in individual metabolic cages. After catheterization, all rats received intravenously 0.9% saline solution at a constant rate by a pump (2 mL/h) with free activity and access to water and chow for 3 d. Then, all rats were randomly divided into Group C infused with 0.9% saline solution (n=16) and group G infused with Ala-Gln-enriched (2% glutamine) 0.9% saline solution (n=16). After 3 d, under urethane anesthesia (intraperitoneal injection, 10 mg/kg) a midline laparotomy was performed and all structures in the portal triad to the liver were clamped for 30 min followed by different periods of reperfusion. Liver tissues and blood were sampled at 1 and 24 h after reperfusion for liver function tests, antioxidant enzyme measurement, Bcl-2 and Bax protein expression and morphological examination.

Liver function tests

Blood samples collected at 1 and 24 h after reperfusion were used to measure the alanine aminotransferase (ALT) and lactic dehydrogenase (LDH) levels with a standard biochemistry automatic analyzer.

Antioxidant enzyme measurement

The content of GSH and activity of SOD in rat liver tissue were measured following instructions of the commercial kit.

Immunohistochemistry

S-P immunohistochemistry was performed to detect Bcl-2 and Bax proteins following instructions of the commercial kit. Only cytoplasmic staining was evaluated and nuclear reaction was interpreted to be nonspecific staining. No positive cells was negative expression. The positive cells were considered weak positive expression when their percentage was lower and scored as (+), moderate positive expression when their percentage was between 1/3 and 2/3 and scored as (++), and strong positive expression when their percentage was higher than 2/3 and scored as (+++).

Histological and electron microscopic analysis

Liver specimens were taken at 1 and 24 h after reperfusion for hematoxylin-eosin staining and light microscopy. The specimens were immediately cut for electron microscopy. The sections were examined under a Philips CM10 electron microscope.

Statistical analysis

The data were presented as mean ± SD. Student's t test was performed for the biochemical parameters and antioxidant enzyme levels. $P < 0.05$ was considered statistically significant. Software SPSS11.0 was used in all statistical analyses.

RESULTS

Hepatic serum enzyme concentration

One hour after reperfusion, the serum ALT and LDH concentrations in group G were significantly lower than those in group C ($P<0.05$). Twenty-four hours after reperfusion, liver enzyme levels in both groups were markedly recovered, the serum ALT and LDH concentrations in group G were also significantly lower than those in group C ($P<0.01$, Figures 1 A and 1 B)

Antioxidant enzyme level

One and 24 hours after reperfusion, the GSH content in the liver tissue of group G was significantly higher than that in group C ($P<0.05$). There was no statistical difference in the activity of SOD between the two groups ($P>0.05$, Figures 2 A and 2 B)

Bcl-2 and Bax expression

One and 24 h after reperfusion, the positive expression rate of Bcl-2 was higher in group G than in group C ($P<0.05$) and the positive expression rate of Bax was lower in group G than in group C ($P<0.05$) (Table 1, Figures 3 A-3D).

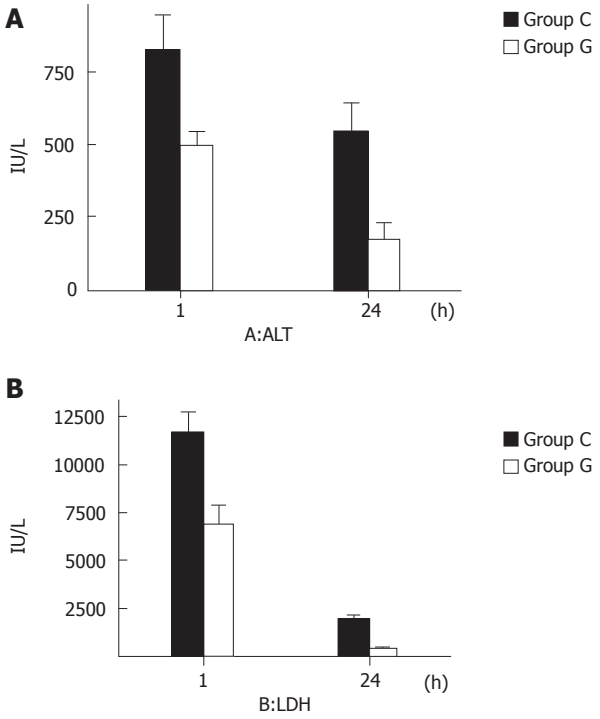

Figure 1 Liver function 1 and 24 h after reperfusion of ALT (**A**) and LDH (**B**) in groups C and G.

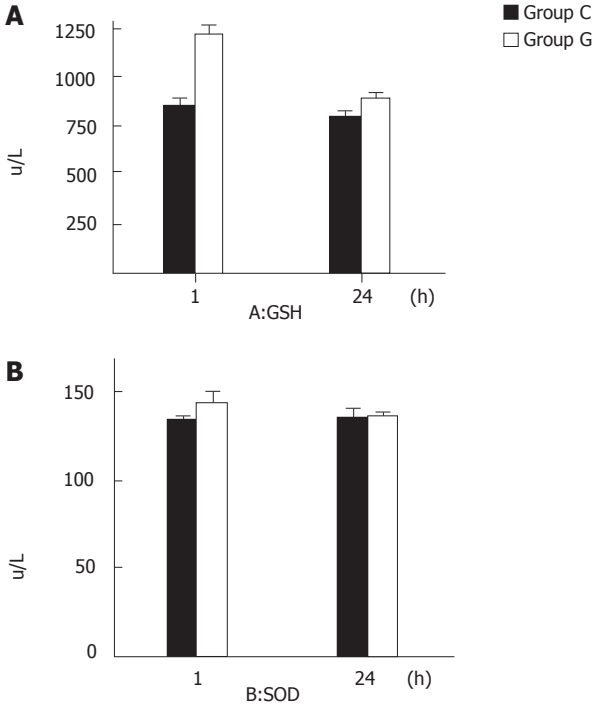

Figure 2 GSH content (**A**) and SOD activity (**B**) 1 and 24 h after reperfusion in groups C and G.

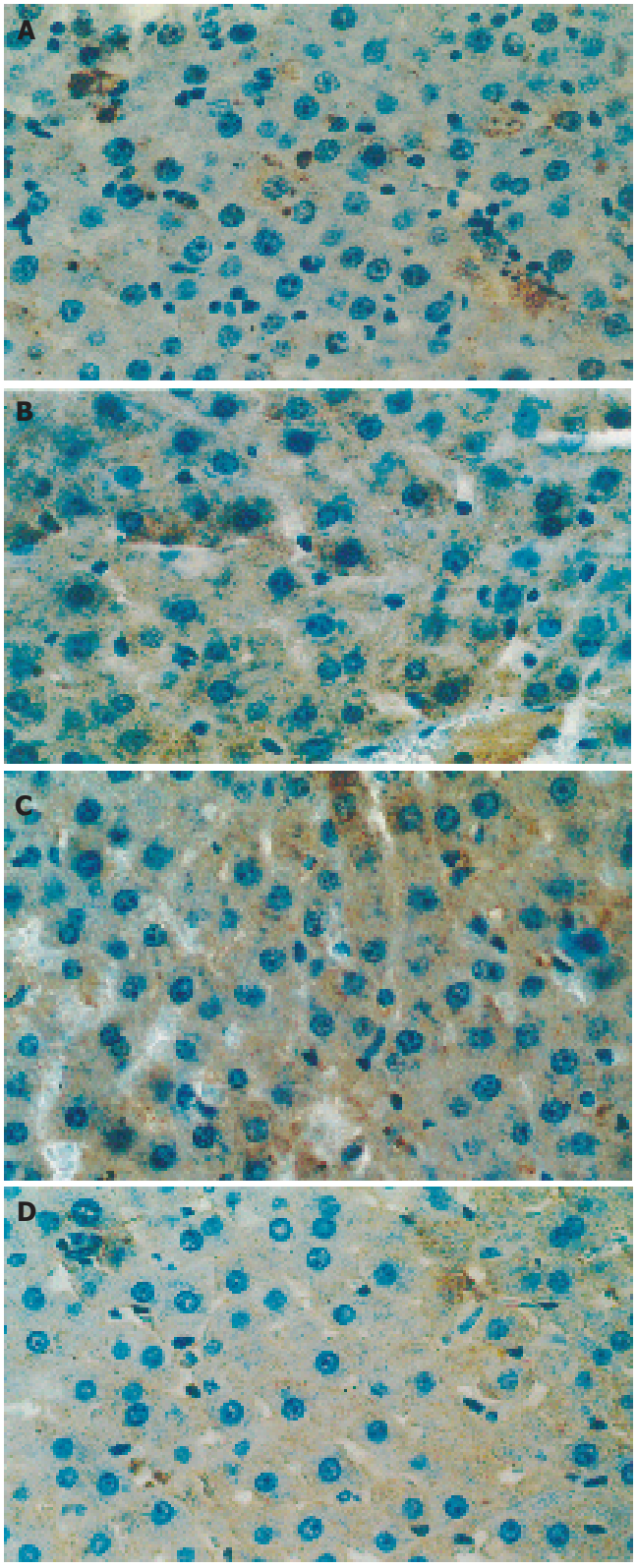

Figure 3 Bcl-2 expression in group C (**A**) and group G (**B**) (post-reperfusion 1 h) (× 400), and Bax expression in group C (**C**) and group G (**D**).

Histological changes

One hour after reperfusion, only slight swelling and less fatty and vacuolation degeneration were found in hepatocytes of group G. However, severe swelling was observed in group C along with more fatty and vacuolization degeneration in hepatocytes. Compartment of hepatic sinus became narrow or disappeared. Infiltration of neutrophils and hemorrhage were also observed. Twenty-four h after reperfusion, the swelling and vacuolization degeneration in group G became worse, but the hepatic cellular structure was still clear and only single-cell necroses were observed. However, derangement of constitution was observed in group C along with a few necroses of hepatocytes.

Table 1 Positive expression of Bcl-2 and Bax proteins in liver tissue

Group	Bcl-2 protein						Bax protein					
	positive expression				total positive expression		positive expression				total positive expression	
	-	+	++	+++	number	percentage(%)	-	+	++	+++	number	percentage(%)
group C (1 h)	5	0	0	3	3	37.5[a]	0	1	3	4	7	87.5[c]
group G (1 h)	0	0	0	8	8	100.0	4	2	1	1	2	25.0
group C (24 h)	5	1	1	1	2	25.0[e]	0	1	4	3	7	87.5[g]
group G (24 h)	1	0	0	7	7	87.5	5	1	1	1	2	25.0

[a]$P<0.05$, [c]$P<0.05$, [e]$P<0.05$, [g]$P<0.05$ *vs* group G.

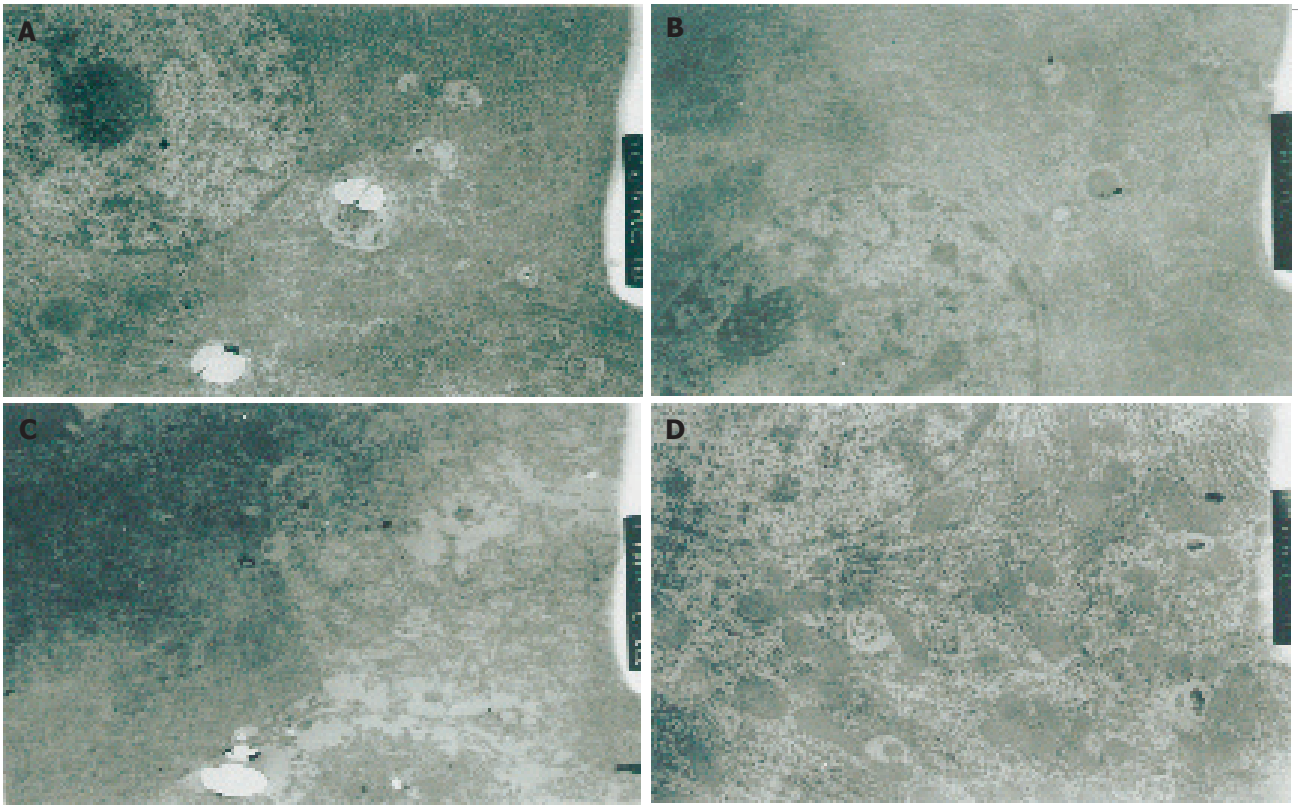

Figure 4 Hepatic ultrastructure 1 h after reperfusion (×1500) in group C (**A**) and group G (**B**), 24 h after reperfusion in group C (**C**) and group G (**D**).

Ultrastructural changes

One h after reperfusion, mitochondria and glycogen granules were observed in hepatic cytoplasm of group G. Rough endoplasmic reticulum was arrayed as lamellar. Mitochondria and rough endoplasmic reticulum were slightly swollen. More primary lysosomes were found. In group C, mitochondria were severe swollen and had a reduction in the number of cristae. Rough endoplasmic reticulum was dilated and ruptured in different degrees. Smooth endoplasmic reticulum increased and glycogen granules decreased. More secondary lysosomes were observed. Twenty-four h after reperfusion, more mitochondria, endoplasmic reticulum and a few of glycogen granules and secondary lysosomes were observed in cytoplasm of group G, along with slightly focal swelling. In group C, hepatocytes only were observed with cellular contour. Severe edema, deformation and necrosis were also observed. The structure of organelles in cytoplasm was not clear. (Figures 4A-4D)

DISCUSSION

Gln is one of the most abundant amino acid in plasma and plays an important role in the metabolism of body. The circulating and tissue concentrations of Gln diminish precipitously after stress. When Gln is exhausted to a certain extent, a large amount of Gln released from skeletal muscle and a small amount from lung are transported to liver and gut. Recently, Gln has been demonstrated to protect against ischemia-reperfusion injury of gut, heart and skeletal muscle[5-7].

The results of our study indicated that after 30 min

ischemia, liver function and morphology were impaired and Gln pretreatment could protect against liver damage.

The exact mechanisms of the protective effect of Gln against ischemia and reperfusion injury of organs and tissues are still incompletely understood. Gln protects against ischemia-reperfusion injury of gut, heart and skeletal muscle by preserving the GSH content in the tissues[5-7]. GSH is an important endogenous antioxidant that protects against oxygen free radical injuries and intravenous GSH administration during reperfusion of ischemic liver can prevent reperfusion injury in rats[13]. The content of GSH is useful for determination of the degree of tissue damage[14]. In this study, Gln pretreatment could obviously increase the GSH content in rat liver tissue, indicating that Gln can protect against hepatic I/R injury by maintaining the relatively high content of GSH in tissue and relieving the cellular oxidant injuries[15].

In addition, Gln pretreatment can also protect against hepatic I/R injury by participating in energy metabolism, increasing the cellular energy metabolism, protecting the structure and function of mitochondria and decreasing the production of oxygen free radicals, leading to less consumption of GSH in liver tissue. A complete inhibition of α-ketoglutarate dehydrogenase activity in the TCA cycle has been observed in hyperoxia-exposed glutamine-deprived cells and Gln could protect α-ketoglutarate dehydrogenase from inactivation under oxidative stress as well as mitochondria from oxidative stress and increase cellular ATP levels[16]. Gln could stimulate the synthesis of glycogen and increase the glycogen store in hepatocytes, reinforcing the ability of liver against cellular oxidant injuries[17]. In this study, mitochondria and glycogen granules were observed in hepatic cytoplasm, mitochondria and rough endoplasmic reticulum were protected.

Gln increases the activity of SOD in liver tissue after stress[18], which was not confirmed in our study due to the model of rat, the number of samples and the dosage of Gln in this experiment.

It was reported that portal triad clamping produces not only ischemic injury of the liver but also portal venous congestion[19]. Acute portal venous congestion for a long period may impair the intestinal mucosal barrier and increase intestinal permeability, causing endotoxemia, bacterial translocation, activation of reactive oxygen radicals and inflammatory cytokines, such as tumor necrosis factor-α (TNF-α)[20-23]. Reperfusion of stagnant portal venous blood with deleterious chemical mediators into the ischemic liver aggravates the liver injury, leading to intra-abdominal sepsis and abscess formation, which is the major cause of postoperative septic complications induced by hepatic I/R injury[24-26]. However, attempts to protect against hepatic I/R injury by alleviating the possible detrimental effects of portal venous congestion have not achieved satisfactory results.

Gln is a precursor for synthesis of nucleic acids and glutathione. It is the main fuel for rapid proliferating and dividing cells such as enterocytes and lymphocytes. It can maintain the metabolism of intestinal mucosal cells directly or indirectly, promote hyperplasia of epithelial cells of ileum and colon, maintain the structure and function of small intestinal mucosal and reduce the increment of intestina permeability[27-30].

Gln can enhance the function of gut-associated lymphoid tissue (GALT) and gut[31]. Gln administration can prevent the depletion of GSH in Peyer's patches of endotoxaemic mice[32] and promote secretion of mucosal secretory immunoglobulin A (S-IgA), a major effector of the gut-associated lymphoid tissue in the intestine, which can bind to bacteria and prevent their adherence to the epithelium and bacterial translocation[33, 34].

Hepatic injury caused by ischemia can be described as necrosis. It was reported that apoptosis of hepatocytes and sinusoidal endothelial cells is a critical mechanism contributing to hepatic I/R injury[8]. The mechanism of apoptosis in ischemia and reperfusion injury usually includes prodtion of oxygen radical species and intracellular calcium overloading[35]. Translocation of bacteria and endotoxin from gut, well documented in hepatic I/R injury, also contribute to the induction of hepatocyte apoptosis. TNF-α induced by endotoxin also can induce hepatocyte apoptosis[36]. A number of genes regulate the apoptotic process. The family of *bcl-2*-related proteins plays a key role in the regulation of apoptosis.

Bcl-2, a member of the *bcl-2*-related protein family, can promote cell survival through protein-protein interactions with other *bcl-2*-related protein family members. Recent studies indicate that overexpression of Bcl-2 protein could reduce hepatocellular apoptosis after reperfusion and protect against hepatic I/R injury[8, 11]. Bax, another member of the *bcl-2*-related protein family, has extensive amino acid homology with Bcl-2 and may form homodimers to accelerate cell death or form heterodimers with Bcl-2 to inhibit cell death. Therefore, changes in the ratio of Bcl-2 and Bax expression may determine survival or death following apoptotic stimuli and attenuate the anti-apoptotic effect of Bcl-2 protein by reducing post-ischemic apoptosis[9, 37].

In this study, up-regulation of Bcl-2 protein expression and down-regulation of Bax protein expression in rat liver were found 1 and 24 h after reperfusion, indicating that Gln can protect against hepatic I/R injury by regulating the expression of Bcl-2 and Bax proteins. Our findings suggest that Gln pretreatment could enhance GSH content and protect against oxidative stress in hepatic I/R injury. It has been proved that reduction in the cellular level of GSH increases degradation of Bcl-2 protein and apoptosis[10]. Gln pretreatment can significantly increase cellular ATP levels and synthesis of glycogen and glycogen store in hepatocytes[16, 17]. Gln can decrease the diminution of liver tissue ATP content and intracellular calcium overloading maintain the activity of Na^+-K^+ and Ca^+-ATPase, and prevent hepatocellular apoptosis. On the other hand, Gln pretreatment can protect gut barrier and decrease the release of enteric endotoxin and inflammatory cytokines (such as TNF-α), thus reducing the direct pro-apoptotic effect of endotoxin and TNF-α.

In conclusion, glutamine pretreatment can protect against hepatic I/R injury by enhancing GSH content and regulating the expression of Bcl-2 and Bax proteins in liver tissue.

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BASIC RESEARCH

In vitro and *in vivo* protective effects of proteoglycan isolated from mycelia of *Ganoderma lucidum* on carbon tetrachloride-induced liver injury

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Abstract

AIM: To investigate the possible mechanism of the protective effects of a bioactive fraction, *Ganoderma lucidum* proteoglycan (GLPG) isolated from *Ganoderma lucidum* mycelia, against carbon tetrachloride-induced liver injury.

METHODS: A liver injury model was induced by carbon tetrachloride. Cytotoxicity was measured by MTT assay. The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined with an automatic multifunction-biochemical analyzer and the levels of superoxide dismutase (SOD) and TNF-α were determined following the instructions of SOD kit and TNF radioimmunoassay kit. Liver sections were stained with hematoxylin and eosin (H&E) for histological evaluation and examined under light microscope.

RESULTS: We found that GLPG can alleviate the L-02 liver cells injury induced by carbon tetrachloride (CCl_4) through the measurements of ALT and AST activities and the administration of GLPG to L-02 cells did not display any toxicity. Furthermore, histological analysis of mice liver injury induced by CCl_4 with or without GLPG pretreatment indicated that GLPG can significantly suppress the toxicity induced by CCl_4 in mice liver. We also found that GLPG reduced TNF-α level induced by CCl_4 in the plasma of mice, whereas increased SOD activity in the rat serum.

CONCLUSION: GLPG has hepatic protective activity against CCl_4-induced injury both *in vitro* and *in vivo*. The possible anti-hepatotoxic mechanisms may be related to the suppression of TNF-α level and the free radical scavenging activity.

Key words: *Ganoderma lucidum proteoglycan* (GLPG); Carbon tetrachloride (CCl_4); Liver injury; Hepatic protective activity

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INTRODUCTION

The mainstream pharmaceutical industry makes use of many plant products[1]. In traditional Chinese medicine (TCM) many products from plants are used in the treatment of a wide range of disorders including cancer[2]. Moreover, the researches on the bioactive ingredients and the investigation of the functional mechanism of natural products used in TCM are becoming increasingly important. Several substances which are successful in tumor therapy, such as betulinic acid and indirubin, have been elucidated by molecular biological and cell biological methods[3, 4].

Fungi are an important source of materials in TCM. Extracts from about 200 species of fungi have been shown to stimulate immunoactivity and inhibit the growth of different kinds of tumors[5-8]. *Ganoderma lucidum* (Leyss. ex Fr.) *Karst* is a medicinal mushroom belonging to the polyporaceae of aphyllophorales. Its fruiting body is called "Lingzhi" in China and "Reishi" in Japan. For hundreds of years, this mushroom has been regarded as a TCM or a folk medicine used in the prevention and treatment of various human diseases, such as chronic bronchitis, hepatitis, hypertension, hypercholesterolemia, tumorigenic disease and immunological disorders in China and other Asian countries[9].

Carbon tetrachloride (CCl_4) is a xenobiotic producing hepatotoxicity in human beings and animals[10-11]. In fact

it has been shown that the trichloromethyl radical formed in the metabolism of CCl_4 via the liver microsomal cytochrome P450 system, reacts rapidly with molecular oxygen to produce trichloromethyl peroxy radicals. These radicals react with unsaturated fatty acids of phospholipids present in cell membranes, initiating lipid peroxidation in liver cells[12]. Hydrogen atoms are removed from unsaturated fatty acids by such radical-created carbon-centered lipid radicals[13]. These lipid radicals quickly add molecular oxygen to form lipid peroxyl radicals which in turn abstract hydrogen atoms from other lipid molecules, thereby propagating the process of lipid peroxidation[14]. Transition metals such as copper and iron can catalyze oxygen free radical reactions that lead to peroxidation of membrane lipids or inactivation of antioxidant defense[15]. Previous studies have also reported involvement of iron as a mediator of CCl_4-hepatotoxicity[16]. When the amount of reactive oxygen species (ROS) production exceeds the capacity of the endogenous cellular antioxidant system, significant cellular injury can occur[17, 18]. Treatment of animals with different antioxidants such as vitamin E[19], vitamin E-like compounds[20], 5-methylthioadenosine[21], colchicines[22] and desferrioxamine[23] can significantly improve hepatic conditions by reducing CCl_4-induced damage.

Ganoderma lucidum contains many biologically active components[24, 25]. Previous studies suggested that *Ganoderma lucidum* polysaccharide (Gl-PS), one of the main efficacious ingredients of *Ganoderma lucidum Karst,* has been under modern pharmacological research in recent 30 years and is effective in modulating immune functions, inhibiting tumor growth, resisting invasion of various viruses[9, 26, 27]. Miyazaki and Nishijima[28] have separated a heteroglycan from the fruit body of Ganoderma lucidum. Moreover, Some researchers have isolated several hypoglycemic glycans from another fraction of the same crude polysaccharide[29].

Though the fruit body and the spores of *Ganoderma lucidum* have been used as medicines for a long time, no data is available about the protective activity of extracts from mycelium on CCl_4-induced liver injury. The main aim of this study was to investigate the effects of GLPG isolated from mycelium of *Ganoderma lucidum* on CCl_4-induced liver injury *in vivo* and *in vitro*, and the possible mechanism of the hepatic protective activity of GLPG.

MATERIALS AND METHODS

Materials and reagents

Ganoderma lucidum (*FR.*) *Karst* (Ganodermataceae) was preserved in our laboratory. RPMI-1640, trypsin, penicillin and streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). SOD kit was purchased from Jiancheng Bioengineering Institute of Nanjing. TNF radioimmunoassay kit was purchased from Jiuding Corporation (Tianjin, China). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), crystal violet and trypan blue were from Sigma (St. Louis, MO). L-02 cells were obtained from China Center for Type Culture Collection (CCTCC, Wuhan, Hubei). All the other commercially available chemicals used were of the highest grade.

Extraction and purification of GLPG

Ganoderma lucidum (Fr.) Karst was extracted as previously described[30]. In brief, fungal mycelia were collected by filtration, dried and disrupted, and then the residue was extracted with 30-40 fold boiling water for 30 min. After centrifugation, the supernatant solution was concentrated under reduced pressure and intensively dialyzed. The retentate was added to three volumes of ice cold EtOH to precipitate the crude extracts. Then the sample was allowed to stand overnight at 4 ℃ and then centrifuged. The precipitate obtained was lyophilized. The lyophilized products were a dark brownish powder of water-soluble substance.

To purify the crude products, a portion of crude polysaccharide fraction was dissolved in double-distilled water and centrifuged to remove the insoluble materials. The supernatant was applied onto the DEAE-cellulose column and eluted with 0.1 N NaCl. Each perk eluent was separately pooled, concentrated, dialyzed, and three volumes of ice cold EtOH was added to precipitate the polysaccharides. The polysaccharide content in each fraction was determined by phenol-sulfuric acid method [31]. The GLPG fraction was lyophilized and further dissolved to indicate the concentrations for subsequent experiments.

Animal treatment and CCl_4-induced hepatotoxicity

Male BALB/c mice weighing 18-22 g (6-8 wk old), were provided by Experimental Animal Center, Wuhan University, and fed with standard diet and tap water. The animals were housed in cages (4-5 each cage) and maintained at 24±2 ℃, under 50-60% relative humidity in a 12 h light/ dark cycle throughout the experiment.

GLPG was diluted with saline and given orally by gavage for 20 d, at daily doses of 300, 600 and 900 mg/kg as aqueous extract. The saline control group received equal amounts of saline given orally for 20 d. During this treatment, carbon tetrachloride was introduced to the mice orally (1600 mg/kg CCl_4, mixed with corn oil) for the last 3 d. The same volume of corn oil was given to vehicle control mice orally for the last 3 d. After 3 d, the mice were euthanized under ether anaesthesia, blood and liver samples were collected.

Cell culture and liver cells injury induction

L-02 cells were cultured in RPMI-1640 medium supplemented with 10 % heat inactived fetal bovine serum (FBS), 100 IU /mL penicillin, 100 μg/mL streptomycin and 250 U/L insulin. The cells were maintained at 37 ℃ in a humidified atmosphere containing 5 mL/L CO_2 and subcultured 2-3 times a week.

Semi-confluent L-02 cells in 24-well culture plate (Falcon, NJ, USA) were divided into control group, dimethyl sulfoxide (DMSO) vehicle group, GLPG group, and CCl_4-induced hepatotoxicity group (20 mmol/L final concentration) with or without GLPG at various concentrations. After 4 h the culture supernatant was collected and stored at -20 ℃.

Cytotoxicity assay

Cytotoxicity was measured by MTT assay. The cells were

seeded in 96-well culture plate (Falcon, NJ, USA) at the concentration of 4×10^3 cells per well in 100 μL medium. After incubation of the cells for 12 h at 37 ℃, various concentrations of GLPG were added and the incubation was continued for 48 h, or at a given concentration of GLPG (200 μg/mL final concentration) the incubation time was prolonged for 72 h. Then the viable cells were determined by MTT reduction assay. In brief, MTT was dissolved in phosphate-buffered saline (PBS) at 5 mg/mL and sterilized by filtration to remove insoluble reside present in some batches of MTT. At the indicated time, the MTT solution (20 μL) was added to each well. After incubation for 5 h, cell culture medium was removed carefully, and 150 μL dimethyl sulfoxide (DMSO) was added to each well and mixed thoroughly to dissolve the dark purple crystals. The plate was incubated for 10 min at room temperature, to ensure that all formazane were dissolved. The plate was thenread on a Perkin-Elmer ELISA reader (HTS7000 plus) at a wavelength of 570 nm.

The effects of GLPG on cell proliferation and viability were compared according to the commonly accepted method of staining cells with trypan blue. L-02 cells were seeded in 96-well plate at the concentration of 2×10^3 cells per well in 100 μL of RPMI-1640 medium. The cells were incubated with or without various concentrations of GLPG for 48 h. Then the cells were trypsinized and collected. The number of cells was determined in a Neubauer hemacytometer using the trypan blue exclusion method, and the mean values were calculated.

Assay of ALT and AST activities in vivo and in vitro

As a marker of hepatocyte necrosis, the activities of ALT and AST were determined with an automatic multifunction-biochemical analyzer (Beckman, USA) in serum and cell culture medium.

Superoxide dismutase (SOD) determination

Wistar rats weighing 80-120 g (7 wk old) were divided into 3 groups, 22 rats each group. GLPG was diluted with saline and given orally for 10 wk at daily doses of 1 000 and 3 000 mg/kg as an aqueous extract. The saline control group received equal amount of saline given orally for 10 wk. After 10 wk the rats were sacrificed by decapitation and the serum was collected to determine the activity of SOD, according to the instructions of the kit used.

SOD measurement method was based on the principle in which xanthine reacts with xanthine oxidase to generate superoxide radicals reacting with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The SOD activity was then measured as previously described [32].

Histopathological examination

Liver was removed, fixed overnight in 10% buffered formalin and paraffin-embedded. The sections were stained with hematoxylin and eosin (H&E) for histological evaluation and examined under light microscope. In brief, 4-μm thick sections of paraffin-embedded mice liver were dewaxed in xylene, rehydrated in graded alcohol series, and washed with distilled water for 2 min. Subsequently, the sections were stained with hematoxylin for 5 min at room temperature. After 15 min, the sections were counterstained with eosin for 2 min, dehydrated in graded alcohol series, washed with xylene, and blocked by rosin. H&E- stained slides were observed under microscope at × 40 magnifications.

Measurement of plasma TNF-α level

Plasma TNF-α level was determined by TNF radioimmunoassay kit. All the mice were introduced orally with CCl_4 with or without GLPG, and the mice in control group were euthanized, then the blood was collected in the tubes with previous addition of 25 μL of heparin solution (4 000 IU). The plasma samples obtained after centrifugation at 3 000 r/min for 10 min at 4 ℃ were stored at -70 ℃ until assay.

The TNF-α levels of plasma samples were measured by sequential-saturation-type assay as previously described [33]. In brief, 200 μL of standard sample, the plasma samples, and the control were added to each tube, then 100 μL anti-TNF-α antibody was added to each tube and mixed thoroughly. After 18 h of incubation at 4 ℃, 100 μL of solution containing radioactive label was added to each tube and mixed. After a further incubation for 3 h at 37 ℃, 1 000 μL of the secondary antibody was added for 15 min. the tubes were centrifuged at 3 600 r/min for 20 min, the supernatant was discarded carefully. The gamma cpm of deposition was measured with a gamma counter, and the plasma TNF-α levels were measured by standard sample curve diagram. TNF-α values were expressed as ng/mL.

Statistical analysis

Data were expressed as mean ± SD. The difference between the means of two groups was evaluated by ANOVA. $P<0.05$ was considered statistically significant.

RESULTS

Effects of GLPG on cytotoxicity

Compared with the L-02 cells administrated with GLPG and the control cells, there was no obvious change occurred in cell growth and morphology (Figures 1A and 1B). Trypan blue exclusion method showed that the total cell number of L-02 cells treated with GLPG was approximately 96 % as compared to that of the control cells Even at the GLPG concentration was 1 024 μg/mL. And we also found that no toxic response and death was occurred *in vivo* experiment (data not shown). And we also found that no toxic response and death was occurred *in vivo* experiment (data not shown).

Detection of liver weight and assay of ALT and AST activities in vivo and in vitro

Compared to the control and GLPG groups, GLPG (300, 600 and 900 mg/kg) markedly ameliorated hepatic injury induced by CCl_4. The liver weight (Table 1) and serum ALT and AST activities (Table 2) were observed in mice 24 and 72 h after CCl_4 administration. The liver weight did not increase compared to CCl_4-treated group. Furthermore, the serum ALT and AST activities in GLPG pretreatment groups induced by CCl_4 were lower than those in CCl_4-treated group. Simultaneously, GLPG treatment reduced

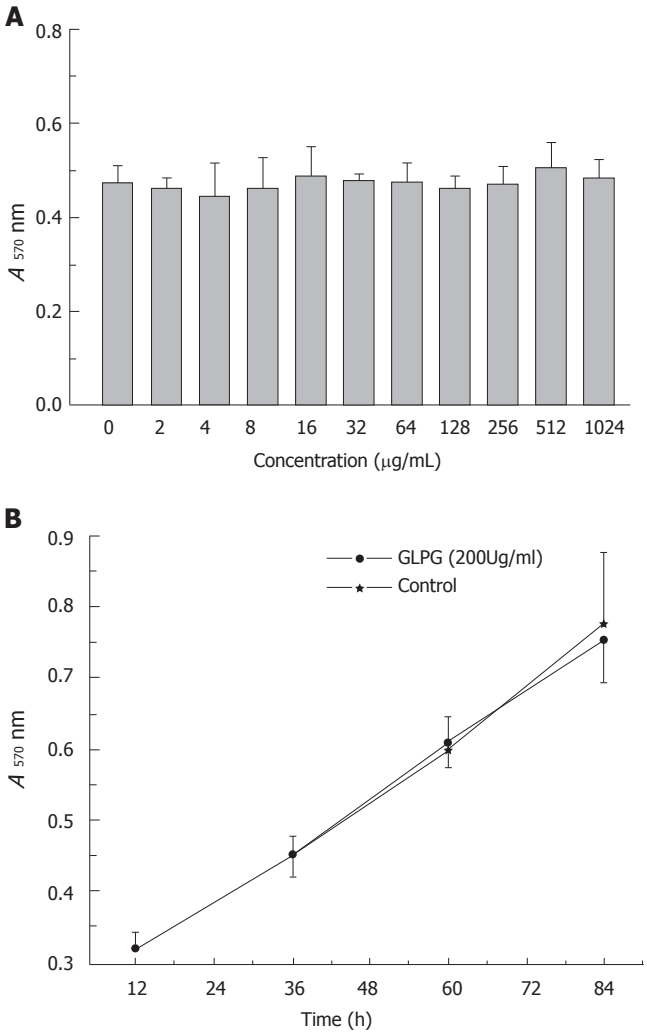

Figure 1 Effects of GLPG on proliferation of L-02 cells treated with GLPG at the concentration of 2-1024 μg/mL for 2 d (A) and at the concentration of 200 μg/mL for 3 d (B). Viable cells were detected every 24 h. The cells untreated with GLPG were used as controls in experiment. Results shown represent the mean ± SD for at least three separate experiments.

Table 1 Effect of GLPG on liver weight in mice with CCl_4-induced hepatic injury after 72 h (mean ± SD)

Group	Liver weight (g)
Control	1.77 ± 0.26
GLPG (500 mg/kg)	1.74 ± 0.21
CCl_4 (1600 mg/kg)	2.22 ± 0.34
CCl_4 (1600 mg/kg) + GLPG(300 mg/kg)	1.67 ± 0.17
CCl_4 (1600 mg/kg) + GLPG(600 mg/kg)	1.57 ± 0.18
CCl_4 (1600 mg/kg) + GLPG(900 mg/kg)	1.52 ± 0.14

[a]$P<0.05$ *vs* CCl_4-treatment group; [b]$P<0.01$ *vs* control group; $n=10$ mice.

the serum ALT and AST activities in a dose-dependent manner within the similar range of concentrations, indicating that GLPG showed anti-hepatotoxic activities on CCl_4-induced liver injury.

Administration of GLPG also showed anti-hepatotoxic activity in L-02 cells injury induced by CCl_4. GLPG at concentrations of 100, 250 and 500 μg/mL suppressed the

Table 2 Effects of GLPG on ALT and AST activities in mice with CCl_4-induced hepatic injury after 24 h and 72 h (mean ± SD)

Group	ALT (U/L)		AST (U/L)	
	24 h	72 h	24 h	72 h
Control	54.38 ± 6.20	-	44.50 ± 34.36	-
GLPG (500 mg/kg)	74.00 ± 17.14	-	51.75 ± 29.43	-
CCl_4 (1600 mg/kg)	642.50 ± 225.33	105.00 ± 39.64	147.50 ± 58.80	180.25 ± 45.51
CCl_4 (1600 mg/kg)+ GLPG (300 mg/kg)	433.38 ± 133.97[b]	96.56 ± 27.84	99.88 ± 40.47[a]	161.13 ± 18.29
CCl_4 (1600 mg/kg)+ GLPG (600 mg/kg)	374.00 ± 107.34[c]	87.30 ± 15.60	61.25 ± 26.50[c]	151.63 ± 32.67
CCl_4 (1600 mg/kg)+GLPG (900 mg/kg)	316.50 ± 98.76[c]	79 .70 ± 17.28[a]	56.75 ± 36.58[c]	114.75 ± 67.13[a]

[a]$P<0.05$ *vs* CCl_4 treatment group; [b]$P<0.01$, [c]$P<0.001$ *vs* control group; $n=20$ mice.

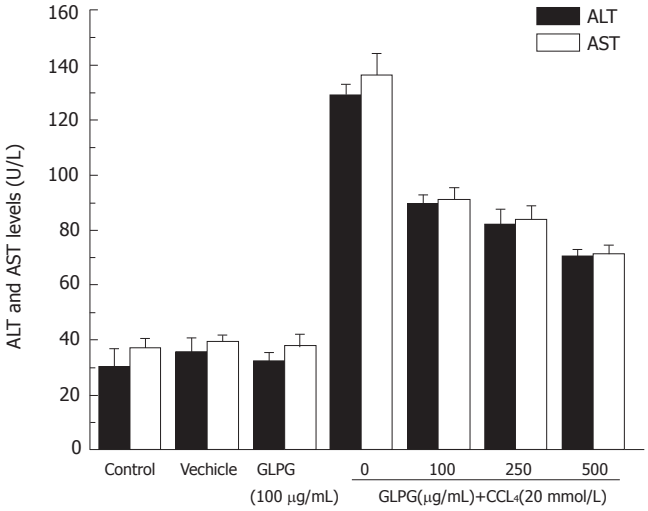

Figure 2 Effects of GLPG on ALT and AST activities in CCl4-induced L-02 cell (n = 3) injury. Compared to control group, GLPG suppressed the activities of ALT and AST in the other groups. Results shown represent the mean ± SD from three separate experiments.

activities of ALT and AST in a dose-dependent manner (Figure 2).

Superoxide dismutase (SOD) assay

According to the results of SOD assay, the activity of SOD in the serum of rats pretreated with GLPG increased significantly compared with that of control group. As shown in Figure 3, compared to the control group, the activities of SOD in the group administered orally with GLPG at the concentrations of 1 000 mg/kg and 3 000mg/kg increased by 57.2 % and 70.6 %, respectively.

Histological analysis

We examined the effects of GLPG at various doses (300, 600 and 900 mg/kg) on histopathological changes of CCl_4-induced liver injury in mice *in vivo*. GLPG showed strong

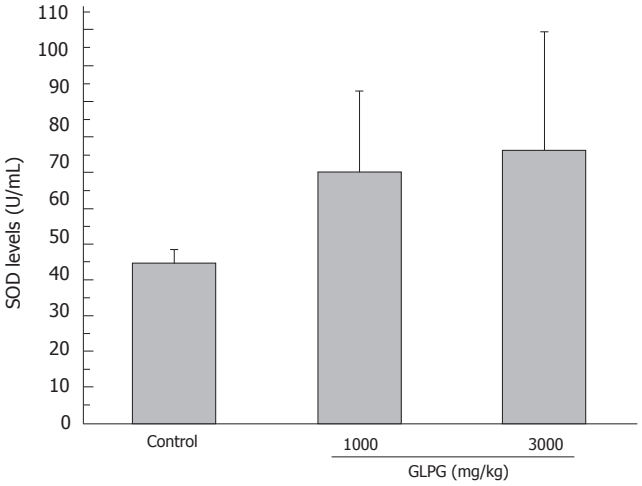

Figure 3 Effect of GLPG on SOD activity in serum of rats. The rats were administered orally with or without GLPG at 1000 mg/kg and 3000 mg/kg. The increment of SOD activities was 57.2 % and 70.6 %, respectively.

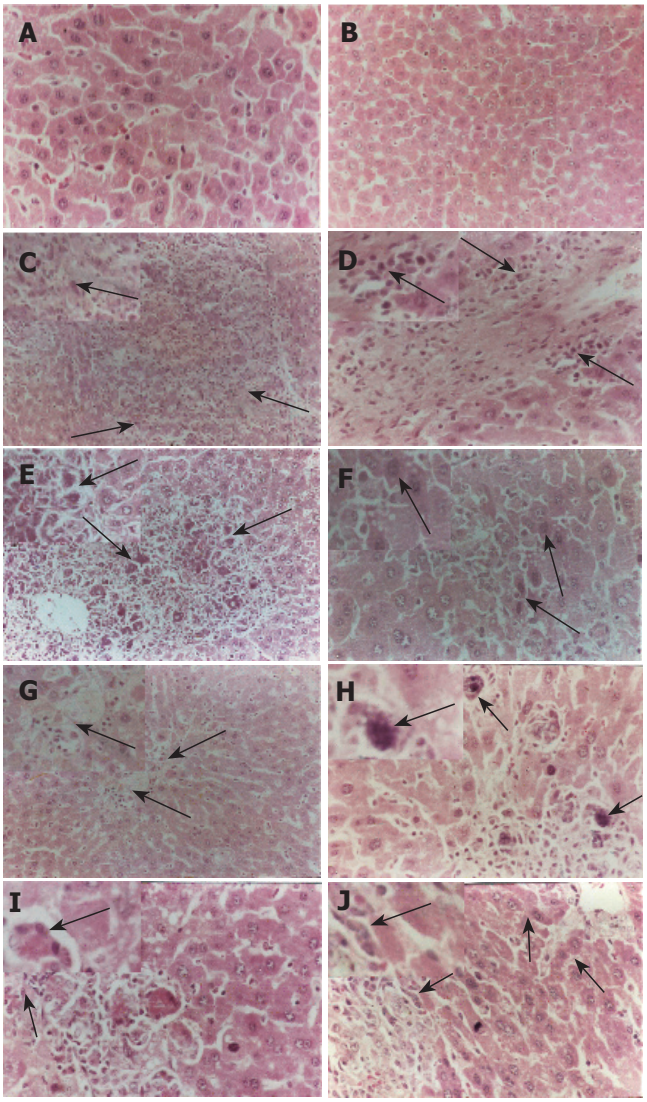

Figure 4 Histological changes (marked by arrows) of CCl_4-induced hepatic injury in the presence or absence of GLPG in mice by hematoxylin-eosin (**H&E**) staining in control group (**A**), GLPG group (**B**), and CCl_4-induced liver injury group (**C-J**) 24 and 72 h after GLPG pretreatment at different doses(0, 300, 600 and 900 mg/kg).

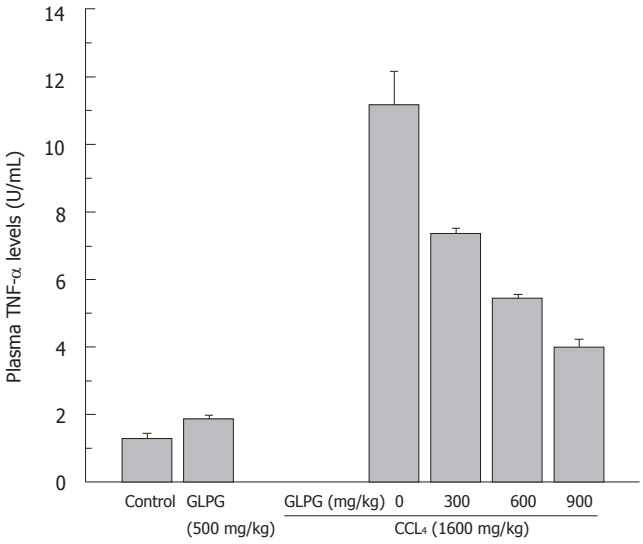

Figure 5 Effect of GLPG on the levels of TNF-α in the plasma of mice, as determined by TNF-α radioimmunoassay kit. Compared with control group, administration of GLPG at various doses could significantly suppress the level of TNF-α in a dose-dependent manner after 48 h. Data are the mean ± SD from 4 determinations.

hepatic protective activity. Significant changes of liver tissue were observed in carbon tetrachloride-induced group after 24 h and 72 h (Figures 4C and 4D) as compared to the control group (Figure 4A) and the GLPG pretreated group (Figure 4B). No obvious histopathological change was observed in control and GLPG groups. and Both the control group and the GLPG group had no infiltrations and hemorrhagic signs. Figure 4C shows the histological changes after 24 h of CCl_4 post-administration with a marked liver injury. After 72 h of CCl_4 post-administration, liver lobule structures disappeared and necrotic hepatic tissues induced by CCl_4 with pretreatment GLPG (300 mg/kg) after 24 h (Figure 4E) and 72 h (Figure 4F) were had no evident recovery. liver injury and necrotic hepatic tissue recovered markedly. As shown in Figure 4G-J, basophilic granules and many double-nuclear regenerative liver cells appeared 24 and 72 h after GLPG pretreatment at different doses (600 mg/kg and 900 mg/kg). These results showed that GLPG could alleviate CCl_4-induced liver injury *in vivo* in mice.

Measurement of plasma TNF-α level

Figure 5 showed that the changes of TNF-α levels in the plasma of experimental groups 48 h after CCl_4 treatment with or without GLPG at different doses. Low TNF-α levels were detected in the plasma of the control group and GPLG group. In contrast, a marked rise of TNF-α level was found in the plasma of CCl_4-pretreatment group. Simultaneously, GLPG (500 mg/kg) can decrease the level of TNF-α in the plasma of mice treated with CCl_4 (data not shown). Administration of GLPG at 300, 600 and 900 mg/kg significantly suppressed the levels of TNF-α in dose-dependent manner after 48 h. The mean values of TNF-α levels at 48 h were 34 %, 51.3 % and 64.2 % at 300, 600 and 900 mg/kg respectively.

DISCUSSION

Rapid lipid peroxidation of the membrane structural lipids has been proposed as the basis of CCl_4 liver toxicity[34-35]. In fact the first step in liver injury induced by CCl_4 is the formation of reactive oxygen species (ROS) that may further lead to membrane peroxidation or cell injury. There is evidence that Kupffer cells as well as hepatocytes have inducible cytochrome P-450 and are capable of metabolizing CCl_4 or other toxic species[36]. Moreover, it was reported that Kupffer cell activation is a crucial step in hepatocyte injury induced by CCl_4 or other toxic agents[37-38]. Transition metal ions like Cu^{+2} and Fe^{+3} play an important role as components of proteins essential for biological functions. However, these metals can also initiate Haber-Weis and Fenton reactions where superoxide anions are transformed into detrimental hydroxyl radicals which are in turn responsible for attack of the membrane polyunsaturated fatty acids[15]. The use of metal chelating agents may have therapeutic effect by reducing the oxidative burst and the consequent membrane lipid peroxidation[39-40]. Previous findings have shown that transition metals can mediate free radical production after CCl_4 administration in rats[16, 41-42]. Treatment of these animals with agents capable of chelating these metal ions can protect hepatocytes against damage by reducing oxidative burst and lipid peroxidation[16, 41-42].

In this study, a water soluble substance, *Ganoderma lucidum* proteoglycan was isolated from mycelia of *Ganoderma lucidum* by EtOH precipitation and DEAD-cellulose column chromatography. GLPG is a proteoglycan consisting of about 86.4 % carbohydrates and has antioxidant activity against CCl_4-induced liver injury. Treatment with GLPG could ameliorate hepatic injury induced by CCl_4. Administration of GLPG significantly decreased ALT and AST activities in CCl_4-induced liver injury *in vivo* and *in vitro* (Table 2 and Figure 2). Histological changes observed in GLPG-pretreatment groups were less than in CCl_4-induced group. Some histological changes, such as hemorrhage, inflammatory infiltration and necrosis in hepatic tissue, were simultaneously improved after pretreatment with GLPG. These results suggested that the hepatic protective activity of GLPG against CCl_4-induced liver injury was in a dose-dependent manner.

Though the scavenging effect of *Ganoderma lucidum* polysaccharide (Gl-PS) has been reported [25, 43-46], no report is availabe about the hepatic protective activity of GLPG. In our study, the activity of SOD after pretreatment with GLPG increased significantly in the serum of rats, while SOD could eliminate peroxide *in vivo*, suggesting that GLPG might scavenge the free radicals induced by CCl_4.

Although it is generally believed that the hepatic protective activity of GLPG is mainly due to its ability to scavenge free radicals induced by CCl_4. However, there may be other mechanisms. A large amount of this cytokine may be interpreted as a progression of hepatic damage[47]. TNF-α induced by CCl_4 may contribute to cellular damage in liver injury. The antioxidant activity of GLPG can reduce TNF-α level in the plasma of mice, thus inhibit inflammation occurrence. Though the mechanism of GLPG down-regulates CCl_4-induced TNF-α level *in vivo* in mice remains unknown, the suppression of GLPG on TNF-α level might play an important role in its hepatic protective activity against CCl4-induced liver injury.

In conclusion, GLPG exhibits strong hepatic protective activity against CCl_4-induced liver injury both *in vivo* and *in vitro*. And the possible anti-hepatotoxic mechanisms may be related to the suppression of TNF-α level and the free radical scavenging activity.

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BASIC RESEARCH

Therapeutic effect of interleukin-10 on CCl_4-induced hepatic fibrosis in rats

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Abstract

AIM: To study the therapeutic effect of exogenous interleukin-10 on CCl_4-induced hepatic fibrosis in rats and its possible mechanisms.

METHODS: Fourty-seven SD rats were randomly divided into control group (group N) and CCl_4-induced hepatic fibrosis model group (group C). After CCl_4 was given for 9 wk, the model group was divided into three groups. Rats in group M were put to death immediately, rats in group T were treated with IL-10 for another three wk and then put to death, rats in group R recovered after three weeks and were then killed. The degree of hepatic fibrosis was measured by HE staining and histological activity index (HAI). Histological activity index (HAI), change of collagen types I and III were measured by Picrosirius staining. The expression of TNF-α, MMP-2 and TIMP-1 in liver tissue was measured by S-P immunohistochemistry.

RESULTS: CCl_4- induced experimental rat hepatic fibrosis model was established successfully. The degree of hepatic fibrosis was markedly lower in group T than in groups M and R, and there was no difference between the two groups. The expression of collagen types I and III was significantly suppressed in group T and was slightly suppressed in groups M and R. The positive levels of TNF-α, MMP-2 and TIMP-1 in group M increased significantly compared to those in group N ($P < 0.01$). The positive signals decreased significantly in groups T and R ($P < 0.01$), but positive score was significantly lower in group T than in group R ($P < 0.01$).

CONCLUSION: Exogenous IL-10 can reverse CCl_4-induced hepatic fibrosis in rats. IL-10 may exert its reversible effects on hepatic fibrosis by blocking CCl_4-induced inflammation, inhibiting expression of MMP-2 and TIMP-1 and promoting resolution of collagen types I and III.

Key words: Rat; Hepatic fibrosis; Interleukin-10; Tumor necrosis factor; Matrix metalloproteinase; Collagen

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INTRODUCTION

Hepatic fibrosis represents the final common pathological outcome for the majority of chronic liver insults. Its final stage is cirrhosis. Liver cirrhosis, the irreversible terminal stage of chronic liver disease, characterized by widespread fibrous scaring, is a major cause of morbility and mortality worldwide, with no effective therapy. Regardless of causes, hepatic fibrosis involves abnormal accumulation of extracellular matix (ECM) components, particularly collagens. Present data indicate that a specific potentially safe orally bioavailable and inexpensive antifibrotic agent is not yet available[1]. Interleukin-10 (IL-10) has anti-inflammatory and immunomodulatory effects and can down-regurate production of proinflammation cytokines, such as interleukin-1, interferon-gama and interleukin-2 from T cells. Previous reports indicate that IL-10 might have antifibrogenic properties by downregulating profibrogenic cytokines, such as TGF-β_1. Many studies indicate that IL-10 may become a new therapeutic target[2]. Therefore, the aim of this study was to evaluate its therapeutic effect on reversing well-established hepatic fibrosis after 9 wk of CCL_4 administration through different markers.

MATERIALS AND METHODS

Materials

Forty-seven clean male Sprague-Dawley rats weighing 180-280 g were provided by Shanghai Experimental Animal Center. All the rats were bred under routine condition (room temperature of 22±2℃, humidity of 55±5%) in a 12 h light/dark cycle with free access to water and food. The food was provided by BK Company (Shanghai,

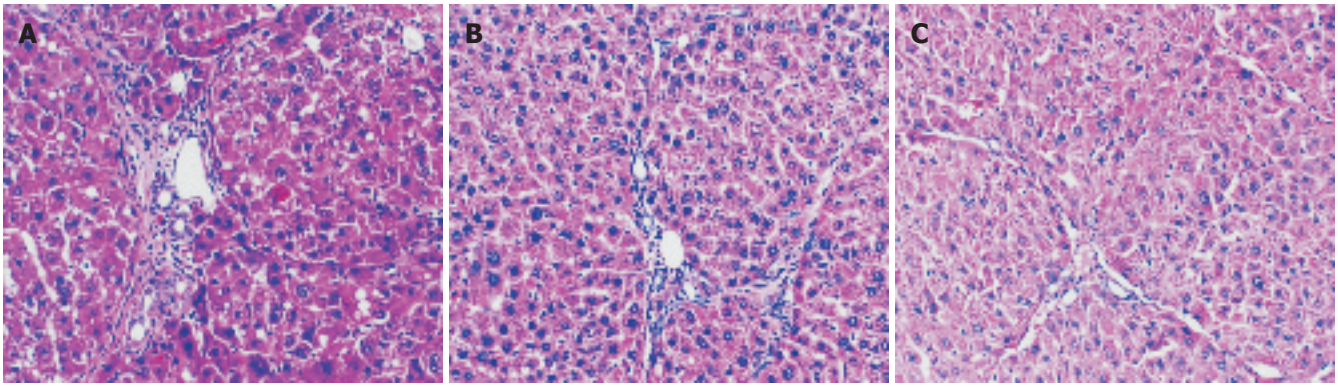

Figure 1 Effects of IL-10 on histology of CC4-induced fibrotic rat liver after treated with CCL4 for 9 wk (**A**), IL-10 for 3 wk (**B**) and spontaneous recovery for 3 wk (**C**).

China). IL-10 was purchased from Jingmei Biotechnology Company of Shenzhen. MMP-2 monoclonal antibody was purchased from NeoMarkers Company. TNF-α, TIMP-1 polyclonal antibodies and S-P immunohistochemical kit were obtained from Zhongshan Company of Beijing.

Preparation of rats

Forty-seven clean SD rats were randomly divided into control group (group N, $n=6$) and CCl_4-induced hepatic fibrosis model group (group C, $n=41$). The rats of group N were injected intraperitoneally with saline (2 mL/kg) twice a week. After 9 and 12 wk, 3 rats of groups N were sacrificed and their livers were taken out. The rats of group C were injected intraperitoneally with 50 % CCl_4 dissolved in castor oil (2 mL/kg) twice a week for nine weeks. After injection group C was divided into three groups. Rats in group M were put to death immediately by the end of week 9, rats in group T were treated with IL-10 (4 ug/kg) three times a week for three weeks and then put to death, rats in group R recovered after three weeks and were then killed.

Histopathological examination

Rats of groups N, M, T and R were sacrificed and their livers were taken out. The specimens were fixed in 10% formalin and embedded with paraffin. The sections stained with hematoxylin and eosin were evaluated by two pathologists. Histological activity index (HAI) was evaluated using a numerical system proposed by Knodell *et al*[3].

Picrosirius staining and collagen measurement

The sections were deparaffinized with xylene and rehydrated with graded ethanol. After rinsing, the sections were washed 3 times with distilled water, stained in 0.1% Sirius red in saturated picric acid solution for 30 min, and put into ethanol for differentiation for 2 min. The sections were then rinsed once in phosphate-buffered saline and twice in water for 30 s each to remove any unbound dye. After drying for two hours, slides were mounted. Quantitative analysis of collagen types I and III was made with the Olympus-BX41 image analyzing system in 5 microscopic fields (40x magnification) of per section. The average of the 5 fields was calculated for assessment of the degree of fibrosis in each case. All sections were examined by the same person. The liver tissue was distinguished from the background according to a difference in light density, which allowed the measurement of the total liver tissue area. Then the amount of connective tissue stained red was measured. Finally, the percentage of collagen on the section was calculated.

Immunohistochemistry

Rat liver tissues were sectioned at the thickness of 4 μm. After deparaffinization with xylene and rehydration in graded ethanol, the sections were incubated in PBS containing 30 mL/L H_2O_2 to remove endogenous peroxidase and in PBS containing 0.1 mol/L citrate to retrieve microwave antigens and then with normal goat serums to block the nonspecific binding sites. After incubation with rabbit anti-rat MMP-2(monoclonal antibody) as well as TNF-α and TIMP-1 polyclonal antibodies respectively, the sections were treated with instant S-P immunohistochemical reagents and then incubated in a buffer solution containing 3, 3-diaminobenzidine tetrahydrochloride (DAB) and H_2O_2 to produce a brown reaction product, dehydrated and coverslipped. Microscopic examination of the sections was then performed as previously described[4].

Statistical analysis

All data were expressed as mean ± SE. The difference between groups was studied with SPSS11.0 by one-way ANOVA. $P<0.05$ was considered statistically significant.

RESULTS

Histological examination

CCl_4-induced experimental rat hepatic fibrosis model was established successfully. After treatment with CCl_4 for 9 wk (group M), the liver had severe pathological damages, such as hepatocyte degeneration and necrosis, mononuclear cells and neutrophil infiltration and collagen deposition. The collagen fibers began to extend with hepatic plate and formed intact fibrous septum and distorted tissue architecture, abnormal hepatic lobules could be observed occasionally (Figure 1A). The liver tissue in group N showed normal lobular architecture with central veins and radiating hepatic cords. The liver of group T showed that

Table 1 Grading and staging of rat liver histopathology

Group	Number	Grading of inflammation					Staging of fibrosis				
		0	1	2	3	4	0	1	2	3	4
N	6	6	0	0	0	0	6	0	0	0	0
M [bf]	9	0	0	9	0	0	0	0	0	0	9
R [bdf]	9	0	7	2	0	0	0	0	0	0	9
I [bdhfi]	9	0	7	2	0	0	0	0	3	6	0

Grading of inflammation: $^{b}P<0.01$ *vs* group N ; $^{d}P<0.01$ *vs* group M; staging of fibrosis: $^{f}P<0.01$ *vs* group N; $^{h}P<0.01$ *vs* group M; $^{i}P<0.01$ *vs* group R.

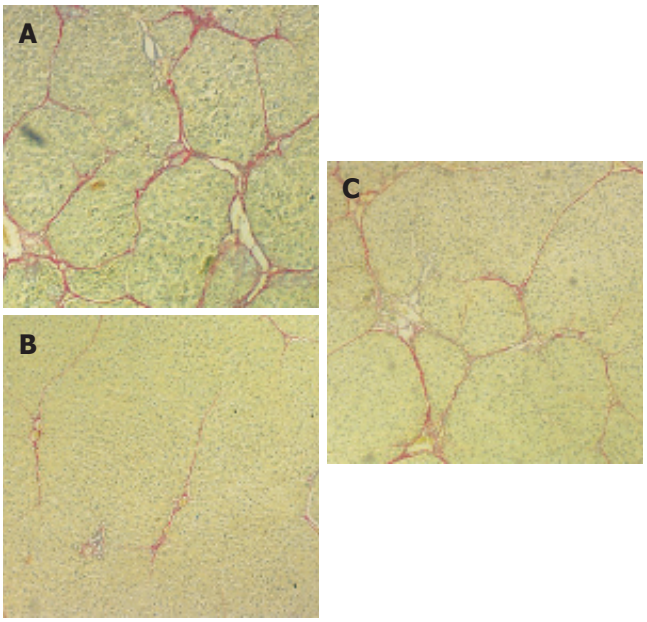

Figure 2 Photomicrographs of liver tissue from rats after treatment with with CCL_4 (**A**), IL-10 for 3 wk (**B**) and spontaneous recovery for 3 wk(**C**) by Picrosirius staining (40x).

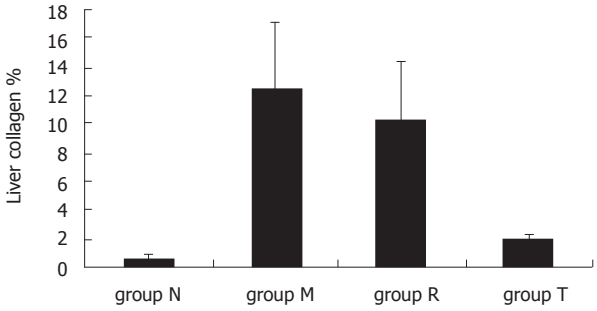

Figure 3 Percentage of collagen types I and III in fibrotic rats.

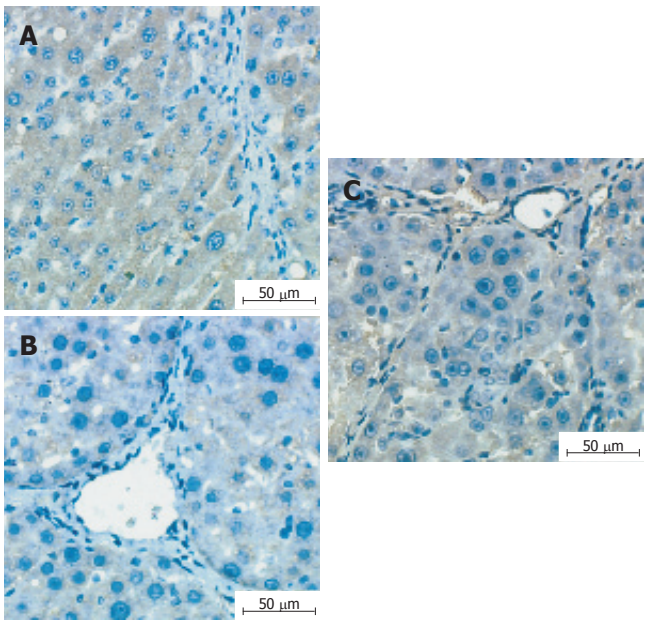

Figure 4 Expression of MMP-2 protein in liver tissue of rats treated with CCL_4 for 9 wk (**A**), IL-10 for 3 wk (**B**) and spontaneous recovery for 3 wk (**C**).

the degree of hepatocyte necrosis and degeneration was decreased markedly, and there were a few inflammatory cell infiltrates around central lobular veins, deposition of collagen fibers was relieved (Figure 1B). In addition, collagen fibers were changed slightly in group R (Figure 1C). According to HAI score, the degree of inflammation of liver decreased markedly in groups T and R compared to group M. The degree of hepatic fibrosis decreased markedly in group T compared to group M ($P<0.01$), but there was no difference between groups R and M ($P>0.05$, Table 1).

Change of collagen types I and III in liver

Collagen types I and III were stained intensely red with the Picrosirius procedure, while the non-collagen tissue was stained yellow. CCL_4 treatment for 9 wk induced a significant deposition of collagen types I and III, leading to severe fibrosis (Figure 2A). Little collagen was deposited around central vain in group N. After IL-10 treatment for 3 wk, the degree of hepatic fibrosis was markedly reduced, the area of collagen types I and III was significantly decreased (Figure 2B). After spontaneous recovery for 3 wk, obvious deposition of collagen types I and III was observed (Figure 2C).

The area of collagen types I and III increased from $0.64\pm0.11\%$ of field area in group N to $12.41\pm4.62\%$ of field area in group M ($P<0.01$), while the areas of collagen dramatically decreased to $2.00\pm0.31\%$ in group T ($P<0.01$, Figure 3). Although there was a trend toward lower values of collagen types I and III in group R ($10.24\pm4.12\%$.) compared to group M, the difference failed to reach any statistical significance ($P>0.05$).

Relative quantities of MMP-2 and TIMP-1 in the liver

Positive expression of MMP-2 was localized in endothelial and hepatic cells. Positive expression of TIMP-1 was localized in cytoplasm of hepatocytes and biliary epithelial cells, but not in nuclei. After treatment with IL-10, the distribution area of MMP-2 and TIMP-1 was smaller and the color was lighter (Figures 4 and 5).

The expression of MMP-2 and TIMP-1 was markedly higher in group M than in group N ($P<0.05$). The expression of these two cytokines in group T and R was significantly reduced compared to those in group M ($P<0.05$). However, the levels of MMP-2 and TIMP-1 decreased markedly in group T compared to those in group R ($P<0.05$). Tissue concentrations of MMP-2 and

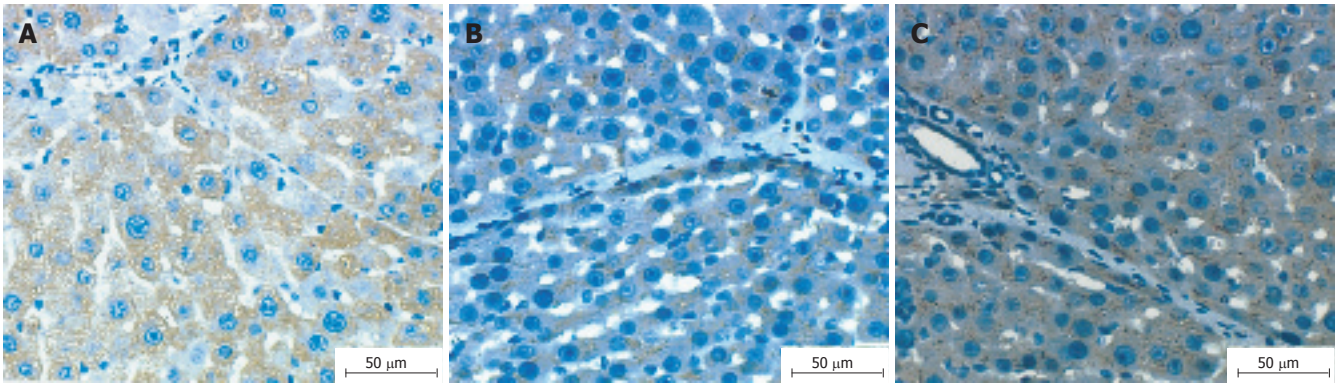

Figure 5 Expression of TIMP-1 protein in liver tissue of rats treated with CCL_4 for 9 wk (**A**), IL-10 for 3 wk (**B**) and spontaneous recovery for 3 wk (**C**).

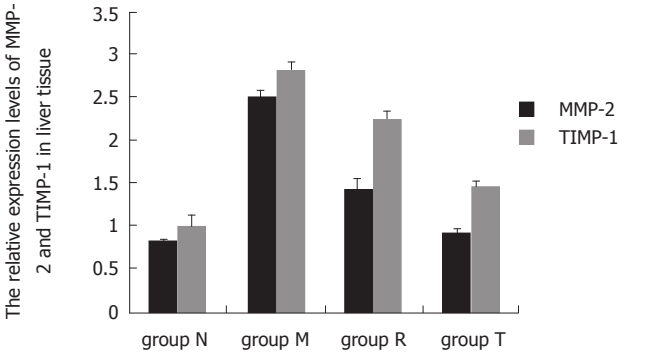

Figure 6 Relative expression levels of MMP-2 and TIMP-1 in liver of different groups.

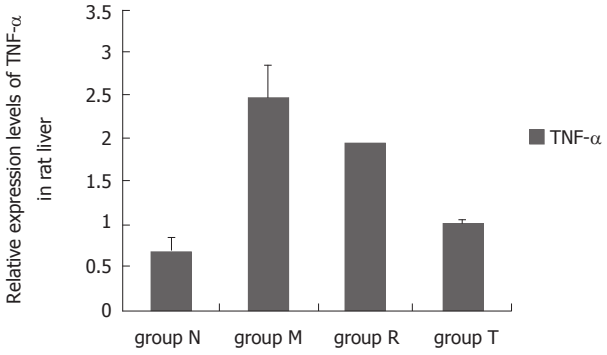

Figure 8 Relative expression of TNF-α in rat liver of different groups.

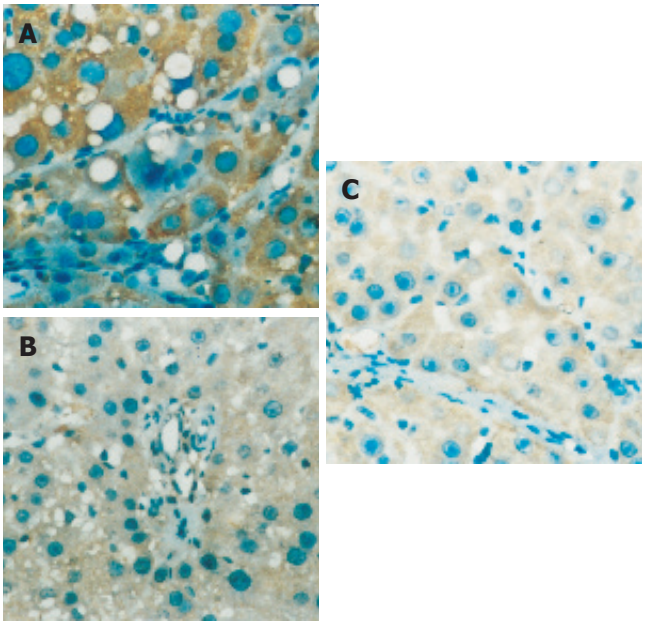

Figure 7 Expression of TNF-a protein in liver tissue of rats treated with CCL4 for 9 wk (**A**), IL-10 for 3 wk (**B**) and spontaneous recovery for 3 wk (**C**).

TIMP-1 were decreased after IL-10 treatment for 3 wk (Figure 6).

Relative quantity of TNF-α in liver

Positive expression of TNF-α was localized in most hepatic cells. After treatment with IL-10, the distribution area was smaller and the color was lighter (Figure 7).

The expression of TNF-α was markedly higher in group M than in group N ($P<0.05$). The expression of this cytokine in group T was significantly reduced compared to group M ($P<0.01$), but the level of TNF-α in group R was lower than that in group M but higher than that in group T ($P<0.01$, Figure 8).

DISCUSSION

Hepatic fibrosisis is a progressive pathological process involving multi-cellular and molecular events that ultimately lead to deposition of excess matrix proteins in the extracellular space. It is generally accepted that hepatic stellate cells (HSCs) are central to the process of fibrosis as the major source of ECM components[5-10]. Advanced fibrosis and cirrhosis are generally considered to be irreversible conditions even after removal of the injurious agent. However, data from the histological assessment of biopsy tissue from patients with liver fibrosis complicating chronic viral infection after successful treatment and from animal models of fibrosis indicate that recovery with remodeling of the excess collagens is possible[11-15], but current therapies targeting at arresting or reversing hepatic fibrosis are largely ineffective and have unacceptable side effects in long-term therapy.

IL-10, initially discovered in 1989, is a cytokine synthesis inhibitory factor for T lymphocytes[16]. IL-10 is produced by other cells of the immune system including

the liver. Within the liver, production of IL-10 has been documented within hepatocytes, sinusoidal cells, kupffer cells, stellate cells and liver-associated lymphocytes[17]. It was reported that endogenous IL-10 can decrease intrahepatic inflammatory response and fibrosis in several models of liver injury[18, 19]. It is well known that HSCs are the principal cells involved in hepatic fibrosis. Our previous studies indicate that exogenous IL-10 down regulates collagen type I in cultured HSCs and up regulates metalloproteinase gene expression *in vitro*[20, 21]. IL-10 may promote apoptosis of HSCs by up-regulating the expression of FasL and Bax and down-regulating the expression of Bcl-2 *in vitro*[22]. It also exerts antifibrogenic effect by down regulating profibrogenic cytokines such as TGF-b1 and TNF-a[23,24]. All these studies indicate that IL-10 might become a new therapeutic target. In this study, exogenous IL-10 was used to treat well-established hepatic fibrosis after 9 wk administration of CCL_4. Liver biopsy, the gold-standard method for detecting changes in liver fibrosis, showed that advanced fibrosis or cirrhosis was established after 9 wk administration of CCL_4, most of the fibrotic septa were resolved, and only small fibrotic fragments could be found after 3 wk of IL-10 treatment. The results indicated that exogenous IL-10 had therapeutic effect on advanced fibrosis. Predominant collagens in fibrotic liver were collagen types I and III. There were massive depositions of collagen types I and III in the peak of fibrosis, while the areas of collagen types I and III were significantly decreased 3 wk after IL-10 treatment. Matrix degradation may occur predominantly as a consequence of the action of a family of enzymes called matrix metalloproteinases (MMPs), and the expression of these enzymes is inhibited by a family of TIMPs[25-27]. To explore the reason why IL-10 results in a significant reduction of hepatic fibrosis, we investigated the effect of IL-10 on expression of MMP-2 and TIMP-1 by immunohistochemistry. The data showed that in the peak of fibrosis, the expression levels of MMP-2 and TIMP-1 were significantly increased. After 3 wk IL-10 treatment, the expression levels of MMP-2 and TIMP-1 were significantly decreased, indicating that collagen types I and III are be associated with the decrease of MMP-2 and TIMP-1 levels.

IL-10 is a pleiotropic cytokine, which has anti-inflammatory inhibitory action on the immune response under various stimuli. TNF-α is a pro-inflammation cytokine and a major endogenous mediator of hepatotoxicity[28]. TNF-α is expressed in chronic liver injuries by both infiltrating inflammatory cells and hepatocytes and plays an important role in tissue damage[29]. In this study, the results of HE staining showed that IL-10 could suppress inflammation induced by CCL4. TNF-α was significantly increased 9 wk after initial CCL_4 treatment, but 3 wk treatment with exogenous IL-10 markedly suppressed the expression of TNF-α in liver, suggesting that IL-10 exerts its inhibitory effect on hepatic fibrosis by blocking the release of inflammatory mediators such as TNF-α, which may consequently suppress HSC activation leading to hepatic fibrosis. A similar result has been reported by Nakamuta *et al*[30].

Issa *et al*[31] studied a model of cirrhosis to determine the mechanisms mediating and limiting spontaneous recovery, and found that micronodular cirrhosis undergoes remodeling to macronodular cirrhosis. Lee *et al*[32] reported that hepatic fibrosis can reverse gradually[32]. In this study, livers were harvested from rats for spontaneous recovery from hepatic fibrosis induced by CCl_4. Histology of liver sections indicated that advanced septal fibrosis observed at peak fibrosis was not remodeled. On the other hand, the degree of inflammation of liver decreased markedly after spontaneous recovery and the expression levels of TNF-α, MMP-2 and TIMP-1 decreased, suggesting that inflammation is decreased, but fibrosis is not significantly changed after removal of CCL_4. The mechanisms need to be elucidated.

In conclusion, the therapeutic effect of IL-10 on hepatic fibrosis is not only related with removal of deposited collagen and expression levels of MMP-2 and TIMP-1, but also related with the degree of inflammation.

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BASIC RESEARCH

Interleukin-1 beta up-regulates tissue inhibitor of matrix metalloproteinase-1 mRNA and phosphorylation of c-jun N-terminal kinase and p38 in hepatic stellate cells

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Abstract

AIM: To study the relationship between interleukin-1beta (IL-1β) up-regulating tissue inhibitor of matrix metalloproteinase-1 (TIMMP-1) mRNA expression and phosphorylation of both c-jun N-terminal kinase (JNK) and p38 in rat hepatic stellate cells (HSC).

METHODS: RT-PCR was performed to measure the expression of TIMMP-1 mRNA in rat HSC. Western blot was performed to measure IL-1β-induced JNK and p38 activities in rat HSC.

RESULTS: TIMMP-1 mRNA expression (1.191 ± 0.079) was much higher after treatment with IL-1β (10 ng/mL) for 24 h than in control group (0.545 ± 0.091) (P<0.01). IL-1β activated JNK and p38 in a time-dependent manner. After stimulation with IL-1β for 0, 5, 15, 30, 60 and 120 min, the JNK activity was 0.982 ± 0.299, 1.501 ± 0.720, 2.133 ± 0.882, 3.360 ± 0.452, 2.181 ± 0.789, and 1.385 ± 0.368, respectively. There was a significant difference in JNK activity at 15 min (P < 0.01), 30 min (P < 0.01) and 60 min (P < 0.01) in comparison to that at 0 min. The p38 activity was 1.061 ± 0.310, 2.050 ± 0.863, 2.380 ± 0.573, 2.973 ± 0.953, 2.421 ± 0.793, and 1.755 ± 0.433 at the 6 time points (0, 5, 15, 30, 60 and 120 min) respectively. There was a significant difference in p38 activity at 5 min (P < 0.05), 15 min (P < 0.01), 30 min (P < 0.01) and 60 min (P < 0.01) compared to that at 0 min. TIMMP-1 mRNA expression trended to decrease in 3 groups pretreated with different concentrations of SP600125 (10 μmol/L, 1.022 ± 0.113; 20 μmol/L, 0.869 ± 0.070; 40 μmol/L, 0.666 ± 0.123). Their decreases were all significant (P < 0.05, P < 0.01, P < 0.01) in comparison to control group (without SP600125 treatment, 1.163 ± 0.107). In the other 3 groups pretreated with different concentrations of SB203580 (10 μmol/L, 1.507 ± 0.099; 20 μmol/L, 1.698 ± 0.107; 40 μmol/L, 1.857 ± 0.054), the expression of TIMMP-1 mRNA increased. Their levels were higher than those in the control group (without SB203580 treatment, 1.027 ± 0.061) with a significant statistical significance (P < 0.01).

CONCLUSION: IL-1β has a direct action on hepatic fibrosis by up-regulating TIMMP-1 mRNA expression in rat HSC. JNK and p38 mitogen-activated protein kinases (MAPKs) are involved in IL-1β-induced TIMMP-1 gene expression, and play a distinct role in this process, indicating that p38 and JNK pathways cooperatively mediate TIMP-1 mRNA expression in rat HSC.

Key words: TIMMP-1; JNK; p38; Signal transduction; Interleukin-1β; Hepatic stellate cells

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INTRODUCTION

Hepatic fibrosis is a common consequence of chronic liver disease and results from the activation of hepatic stellate cells (HSC). After liver tissue damage, HSC undergo a transition from quiescent to activated phenotypes and increase proliferation and synthesis of extracellular matrix (ECM)[1-4]. Activated HSC express matrix metalloproteinases (MMPs), the key enzyme in the degradation of ECM, but also expresses the tissue inhibitors of matrix metalloproteinases (TIMMPs). Many cytokines may affect the activation of HSCs and regulate the secretion of MMPs and TIMMPs[5,6].

Mitogen-activated protein kinase (MAPK) plays an important role in the transduction of extracellular signals to the nuclei[7,8]. Four groups of mammalian MAPK-family have been characterized, namely extracellular signal-regulated kinases (ERK), c-jun N-terminal kinase (JNK),

p38 and ERK5. Studies indicate that JNK and p38 are essential members of MAPK supper family and play a role in the responses of HSC to hepatic injury and inflammation[9,10]. JNK and p38 are activated by lipopolysaccharide endotoxin (LPS), tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1). Activated JNK translocates to the nuclei, activates transcription factors and involves a wide range of cellular events, including cell proliferation, differentiation and apoptosis[11,12].

IL-1 is a pro-inflammatory cytokine that has a key role both in the inflammatory response and in autoimmune diseases[13-17]. Although HSC activation is insufficiently documented, IL-1 is accepted as a potent cytokine for fibrosis of other organs such as the heart, gingival tissue, and kidney[15,18,19]. Recent findings suggest that activation of MAPKs participates in intracellular signaling events induced by IL-1. In this study, we demonstrated the relationship between effects of interleukin-1β up-regulating TIMMP-1 mRNA expression and MAPK signal transduction in rat HSC.

MATERIALS AND METHODS

Reagents

RPMI 1640 was purchased from GIBCO BRL. SP600125, SB203580 and HEPES were purchased from Sigma Chemicals International. IL-1β was obtained from PeproTech INC. Mouse anti-phospho-JNK antibody and mouse anti- phospho-p38 antibody were purchased from Santa Cruz Company. Rabbit anti-β-actin polyclonal antibody was obtained from Zhongshan Company. RNasin was purchased from Promega Company.

Cell culture of rat HSC

HSC cell line (CFSC) was established and presented by Professor Greenwell, Marion Bessin Liver Research Center, Albert Einstein College of Medicine. The phenotype of CFSC was activated HSC obtained from CCl_4-induced cirrhotic liver of rats after spontaneous immortalization in culture. Cells were seeded and grown at 37℃ in 50 mL/L CO_2 RPMI 1640 supplemented with 10% fetal calf serum (FCS), 4 mmol/L L-glutamine, 1 mmol/L HEPES and 100 U/mL penicillin/streptomycin. When the cells were 80%-90% confluent, HSC were serum-starved in medium containing 1% FCS for 12 h. HSC of IL-1β groups were treated with IL-1β (10 ng/mL) for 24 h, but HSC of control group were treated with nothing, then TIMMP-1 mRNA expression in rat HSC was examined. After stimulation with IL-1β (10 ng/mL) for 0, 5, 15, 30, 60, and 120 min, the activities of JNK and p38 were examined. To study the relationship between IL-1β up-regulating TIMMP-1 mRNA expression as well as JNK and p38 signal pathway, JNK inhibitor SP600125 and p38 inhibitor SB203580 were used to inhibit JNK and p38 activities. When HSC were pretreated for 30 min with 1% dimethyl sulfoxide (DMSO, a solvent of SP600125 and SB203580), SP600125 or SP203580 was then treated with IL-1 beta (10 ng/mL) for 24 h, and subsequently TIMMP-1 mRNA expression in rat HSC was examined. SP600125 and SB203580 were prepared as previously described[20, 21].

Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR was performed to measure the expression level of TIMMP-1 mRNA in rat HSC. The sequences of the primers used for TIMMP-1 sense and antisense are 5' -TCC CCA GAA ATC ATC GAG AC-3' (sense) and 5' -ATC GCT GAA CAG GGA AAC AC-3' (antisense), a 329 bp fragment was amplified. The sequences of the primers used for GAPDH sense and antisense are 5'-GGC CCC TCT GGA AAG CTG TG -3' (sense) and 5'-CCG CCT GCT TCA CCA CCT TCT-3' (antisense), a 239 bp fragment was amplified. Total RNA was extracted from cultured HSC using TRIzol reagent following the manufacturer's instructions. Then 2 μg RNA of each sample was reverse-transcribed using random primer and reverse transcriptase in 25 μL of volume. Subsequently PCR was carried out in 25 μL reaction mixture containing 5 μL cDNA template, 2.5 μL 10×PCR buffer, 1 μL 10 mmol/L dNTPs, 1.5 μL 15 pmol/L TIMMP-1 or GAPDH primers, 2.5 U Taq DNA polymerase. Thirty-five cycles of PCR amplification for TIMP-1 and 30 cycles of PCR amplification for GAPDH were carried out for 5 min at 94℃ for initial DNA denaturation, followed by individual cycles of denaturation (at 94℃ for 45 s), annealing (at 56 ℃ for 35 s), polymerization (at 72℃ for 45 s) and then a final extension at 72℃ for 5 min. PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide (EB) and quantitated using Gel-Pro Analyzer Version 3.0. The band intensity of TIMMP-1 was compared to that of GAPDH, and the amount of TIMMP-1mRNA was estimated.

Western blot analysis

Western blot was performed to measure IL-1β-induced JNK and p38 activation in rat HSC. The HSC were lysed on ice by lysis buffer containing 50 mol/L Tris-HCl (pH 7.5), 150 mol/L NaCl, 10% glycerol, 1% Nonidet P-40, 1% SDS, 0.5% deoxycholate, 1.0 mol/L PMSF, and 1 mol/L sodium orthovanadate for 30 min. The cell lysate was centrifuged at 10 000 r/min for 10 min and the supernatant was collected for Western blot analysis. Protein concentration was measured using Coomassie brilliant blue G-250 (CBB) kit following the manufacturer's instructions. Protein samples (50 μg) were subjected to 10% SDS-PAGE gel electrophoresis and then transferred onto a nitrocellulose membrane by electro-blotting. The membrane was incubated at 4 ℃ overnight in Tris-buffered saline/Tween 20 (20 mol/L Tris-HCl, pH 7.4, 150 mol/L NaCl, 0.05% Tween 20) with 5% nonfat milk. After blocking, the membrane was incubated for 5 h at room temperature in TBS buffer (50 mol/L Tris-HCl, 150 mol/L NaCl) containing an 1:100 dilution of mouse anti-phospho-JNK monoclonal antibody, anti-phospho-p38 monoclonal antibody or rabbit anti-β-actin polyclonal antibody. Then the membrane was incubated for 2 h at room temperature in TBS containing an 1:300 0 dilution of anti-mouse IgG (H+L) / HRP, 1:100 0 dilution of anti-mouse IgM (H+L) /HRP or 1:300 0 dilution of anti-rabbit IgG (H+L) /HRP antibody. Specific binding of the antibody was visualized by the enhanced chemiluminescence (ECL) detection system following the manufacturer's instructions. The intensity of

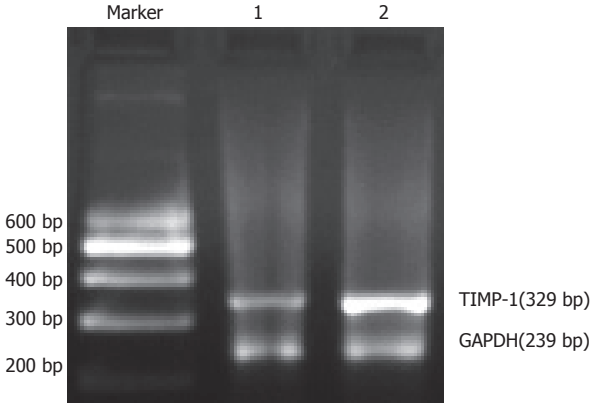

Figure 1 Expression of TIMMP-1 mRNA in HSC detected by RT-PCR. Marker: 200-600 DNA marker; lane 1: control group (without IL-1beta treatment); lane 2: IL-1β group (with IL-1β treatment)

the bands was determined by scanning video densitometry using Gel-Pro Analyzer Version 3.0. The levels of phospho-JNK protein and phospho-p38 protein were normalized to the level of β-actin protein.

Statistical analysis

The data were presented as mean ± SD. Statistical analysis was performed by the statistical software SPSS 11 using independent-sample *t* test and one-way ANOVA test. $P<0.05$ was considered statistically significant.

RESULTS

IL-1β up-regulated mRNA expression of TIMMP-1

We examined the mRNA expression of TIMMP-1 in rat HSC with RT-PCR. The ratio of TIMMP-1/GAPDH represented the expression of TIMMP-1 mRNA. The data showed that the TIMMP-1 mRNA expression (1.191 ± 0.079) in the group treated with IL-1β (10 ng/mL) for 24 h was much higher than that in the control group (0.545 ± 0.091). There was a statistical significance between the two groups ($P<0.01$, Figure 1).

IL-1β activated JNK and p38 in a time-dependent manner

After stimulation with IL-1β for 0, 5, 15, 30, 60 and 120 min, the activities of JNK, p38 and β-actin were measured. The intensity of the two bands at 46 KD and 55 KD of JNK and the band at 38 KD of p38 were compared to that of β-actin and the ratio represented JNK and p38 activity. At the 6 time points, the JNK activity was 0.982 ± 0.299, 1.501 ± 0.720, 2.133 ±0.882, 3.360 ± 0.452, 2.181 ± 0.789, and 1.385 ± 0.368 , respectively. There was a significant difference in JNK activity at 15 min ($P<0.01$), 30 min ($P<0.01$) and 60 min ($P<0.01$) compared to that at 0 h. The data showed that JNK activity increased slightly after stimulation with IL-1β for 5 min, but the difference was not significant when compared to that at 0 h (without IL-1β treatment). An apparently increased phosphorylation of JNK was first detected at 15 min in HSC and reached its peak at 30 min after IL-1β treatment. The values restored to the original levels at 120 min (Figure 2). On the other hand, the p38 activity was 1.061 ± 0.310, 2.050 ± 0.863, 2.380 ± 0.573, 2.973 ± 0.953, 2.421 ± 0.793, and 1.755 ± 0.433, respectively at the 6 time points. There was a significant difference in p38 activity compared to that at 0 h, 5 min ($P<0.05$), 15 min ($P<0.01$), 30 min ($P<0.01$) and 60 min ($P<0.01$). A significant increase was first observed at 5 min and peaked at 30 min. The values restored to the original levels at 120 min (Figure 2).

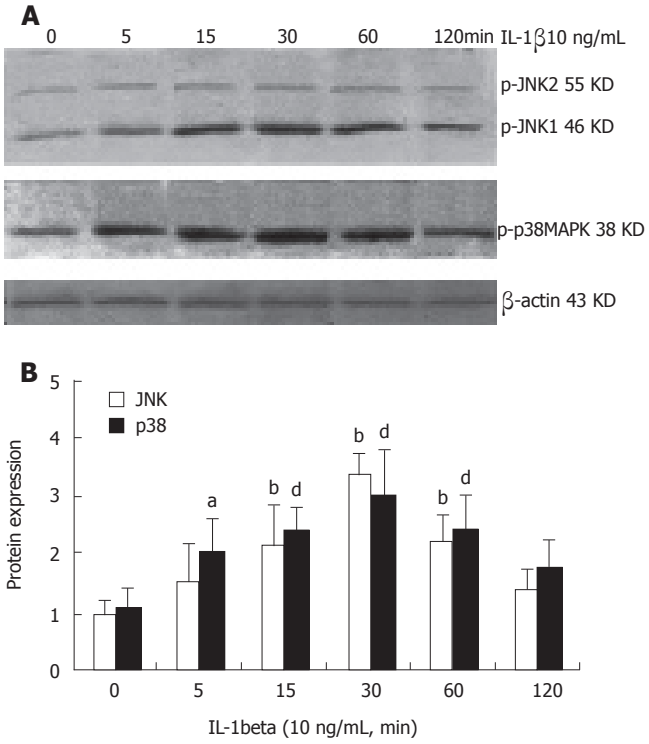

Figure 2 IL-1β activates JNK and p38 in a time-dependent manner in rat HSC. **A:** Representative Western blot results of JNK and p38; **B:** Densitometry of Western blot analyzed by Gel-Pro software. *n*=6. $^bP < 0.01$ *vs* 0 h of JNK, $^aP < 0.05$ *vs* 0 h of p38, $^dP < 0.01$ *vs* 0 h of p38.

Effect of SP600125 and SB203580 on IL-1β-induced expression of TIMMP-1 mRNA in rat HSC

TIMMP-1 mRNA expression induced by IL-1β trended to decrease in groups pretreated with different concentrations of SP600125 (10 μmol/L, 1.022 ± 0.113; 20 μmol/L, 0.869 ± 0.070; 40 μmol/L, 0.666 ± 0.123). When the concentration of SP600125 was increased, the expression of TIMMP-1 mRNA was gradually reduced. Compared to control group (without SP600125 treatment) (1.163 ± 0.107), there was a significant difference ($P<0.05$, $P<0.01$, $P<0.01$) (Figure 3). However, the expression of TIMMP-1 mRNA trended to increase in groups pretreated with different concentrations of SB203580 (10 μmol/L, 1.507 ± 0.099; 20 μmol/L, 1.698 ± 0.107; 40 μmol/L, 1.857 ± 0.054). When the concentration of SB203580 was increased, the expression of TIMMP-1 mRNA increased gradually. In comparison to control group (without SB203580 treatment) (1.027 ± 0.061), the difference was significant ($P<0.01$, Figure 3).

DISCUSSION

Hepatic fibrosis represents a repairable process of chronic hepatic damages including viral infection, toxic damage, alcohol, as well as autoimmune reactions. In response to

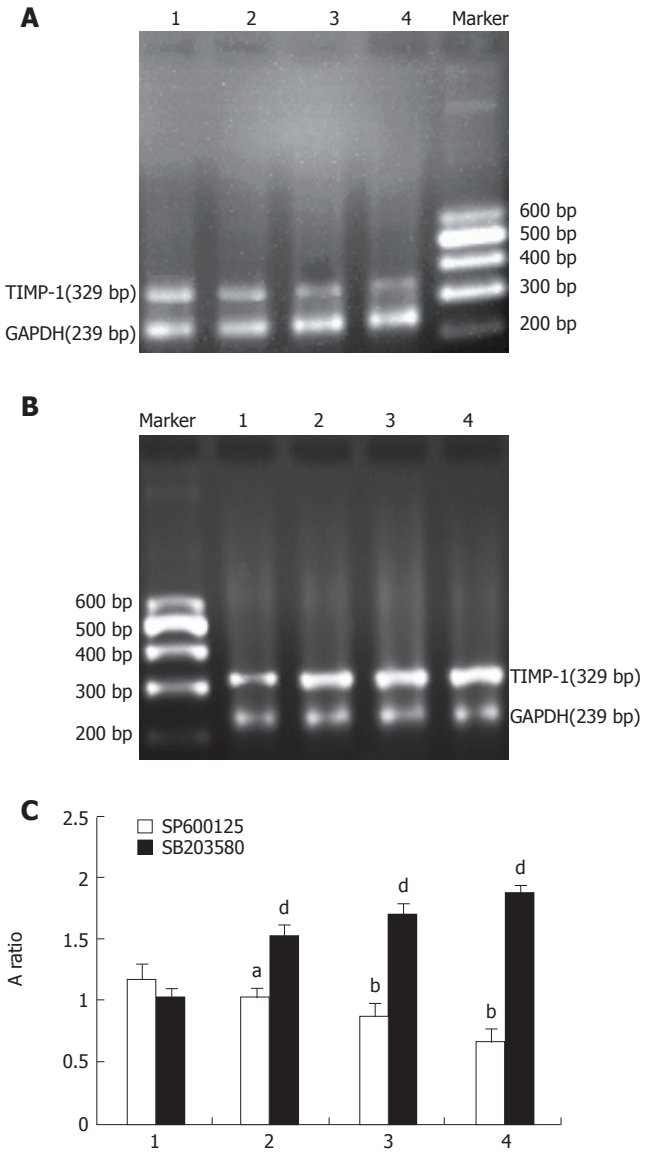

Figure 3 Effect of SP600125 and SB203580 on IL-1β-induced expression of TIMMP-1 mRNA in rat HSC. **A:** Representative photos of different concentrations of SP600125 of RT-PCR; **B:** Representative photos of different concentrations of SB203580 of RT-PCR. **C:** TIMMP-1 mRNA expression in **A** and **B**. 1: IL-1β + DMSO; 2: IL-1β + SP600125 or SB203580 (10 μmol/L); 3: IL-1β + SP600125 or SB203580 (20 μmol/L); 4: IL-1β + SP600125 or SB203580 (40 μmol/L).*n* = 6.^{a}P < 0.05 *vs* Il-1β+SP600125, ^{b}P < 0.01 *vs* IL-1β+SP600125, ^{d}P < 0.01 *vs* IL-1β + SB203580.

liver injury of any etiology, the normally quiescent HSC undergo a progressive process of trans-differentiation into α smooth muscle action (α-SMA) on positive myofibroblast-like cell-activated HSC. By increasing secretion of extracellular matrix proteins (TIMMP-1 and TIMMP-2), activated HSC is responsible for deposition and accumulation of the majority of excess ECM in the fibrotic liver. Furthermore, activated HSC can contribute to the fibrogenic process through their ability to secrete and respond to a wide range of cytokines and growth factors, such as IL-1, IL-6, transforming growth factor β(TGF-β) and platelet-derived growth factor (PDGF). MMPs are a family of zinc metalloendopeptidases and responsible for the turnover of all the ECM components. TIMMPs, specific inhibitors of MMPs, are the key regulators of MMP activity and ECM degradation. Some studies have shown that TIMMP is a very important promoting factor for hepatic fibrosis and inhibits MMPs to decompose ECM. In the liver, TIMMP-1 and TIMMP-2 have been identified and TIMMP-1 plays a more important role in the pathological process of hepatic fibrosis than TIMMP-2[22-26].

Inflammation is a key component of chronic liver disease. IL-1 is one of the major mediators regulating inflammatory response[27, 28]. There are two forms of IL-1, namely IL-1alph and IL-1beta with indistinguishable biological activities. IL-1 may be involved in hepatic fibrosis, causing hepatic tissue injury which induces the fibrotic response and participating in hepatic fibosis by promoting the deposition of ECM[5,7,29,30]. In the present study, TIMMP-1 mRNA expression after treatment with IL-1beta for 24 h was much higher than that in control group. Strong expression of TIMMP-1 inhibits the degradation of collagen by MMPs, thus promoting the deposition of ECM. The continuous deposition of ECM in the liver finally results in hepatic fibrosis, suggesting that IL-1beta has a direct action on hepatic fibrogenesis through stimulating TIMMP-1 production in activated HSC.

As we known, IL-1 could activate the MAPK cascades including ERK, p38 and JNK[31]. In 3 groups of the MAPK family, the role of ERK has been studied in HSC[9,10,30,32], but the role of p38 and JNK in regulating TIMMP-1 expression in HSC is poorly understood. The aim of our study was to evaluate the effect of p38 and JNK on TIMMP-1 mRNA expression induced by IL-1beta in HSC. Our data demonstrated that following stimulation with IL-1beta, the 2 MAPK pathways including p38 and JNK were all activated. In comparison to control group (without IL-1beta treatment), phosphorylation of JNK was first detected at 15 min in HSC and reached its peak at 30 min. The values restored to original levels at 120 min. In comparison to control group (without IL-1beta treatment), a significant increase of p38 activity was first observed at 5 min for p38 peaked at 30 min. The values returned to control levels at 120 min (Figure 2). The data showed that IL-1beta could activate JNK and p38 MAPKs in a time-dependent manner in rat HSC.

To study the relationship between IL-1beta up-regulating TIMMP-1 mRNA and phosphorylation of JNK and p38, JNK inhibitor SP600125 and p38 inhibitor SB203580 were used to inhibit JNK and p38 activities. Then TIMMP-1 mRNA expression in rat HSC induced by IL-1beta was observed. Our study clearly showed that blocking JNK could result in inhibition of TIMMP-1 mRNA expression in HSC, but inhibition of p38 in HSC increased TIMMP-1 mRNA expression. When the concentration of SP600125 was increased, the expression of TIMMP-1 mRNA was gradually reduced, but when the concentration of SB203580 was increased, TIMMP-1 mRNA expression increased gradually, indicating that the 2 MAPKs cooperatively modulate the TIMMP-1 mRNA expression in HSC when they are activated simultaneously by IL-1β. As a result, interaction between JNK and p38 pathways up-regulates TIMMP-1 mRNA expression in rat HSC induced by IL-1beta.The signal transduction in HSC induced by IL-1beta is very complex. Following the treatment with

IL-1beta, whether TIMP-1 has any other pathways such as JAK/STAT and PI3-K to promote hepatic fibrosis is still unknown. Further studies are needed to elucidate its mechanism.

In summary, differential signal transduction pathway triggered by IL-1β can lead to TIMMP-1 gene up-regulation in rat HSC. A better understanding of these pathways may contribute to the development of more rational therapies to counteract the devastating effects of hepatic fibrosis.

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RAPID COMMUNICATION

Sonographic signs of neutropenic enterocolitis

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Abstract

AIM: To investigate the sonographic features at time of diagnosis and follow-up in patients with neutropenic enterocolitis.

METHODS: The sonographic findings in 14 patients with neutropenic enterocolitis were described and evaluated regarding symptoms and clinical outcome.

RESULTS: In all patients with neutropenic enterocolitis, the ileocoecal region was involved with wall thickening >10 mm. A transmural inflammatory pattern, hypervascularity of the thickened bowel wall and free abdominal fluid were the common findings. The sonographically revealed thickness of the bowel wall was associated with lethal outcome ($P < 0.03$). In the 11 surviving patients, the improvement of clinical symptoms was accompanied by progressive reduction of intestinal wall thickness.

CONCLUSION: High-end sonography of the bowel is a helpful tool for diagnosis, assessment of prognosis and follow-up of patients with neutropenic enterocolitis. The ultrasonographically revealed bowel thickness reflects the severity and the course of the disease, and seems to be predictive for the clinical outcome.

Key words: Neutropenia; Bowel wall thickness; Ultrasonography

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INTRODUCTION

Neutropenic colitis, also termed necrotizing enterocolitis or neutropenic typhlitis (Greek: *typhlon* = cecum), was first described by Cooke in 1930[1,2]. It is a necrotizing inflammatory disease of the ileocoecal region. The pathogenesis is not entirely known, although bacterial infection of damaged mucosa, often with superinfection, e.g., *clostridia* (especially *Clostridium septicum*) and other bacteria, seems to play an important role[3-7].

Neutropenic colitis is a complication of severe neutropenia, often in patients after (high-dose) chemotherapy. Although most reported cases occurs in patients, who received chemotherapy for leukemia or solid tumors, it has been seen in a variety of other conditions, e.g. aplastic anemia, benign cyclic neutropenia, AIDS and allergic or toxic agranulocytosis as well[8,9].

Neutropenic colitis has a wide spectrum of severity, ranging from mild gastrointestinal symptoms to peritonism and sepsis with lethal outcome. As abdominal discomfort and diarrhea are common in patients receiving chemotherapy, the prevalence of this condition is not well known. The finding of thickened bowel wall, in association with a clinical syndrome characterized by fever, diarrhea, and abdominal pain, confirms the clinical diagnosis of neutropenic enterocolitis in neutropenic patients who have completed intensive chemotherapy.

A Recent report[10] has shown that sonographic findings might be helpful in this condition and can be easily repeated in these severely ill patients. Herein we report on 14 neutropenic patients with sonographic features of neutropenic colitis, evaluating the clinical findings, sonographic features and outcome of these patients and reviewing of the literature regarding this condition.

MATERIALS AND METHODS

Patients

We reviewed the clinical records of 14 neutropenic patients (absolute neutrophil count $< 500/mm^3$) with sonographic signs of neutropenic colitis who presented at the Department of Gastroenterology for sonography during a period of three years. The patients consisted of 8 men and 6 women [age = 48 (22-66) years; body mass index = 23.5 (17.8-28.2) kg/m^2; weight loss = 3.5 (0-14) kg]. Neutropenia occurred due to intensive chemotherapy in 13 patients (six patients with non-Hodgkin lymphoma, four patients with acute lymphatic leukemia, two patients with acute lymphoblastic leukemia, and one patient with chronic myeloid leukemia). One patient had allopurinol-in-

Table 1 Frequency of clinical symptoms, laboratory, and microbiological findings in 14 patients with neutropenic enterocolitis.

Clinical symptoms	Frequency
Abdominal pain	14 (100%)
Fever	14 (100%)
Diarrhea	10 (71.4%)
Hypotension	3 (21.4%)
Right lower quadrant pain	10 (71.4%)
Peritonism	6 (42.9%)
Abdominal distension	10 (71.4%)
Vomiting	9 (64.3%)
Bloody diarrhea	6 (42.9%)
Ileus	6 (42.9%)
Reduced clinical condition	12 (85.7%)
Palpable resistance	7 (50%)
Daily bowel movements	4 (1-7)
Laboratory findings	
C-reactive protein [mg/dL]	26.3 (6.6-64.0)
Haemoglobin [g/dL]	9.1 (6.9-11.4)
Haematocrit [%]	25.8 (21.1-33.9)
Platelets [/nL]	33 (19-43)
Microbiological findings	6 patients with negative cultures 3 patients with *Streptococcus epidermidis* in blood culture 2 patients with *Enterococcus* in blood culture 1 patient with *Pseudomonas aeroginosa* in blood culture 1 patient with *Streptococcus*, *Enterobacteriaceae*, Enterococcus, and *Escherichia coli* in blood culture 1 patient[1] with *Candida albicans* in bronchio¬alveolar lavage, *Candida glabrata* and *Entero¬coccus faecium* in blood culture, *Pseudomonas aeroginosa* and *Candida albicans* in urine culture

[1]Patients did not survive.

duced severe neutropenia. All patients were monitored by performing blood and feces cultures. In addition, sputum, nasopharynx, oropharynx, and external-genital cultures were performed when appropriate. Clinical symptoms, chemotherapeutic and antibiotic agents, and microbiological findings were documented and evaluated in relation to sonographic results and outcome.

Examination technique

Real-time ultrasound scanning of the bowel wall was performed using a 7 MHz linear transducer (Siemens Elegra, Erlangen, Germany or Acuson Sequoia, Siemens Erlangen, Germany). Bowel wall thickness (outer wall to luminal surface) was measured at terminal ileum, cecum, sigmoid colon, and small intestine by high resolution sonography with moderate pressure as previously described[11,12]. A thickness of less than 2 mm was considered normal, more than 2 mm and less than 5 mm was considered unspecific bowel wall thickening, and a thickness of more than 5 mm was considered abnormal confirming the diagnosis of neutropenic enterocolitis. The morphology and the five layers of ileum and colon were evaluated assessing a mucosal or transmural pattern of inflammation. Bowel wall vascularity was examined by color Doppler imaging and graded as ischemic (no flow pattern), normal or hypervascular[12,13] as recently shown for graft *versus* host disease (GvHD) as a prognostic factor.

Statistical analysis

Data were expressed as median and range. χ^2 test was used to evaluate differences in sonographic findings (ascites, bowel wall vascularity, extension of inflammation), symptom presentation and death rate. Differences in bowel thickness and laboratory data between surviving and non-surviving patients were analyzed using the nonparametric Wilcoxon rank sum two-tailed test. 95% confidence intervals were calculated. $P < 0.05$ was considered statistically significant.

RESULTS

The clinical symptoms and laboratory findings of the 14 neutropenic patients with sonographic signs of neutropenic enterocolitis are mentioned in Table 1.

In all 14 patients, treatment consisted of multiple antibiotic therapy. The antibiotic treatment usually started using beta-lactamic combined with aminoglycoside. Vancomycin was added if the fever persisted. In 9 cases, antimycotic agents were administered due to suspected invasive fungaemia.

G-CSF was given in 8 patients to shorten the time of neutropenia. Supportive treatment consisted of bowel wall rest and total parenteral nutrition. Packed RBC's, platelets, and albumin were infused when appropriate.

Intensive chemotherapies, according to the protocols for the underlying malignant diseases, preceded in 13 patients with the onset of symptoms. The chemotherapies were intended to induce (first) complete remission. In all protocols, drugs were administered in daily doses which are known to be toxic for the gastrointestinal mucosa (500 to 3 000 mg/m^2 high dose cytarabine instead of the standard dose of 100 to 200 mg/m^2, 100 to 200 mg/m^2 etoposide, 10 to 12 mg/m^2 idarubicin, 10 mg/m^2 mitoxantrone, 60 mg/m^2 daunorubicin, 50 mg/m^2 doxorubicin, or 500 to 3 000 mg/m^2 methotrexate).

Three patients died due to sepsis with multiorgan failure. High C-reactive protein ($P < 0.001$) and the sonographically revealed significantly thickened bowel wall ($P < 0.03$) were associated with the lethal outcome.

The surviving 11 patients recovered from neutropenic enterocolitis. The reduction of abdominal symptoms was accompanied by progressive decrease in intestinal mural thickening in follow-up ultrasound examinations. The sonographic findings in 14 patients with neutropenic colitis are summarized in Table 2. Nearly all patients with neutropenic enterocolitis (92.9%, 13/14) presented a transmural inflammatory pattern of the thickened bowel wall. In all patients, the ileocoecal region was involved. The thickness of the inflammated bowel section was at least 10 mm. Hypervascularity on color Doppler imaging and the detection of small amounts of free fluid in the abdomen were documented in the majority of patients with neutropenic enterocolitis. Free abdominal air, fistula or abscesses were not observed in our collective. Typical sonographic charac-

Table 2 Sonographic findings in 14 patients with neutropenic enterocolitis (frequency of findings or median and ranges are given)

Localisation of inflammation	13 in ileocoecal region 1 in ileocoecal region and colon transversum
Pattern of inflammation	10 transmural 4 mural
Longitudinal extension of inflammation	5-15 cm in all 14 patients
Bowel wall thickening (mm)	12 (10-20)
Ascites	11 of 14
Hypervascularisation	84.61% (13/14)
Fistula	None
Abscess	None

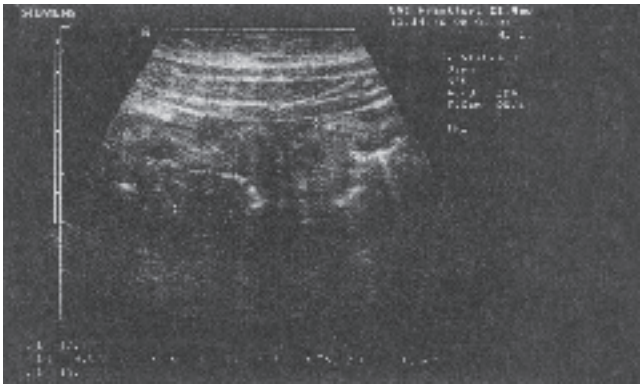

Figure 1 Characteristic sonographic findings in neutropenic enterocolitis showing asymmetric, echogenic (between markers) and inhomogenous bowel wall thickening with areas of hypoechogenity (between markers).

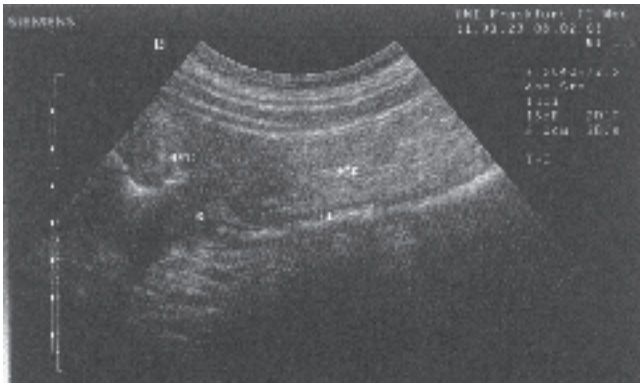

Figure 2 Asymmetric and inhomogenous wall thickening, transmural inflammatory reaction in a patient with neutropenic enterocolitis (NPC). The ileocoecal valve of Bauhin (**B**) and the terminal ileum (not affected, TI) are also indicated. Typically, the surrounding omentum shows echogenic reaction (UGR).

teristics in neutropenic enterocolitis are depicted in Figures 1 and 2.

DISCUSSION

Epidemiology

The incidence of neutropenic colitis in cytopenia patients ranges from 2.6[14] to 33%[15] with a pooled incidence rate from 21 studies of 5.3%[16]. With the use of more intensive chemotherapy regimens, especially after autologous and allogeneic stem-cell transplantations, a higher incidence of neutropenic colitis should be expected. The tendency to relapse has been reported to range from 27% to 83%[17].

Etiology and pathogenesis

Various factors are in discussion to play a role in the pathogenesis of neutropenic colitis. Apart from direct damage to the mucosa by leukemic of lymphatic infiltrates, toxic effects of chemotherapeutic agents contribute to the pathogenesis. The first step is the severe initial damage to the mucosa caused by release of pro-inflammatory cytokines from macrophages and monocytes, followed by a near complete arrest of the cell cycle, inhibition of mechanisms of repair and finally apoptosis. Several cytotoxic agents and combinations of chemotherapeutic agents, depending on their dose, have a high toxic potential[1,17-19].

According to the literature, neutropenic colitis is mainly localized in the ileocoecal region, although other parts of the bowel can be affected as well. The high concentration of lymphatic tissue in this area and the special anatomy of the terminal branches of the superior mesenteric artery with consecutive less vascular perfusion may contribute to ischemia. The cecum represents an area of relative stasis of bowel content and is easily distensible, causing a high intramural pressure and insufficient blood supply[7].

The role of bacteria and viruses in the pathogenesis of neutropenic colitis has been discussed controversially in the literature. Infection is thought to be largely secondary (e.g., Clostridial bacteria, especially *Clostridum septicum*). Abnormal bacterial colonisation in connection with the neurotoxic effects of vincristine is thought to contribute to the pathomechanism[20]. Although *Clostridium septicum* is found in stool cultures of healthy persons only in 2%, it is detectable in a higher percentage in the ileocoecal region, especially in the healthy appendix (63%)[2]. Clostridia produce a number of tissue degrading enzymes, which may play a significant role in the development of mucosal injury. In the absence of neutrophil granulocytes which produce toxin degrading proteases, an important defence mechanism is missing. Similar pathomechanisms have been described for other Clostridial species (e.g., *Clostridium perfringens*, *paraperfringens* and *Clostridium tertium*).

Symptoms

The initial symptoms are not specific and usually occur during the nadir with rapid improvement after neutrophil recovery. The symptoms very often consist of a combination of crampy abdominal pain (sub-ileus symptoms), a palpable mass and tenderness in the right lower quadrant with rebound tenderness (a sign of peritonism) and fever. Diarrhea, occasionally bloody diarrhea, may be present, but the leading symptom is abdominal pain in the right lower quadrant. Sepsis and signs of perforation with peritonism, as well as profuse bleeding are life-threatening complications.

A localized tenderness with rebound tenderness above the affected area is very often the only clinical sign in these severely ill patients. Recurrent abdominal pain, caused by mechanical obstruction of the ileocoecal area, indicates

Table 3 Clinical features of neutropenic colitis (review of the literature)

Abdominal pain	Fever	Diarrhea	Peritonism	Bloody diarrhea	Number of patients	References	
+	+	+			3	Abbasoglu *et al.*	[27]
+	+	+			3	Alexander *et al.*	[22]
+					2	Alt *et al.*	[7]
+			+		2	Avigan *et al.*	[5]
+		+	+		6	Benz *et al.*	[17]
+	+	+		(+)	3	Boggio *et al.*	[29]
+	+	+			1	Capria *et al.*	[30]
+	+	+	(+)	(+)	44	Cartoni *et al.*	[10]
+		+		+	1	Coleman *et al.*	[31]
+		+			1	Dudiak *et al.*	[9]
+	+				4	Frick *et al.*	[23]
			+		1	Frankel *et al.*	[32]
+	+	+		+	2	Furonaka *et al.*	[19]
+		+			2	Gandy *et al.*	[25]
+	+	+		+	1	Glass-Royal *et al.*	[33]
+	+	+	(+)	(+)	18	Gomez *et al.*	[34]
+	+		+		3	Hammerstrom *et al.*	[35]
+	+				1	Hopkins *et al.*	[36]
+	+	+			9	Hsu *et al.*	[37]
+	(+)	(+)	(+)	(+)	11	Jain *et al.*	[38]
+	+				3	Koea *et al.*	[26]
+	+	+	+	+	5	Koroussis *et al.*	[18]
+	+	+			1	Kronawitter *et al.*	[39]
+	+				2	Lev *et al.*	[20]
+	+	+			4	Mulholland *et al.*	[40]
	+				2	Newbold *et al.*	[6]
+	+	+	+		1	Rexroth *et al.*	[2]
+	+				1	Rodgers *et al.*	[41]
+	+	(+)	(+)	(+)	25	Shamberger *et al.*	[15]
+	+				1	Shandera *et al.*	[42]
+					14	Song *et al.*	[43]
+	+	+	+		1	Thaler *et al.*	[44]
+	+	+			1	Verbeeck *et al.*	[45]
+	+	+	(+)	(+)	6	Wach *et al.*	[28]
+	+		(+)		22	Wade *et al.*	[46]
+	+		+		2	Weinberger *et al.*	[47]

symptoms of ileus with dilatation of the bowel loops of the small intestine. Other more unspecific symptoms like abdominal distension, nausea, vomiting and meterorism *etc.* have also been described in the literature. Table 3 summarizes the symptoms mentioned in 36 case reports (including 209 patients overall). Abdominal pain was described in nearly all patients (98%), followed by fever in 87%, diarrhea in 61%, and peritonism in 30% of the patients with neutropenic enterocolitis (Table 3).

Differential diagnosis

Appendicitis is very often the main differential diagnosis. Because of the high perioperative mortalitiy in these patients, the operative approach should be avoided. The perioperative mortality rate in the literature varies widely and ranges between 0%-100%, depending on the case reports or the studies[1]. Besides appendicitis, other acute or chronic inflammatory diseases of the ileocoecal area, e.g., bacterial ileocecitis, cytomegalovirus (CMV) infection, Crohn's disease, pseudomembranous and ischemic colitis, should be taken into account. In patients after allogeneic stem-cell transplantation, one has to think of graft *versus* host disease, although this usually occurs after engraftment.

A neoplastic (lymphocytic leukemia) infiltration of the ileocoecal region must be excluded especially in case of a palpable mass in this area. In pancytopenic patients, one has also to think of an acute hemorrhage into the mucosal wall.

Diagnosis

Besides the routine laboratory and microbiological tests for bacteria (e.g., *Clostridium difficile* and toxin) viruses and parasites, one should perform the CMV PCR- and CMV early (pp65) antigen-test. The endoscopic approach during pancytopenia is relatively contraindicated, although the definitive diagnosis of CMV-colitis, leukemic or neoplastic infiltrates can be definitively diagnosed only by histological examination.

High resolution sonography The characteristic sonographic features of neutropenic colitis are echogenic, asymmetric thickening of the mucosal wall[10,21,22] with transmural inflammatory reaction and areas of different echogenity caused by edema, necrosis and/or circumscript hemorrhages. Intramural air suggests an infection with anaerobic bacteria. Pericolic fluid is a sign of a (possible) perforation.

Sonography may demonstrate free abdominal air which is usually right sided, e.g. perihepatic. In advanced disease with catastrophic prognosis, air bubbles in the vena porta may be demonstrated, as seen after application of contrast enhancing agents. Another feature may be pneumatosis cystoides intestinalis, as seen in premature infants with

necrotizing enterocolitis. It is mentionable that in these patients, the hydrogen content of the expiration air is massively increased.

Computed tomography Although most authors favor the computed tomography as the most sensitive diagnostic tool to diagnose neutropenic colitis[23,24], high resolution sonography is of advantage. In contrast to CT, sonography can be easily performed and repeated (e.g., at the bedside) even in severely ill patients in intensive care or transplantation units.

Abdominal X-ray The findings on abdominal X-ray are often nonspecific and may show small bowel ileus, an ill-defined soft tissue density in the region of the cecum, thickened air-filled loops of bowel or signs of pneumatosis intestinalis.

Barium enema As barium enema should not be performed, when perforation is expected, and as it increases the pressure in the ileocoecal area and therefore may produce ischemia, this diagnostic tool is relatively contraindicated to diagnose neutropenic colitis.

Enteroclysis The oral application of radiopaque medium, such as the barium enema, is not without risk, as large amounts of contrast medium increase the pressure and the risk of perforation. This method is, therefore, also relatively contraindicated.

Other methods Other methods, such as gallium-scintigraphy, or indium-labeled granulocytes, are not routinely used in clinical practice. In certain circumstances, they might give additional informations[25].

Histopathology The macroscopic findings are dilated, edematous thickened bowel wall with areas of hemorrhage and necrosis. The characteristic histological lesions are mucosal ulceration without accompanying inflammatory response, which might progress to gangrene. Often thrombosis of intestinal veins and extensive macroscopic thrombosis of adjacent mesenterial veins in some cases are present, which are probably caused by endotoxins. The main histologic features of neutropenic colitis are edema, hemorrhage and necrosis. Inflammatory, fungal, leukemic or neoplastic infiltrates, as well as frank perforation are occasionally seen.

Therapy

The conservative approach, total parenteral nutrition, antibiotic and antifungal treatment should be placed in forefront. As neutropenia represents the "sine qua non" of neutropenic colitis, time of neutropenia should be shortened, e.g. with granulocyte-colony-stimulating factors or granulocyte transfusions[7, 25].

As the perioperative mortality in these patients is very high, surgical intervention should be placed into the background. On the other hand, the right time for surgery should not be missed, therefore, a close clinical evaluation of the patient by physicians and surgeons is mandatory. The indications for surgical intervention are the same as in immunocompetent patients: Persistant gastrointestinal bleeding after resolution of neutropenia and thrombocytopenia and correction of clotting abnormalities, evidence of intraperitoneal perforation, clinical deterioration requiring support with vasopressors, or large volumes of fluid, suggesting uncontrolled sepsis[7,26].

Selective or complete bowel decontamination as well as prophylactic granulocyte transfusions, especially in patients who had previous episodes of neutropenic colitis, are possible preventive measures.

Prognosis

The prognosis depends on the underlying disease and on the clinical conditions of the patient. The mortality rate in patients with signs of perforation, sepsis and multi-organ failure is higher than 50%[27]. The main prognostic factor is neutrophil recovery and overall time of neutropenia, as neutropenia allows continuous bacterial invasion of the bowel, perpetuating the lesion, with possible necrosis and perforation[28].

In our study, we observed an involvement of the ileocoecal region in all patients, only one patient showed an additional inflammatory reaction of the transverse colon. The sonographically revealed thickness of the bowel wall was associated with poor prognosis and also proved to be a useful tool for monitoring the clinical follow-up by showing the decreasing bowel wall thickening in responding patients. The results of our study agree with the findings of Cartoni *et al*[10] who described an increased mortality in patients with bowel thickness of more than 10 mm. However, all of our patients were classified into this group. We also could demonstrate an increased death rate in patients with thicker bowel walls in the sonographic examination. Although nearly all patients showed a transmural pattern of inflammation, fistulas or abscesses were not observed. This might be explained by the neutropenic condition with restricted defense mechanisms. In addition, sonography might indicate complications of the disease by detection of free abdominal air or intramural hemorrhage. These complications, however, did not occur in our patients.

In conclusion, the high-end sonography of the bowel proved to be a helpful tool in diagnosis, prognosis and follow-up of patients with neutropenic enterocolitis. The ultrasonographically revealed bowel thickness reflects the severity and course of the disease, and seems to be predictive for the clinical outcome.

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RAPID COMMUNICATION

Long-term albumin infusion improves survival in patients with cirrhosis and ascites: An unblinded randomized trial

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Abstract

AIM: To investigate the effects of long-term albumin administration on survival, recurrence of ascites and onset of other complications.

METHODS: One hundred consecutive patients admitted for first-onset ascites were randomized to receive diuretics plus human albumin 25 g/wk in the first year and 25 g every two wk thereafter (group 1) or diuretics alone (group 2). The primary endpoint was survival without liver transplantation. Secondary endpoints were recurrence of ascites and occurrence of other complications.

RESULTS: Median follow-up was 84 (2-120) mo. Albumin-treated patients had significantly greater cumulative survival rate (Breslow test = 7.05, $P = 0.0078$) and lower probability of ascites recurrence (51% *versus* 94%, $P < 0.0001$). Chronic albumin infusion resulted in a mean increase in survival of 16 mo.

CONCLUSION: Long-term albumin administration after first-onset ascites significantly improves patients' survival and decreases the risk of ascites recurrence.

Key words: Cirrhosis; Ascites; Human albumin; Circulatory dysfunction; Plasma expansion; Effective plasma volume; Diuretics; Renin-aldosterone axis

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INTRODUCTION

Ascites is one of the most frequent complications of cirrhosis, occurring in more than 50% of patients within 10 years of the diagnosis of cirrhosis[1-3]. The development of ascites in cirrhosis severely affects patient's prognosis and heralds more severe complications, such as spontaneous bacterial peritonitis and the hepatorenal syndrome[1,3]. Two factors are of major importance in the pathogenesis of ascites: sodium retention, leading to extracellular fluid volume expansion; and imbalance in Starling's equilibrium in liver sinusoids and splanchnic capillaries, which is responsible for translocation of fluid from the intravascular compartment to the abdominal cavity[4,5]. The pathogenesis of sodium retention in cirrhosis is still debated. However, there is no doubt that even if total plasma volume is normal or even increased in cirrhosis, patients with cirrhosis and ascites have a decreased effective arterial blood volume and activation of the renin-angiotensin-aldosterone and sympathetic nervous systems, which stimulate the kidney to retain sodium[4-7]. Starling's forces in the splanchnic compartment are altered because of the increased hydrostatic pressure due to portal hypertension and the low oncotic pressure due to hypoalbuminemia.

Treatment of grade 2 (moderate) ascites is based on sodium restriction and administration of an aldosterone antagonist, with addition of a loop diuretic in the non-responding patients[8]. The same regimen plus paracentesis is recommended in patients with grade 3 (tense) ascites[8]. At present, the main indications to intravenous albumin in cirrhosis are type I hepatorenal syndrome[8,9] and spontaneous bacterial peritonitis[8-10].

In a previously published, controlled study performed at our institution[11], administration of human albumin to a series of patients with ascites improved the response rate to low-sodium diet and diuretics and reduced the cumulative probability of developing ascites during a 20-mo follow-up, but did not affect survival after a mean follow-up of 20 mo.

We report herein the results of an another randomized study aimed at investigating the effects of long-term human albumin administration in a series of consecutive cirrhotic patients with first-onset ascites. The primary endpoint of this study was survival without liver transplantation. Secondary endpoints were recurrence of ascites and occurrence of other complications.

MATERIALS AND METHODS

Patients

The study was performed at the Liver Unit, Department of Internal Medicine, University of Florence, in accordance with the Declaration of Helsinki. It was approved by the University of Florence Ethics Committee in October, 1992 and revised periodically thereafter. One hundred consecutive cirrhotic patients admitted from January 1, 1993 to June 30, 2003 because of first-onset, clinically detectable ascites (i.e., grade 2-3 ascites, according to the more recent definition by Moore *et al*[8]) were included in this randomized, unblinded trial. Exclusion criteria were: age below 35 or over 70 years; active alcohol abuse; previous episode(s) of grade 2-3 ascites; cardiac, respiratory or renal impairment (serum creatinine $\geqslant$1.5 mg/dL); diabetes; refractory ascites; hepatocellular carcinoma or other malignancies; present or previous hepatic encephalopathy of any degree; gastrointestinal bleeding at admission (endoscopy); infections; intravascular coagulation; impossibility or unwillingness to return for follow-up; and refusal of informed, written consent. Diagnosis of cirrhosis was based on history, clinical and biochemical data, abdominal ultrasound, gastrointestinal endoscopy and liver biopsy, when not contraindicated.

At admission, eligible patients were randomly assigned to either group 1 (albumin) or group 2 using sealed envelopes containing the treatment assignments. Allocation schedule was generated using a computed random number generation system. Patients from both groups received low-sodium diet and diuretics in increasing dosage, as commonly used at our institution[12] and later recommended by the International Ascites Club[8]. Briefly, they received low (80 mEq/d) sodium diet, spironolactone (100 - 400 mg), frusemide (25-150 mg), as appropriate. Nineteen group 1 and 16 group 2 patients had tense ascites at admission and had 4 L therapeutic paracentesis without plasma volume expansion. After disappearance of ascites, all patients were given low-sodium diet, diuretics and other drugs (e.g. β-blockers), as appropriate, to prevent ascites accumulation and other complications. In addition, group 1 patients intravenously received 25 g albumin per wk in the first year and 25 g every two wk thereafter. Albumin was administered in the outpatient clinic at our institution. All patients were followed in the outpatient clinic every 1-3 mo by members of the clinical staff not involved in this investigation to monitor the development of ascites or other complications of cirrhosis and modify therapy, as needed. Development of grade 2 - 3 ascites during the follow-up was assessed by clinical examination and confirmed by ultrasound and diagnostic paracentesis. Patients with recurrent ascites or other complications received adequate treatment at our institution as inpatients or outpatients, as indicated. In particular, patients with refractory ascites received therapeutic paracenteses of less than 4 L with no intravenous fluid infusion. The following parameters were recorded and analyzed: liver transplant-free survival; causes of death; recurrence of ascites; and onset of other complications.

Statistical analysis

The data were analyzed using the SPSS 10 (SPSS Inc., Chicago, IL). Being the follow-up loss limited to the first month for withdrawing consent to experimental design, the per protocol analysis was performed as previously described[13]. Student's t test, Fisher's exact test and χ^2 test were employed for the analysis of data, as appropriate. Dependence of survival from other disease parameters besides albumin treatment was analyzed using univariate analysis. Survival time was analyzed by the Kaplan-Meier method and differences were tested by the Breslow test. Data were expressed as median (range). $P<0.05$ was considered statistically significant.

RESULTS

Of the 100 consecutive patients included in this study, 54 were assigned to group 1 (albumin), the remaining 46 patients to group 2 (no albumin). The clinical characteristics of patients at the time of inclusion are shown in Table 1. No significant differences were observed between the two groups with respect to any of the measured biochemical parameters. However, group 1 (albumin) patients had significantly greater Child-Pugh score; as a consequence, significantly more patients in this group were in Child-Pugh class C (Table 1). No patient died during hospital stay. Nine patients in group 1 and eight in group 2 withdrew their consent at discharge or were lost at follow-up immediately afterwards. Median follow-up was 84 (range 2-120) mo.

Transplant-free survival

Among the 100 patients recruited, 81 were still alive at 2 years and 57 at 5 years, survival rates being 81% at 2 years and 57% at 5 years. Univariate analysis showed that the most important factor affecting survival was albumin treatment (R-squared = 0.975) ($F=16.55$, $P=0.005$), followed by Child-Pugh score ($F=6.363$, $P=0.021$) and age ($F=2.783$, $P=0.082$). Albumin-treated (group 1) patients had a significantly greater cumulative survival rate than those in group 2 (group 1: median 108, range 99.55-116.45 mo; group 2: median 36, range 22-50 mo, Breslow test = 7.05, $P=0.0079$, Figure 1). In particular, 34 out of the 54 patients randomized to receive albumin (75%) were still alive at 24 mo, 31 (69%) at 48 mo, 28 (62%) at 60 and 2 (4%) at 120 mo, while the corresponding figures for group 2 patients were 26 (68%), 11 (29%), 10 (26%) and 3 (8%), respectively.

Causes of death

Fifty-four patients (29 in group 1 and 25 in group 2) died during the follow-up. The causes of death in group 1 and group 2 patients are indicated in Table 2. No significant differences were observed between the two groups with

Table 1 Clinical data of the 54 group 1 and 46 group 2 patients at recruitment

Parameters	Group 1 (diuretics plus albumin)	Group 2 (diuretics alone)	*P* value
Age (yr)	63 (44 - 70)	63 (47 - 70)	NS
Sex (M/F)	33/21	29/17	NS
Etiology			
HBV	7	8	NS
HCV	36	28	NS
Alcohol	1	1	NS
Cryptogenic	10	9	NS
Child-Pugh score	10 (8 -11)	9 (8 - 14)	$P<0.0001$
Child class (B/C)	15/39	31/15	$P<0.0001$
Hematocrit	0.33 (0.32 - 0.40)	0.34(0.25 - 0.41)	NS
Serum albumin (g/L)	30.6 (27.0 - 38.0)	31.7 (24.0 - 37.0)	NS
Serum bilirubin (mg/dL)	3.15 (1.30 - 4.90)	2.2 (1.25 - 5.50)	NS
Prothrombin activity (%)	57 (51 - 70)	62 (30 - 70)	NS
MAP (mmHg)	95 (93 - 96)	96 (94 - 98)	NS
Serum creatinine (mg/dL)	0.97 (0.93 - 1.00)	0.98 (0.95 - 0.99)	NS
Creatinine clearance (mL/min)	69 (64 - 85)	71 (65 - 75)	NS

Continuous data are given as median (range); MAP = mean arterial pressure; NS = not significant.

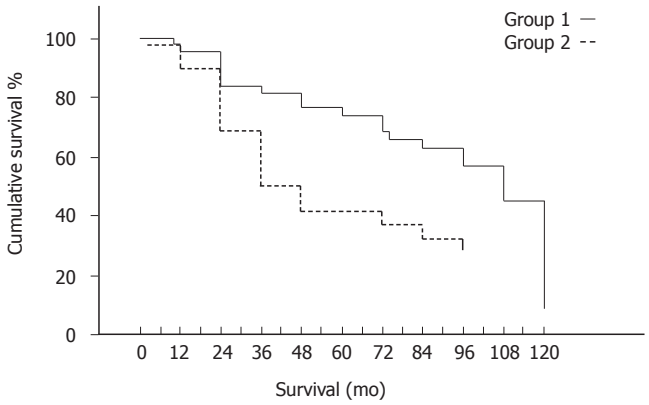

Figure 1 Cumulative survival rate in group 1 (albumin) and group 2 patients. Breslow test = 7.05; *P* = 0.0079.

Table 2 Causes of death in the 25 group 1 and 29 group 2 patients who died during the follow-up

Causes of death	Group 1	Group 2	*P* value
Hepatocellular carcinoma	9	14	NS
Other neoplasms	2	0	NS
Hepatorenal syndrome	1	0	NS
Variceal bleeding	5	4	NS
Liver Failure	10	6	NS
Heart Failure	0	1	NS
Sepsis	2	0	NS

NS = not significant.

respect to causes of death. Liver transplantation was performed in one patient in the albumin group because of hepatocellular carcinoma, and in three patients in group 2 because of progressive hepatic failure (2 patients) and chronic encephalopathy.

Recurrence of ascites

Recurrence of grade 2-3 ascites was markedly lower in group 1 patients (38.88%, 21/54) as compared with group 2 patients (84.78%, 39/46, $P<0.0001$). In group 1, 4 patients had 3 episodes and 2 patients 2 episodes of ascites, the total number of ascitic episodes being 31; whereas in group 2, ascites recurred 4 times in 1 patient, 3 times in 2 and 2 times in 8 patients, the total number of ascitic episodes being 54 ($P<0.001$). Five patients in each group developed refractory ascites ($P>0.05$), while 3 patients in group 1 and two in group 2 developed SBP ($P>0.05$).

Other complications

During the follow-up, there were 19 episodes of hepatic encephalopathy in 11 (20%) patients in group 1 and 15 episodes in 11 (24%) patients in group 2. Variceal bleeding occurred in 5 patients in group 1 and 10 patients in group 2 and hepatic failure in 5 patients in group 1 and seven patients in group 2 ($P>0.05$). One patient in the latter group received transjugular intrahepatic porto-systemic stent shunt (TIPSS) to stop intractable hemorrhage. Surgical porto-systemic shunt was performed in 1 patient in group 1 and 1 patient in group 2 to control severe chronic anemia due to portal hypertensive gastropathy.

Side effects of albumin

No side effects caused by administration of albumin were observed during the whole study period.

DISCUSSION

At the beginning of the 1940's, when salt-poor albumin became available, it was widely used in the management of cirrhotic patients with ascites in an attempt to correct the unbalanced Starling's forces in the splanchnic circulation, so reducing ascites formation, and to improve circulatory and renal function[13,14]. However, few studies investigated the role of albumin in the treatment of ascites and prevention of its recurrence, however, these investigations were uncontrolled and/or included a low number of patients. Albumin infusion usually produced a subjective feeling of "well-being", as also observed in our previous[11] and current studies, but had variable effects on ascites and

edema[14-17].

With the introduction of effective diuretics, the use of albumin for the treatment of ascites in cirrhosis was almost totally abandoned at least in the US. At present, the AASLD recommends albumin only in patients with SBP[18]. Albumin is superior to dextran 70 and polygeline in preventing circulatory dysfunction after paracentesis involving the removal of more than 5 liters of fluid[9] and is therefore widely used outside the US; however, randomized studies have shown no significant difference in survival between patients treated with albumin and those treated with other plasma expanders[19-22].

Despite no evidence from randomized studies support the long-term administration of albumin in patients with cirrhosis and ascites, this practice is widely used at least in Italy, as indicated by a recent study aimed at reaching a consensus among Italian hepatologists as to the use of albumin in patients with cirrhosis and ascites that involved 68 hepatology centers[23]. In the opinion of most experts, long-term use of albumin can help to improve the patient' s general conditions and well being. Seventy-seven percent of the experts involved in this survey agreed that albumin administration can shorten hospital stay or reduce the number of hospital admissions. As a matter of fact, 79% out of the 39 gastroenterological centers participating to a survey organized by the Italian Association of Hospital Gastroenterologists and Endoscopists (AIGO, available on the web site of this association) stated that they usually administer albumin to patients with cirrhosis and ascites.

In a previous randomized study from our department, which included 81 cirrhotic patients with ascites (38 treated with diuretics and 43 with diuretics plus human albumin), and had a follow-up of 20.0 ± 1.9 (range 6-36) mo, patients receiving human albumin had greater rate of ascites disappearance (90.5% *versus* 74.7%, $P < 0.05$) and lower cumulative probability of developing ascites, but no improvement in survival[11].

In the current investigation, we extended the follow-up period up to 62.7 ± 4.2 (mean $\pm$ SE) mo. The main result of our study was that human albumin administration to patients affected by liver cirrhosis (25 g/wk in the first year and 25 g every two wk thereafter) resulted in a significantly greater cumulative survival rate. In addition, as in the previous investigation, albumin administration markedly reduced ascites recurrence.

The patients in group 1 and group 2 were quite similar with respect to arterial pressure and the measured parameters of liver and renal function. However, despite our attempt to reduce variability by including only cirrhotics with first-onset ascites, patients randomized to receive albumin had a slightly greater Child-Pugh score (Table 1), with more patients in this group belonging to Child class C (39 *vs* 15). It is conceivable that the effects of any therapeutic intervention, as albumin infusion in the current investigation, will be quite different in patients with different baseline characteristics. This is confirmed by the results of univariate analysis, showing that transplant-free survival was related to treatment, but also to Child-Pugh score. Albumin administration resulted in a gain of 16 mo of mean survival time (88.63 *vs* 61.39 mo).

Other aspects of this investigation deserve consideration. First, group 2 patients did not receive placebo. This was made to avoid the expenses, discomfort and risks of coming to the outpatient clinic and receive an unnecessary intravenous infusion every 7 - 14 d. On the other hand, patients not receiving albumin are easily identified since they have lower plasma albumin levels. Second, we used the same protocol proved effective in reducing ascites recurrence in our previous investigation[11]. Whether different amounts of albumin would be equally or even more effective in all ascitic patients remains to be established.

The mechanisms determining a better survival in cirrhotic patients with ascites receiving long-term human albumin were not specifically investigated in this study. Albumin is responsible for about 70% of plasma oncotic pressure and therefore plays a major role in modulating the distribution of fluid between compartments. In a hemodynamic study by Brinch *et al*[24], acute administration of albumin (40 g) expanded plasma and blood volume in cirrhosis, although central blood volume only increased in Child class A patients. In addition, arterial compliance increased and plasma renin activity decreased in Child C patients, indicating that albumin is able to improve the low effective arterial blood volume of cirrhosis, so reducing baroreceptor-induced activation of the renin-aldosterone axis and probably of the other vasoconstriction and sodium-retaining factors. Albumin prevented circulatory dysfunction in patients submitted to large-volume paracentesis[19-21], and circulatory dysfunction was found to be related to a shorter survival[22]. It also proved valuable in the treatment of diuretic-induced hepatic encephalopathy[25] and hyponatremia[26], improved survival in patients with spontaneous bacterial peritonitis and was effective in reversing the hepatorenal syndrome when administered together with vasoconstrictors[8-10,18]. It is tempting to speculate that the favorable effect of human albumin on survival observed in this study could be related to its ability to improve the circulatory dysfunction of liver cirrhosis and ascites, so reducing the degree of activation of the main vasoactive systems and antinatriuretic factors that characterize this particular group of patients and are associated with a poor prognosis[8,27].

The use of albumin has been criticized due to its high costs. In Italy, one vial of albumin (10 g) costs € 43.5; so, expenses for albumin were € 4 524 per patient in the first year and € 2 262 thereafter. However, these charges are comparable or even lower than those of other treatments showed to improve survival, such as antiviral treatment of HBV-HCV-related hepatitis, or immunoprophylaxis of HBV reinfection of liver grafts. The advent of recombinant human serum albumin[28] will probably overcome these economic considerations.

Whether albumin should be administered to all patients with cirrhosis and ascites is a problem related to the overall allocation of available resources. In view of the net gain of 16 mo of adjusted survival time demonstrated in the present investigation, in our opinion albumin can be especially useful in patients on waiting list for liver transplantation.

In conclusion, long-term albumin administration to patients with cirrhosis after the first ascitic episode significantly improves patient survival and decreases the

risk of ascites recurrence.

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RAPID COMMUNICATION

Detection of carcinoembryonic antigen mRNA in peritoneal washes from gastric cancer patients and its clinical significance

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Abstract

AIM: To establish a more sensitive method for detection of free cancer cells in peritoneal washes from gastric cancer patients during surgery and to evaluate its clinical significance.

METHODS: The carcinoembryonic antigen (CEA) mRNA levels in peritoneal washes from 65 cases of gastric cancer were detected by real-time RT-PCR. Peritoneal lavage cytology (PLC) was applied simultaneously to detection of free cancer cells. Negative controls included peritoneal washes from 5 cases of benign gastric disease and blood samples from 5 adult healthy volunteers.

RESULTS: There was no CEA mRNA in peritoneal washes from benign gastric disease patients and in blood of adult healthy volunteers. The positive percentage of free cancer cells detected by real-time RT-PCR was 47.7% and only 12.3% by PLC. The positive rate of CEA mRNA was significantly related with serosa invasion between peritoneal metastasis and stage of gastric cancer.

CONCLUSION: Real-time RT-PCR is a sensitive and rapid method for the detection of free cancer cells in peritoneal washes. The presence of free cancer cells in peritoneal washes is related to the pathologic stage of gastric cancer.

Key words: Real-time RT-PCR; Peritoneal washes; Gastric cancer; Carcinoembryonic antigen

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INTRODUCTION

Gastric cancer is one of the most common causes of cancer death in China. The postoperative survival rate of patients with advanced gastric cancer remains very low[1-3]. About 50-60% of gastric cancer patients with serosal invasion after curative resection eventually develop peritoneal metastases[4, 5]. Free cancer cells in the abdominal cavity can predict the prognosis of gastric cancer patients[6-8]. Peritoneal lavage cytology (PLC) has been used to examine cancer cells in peritoneal washes for more than a decade. But it lacks sensitivity and is time consuming[9, 10]. Since CEA is a specific marker of gastric cancer cells, detecting CEA mRNA in peritoneal washings from gastric cancer patients may determine the treatment strategy[11]. To date, many methods to detect CEA mRNA of cancer cells are available, but their sensitivity remains to be improved[11-13]. Recently real-time reverse transcription polymerase chain reaction (RT-PCR) has been used to detect CEA mRNA of free cancer cells in peritoneal washes from gastric cancer patients and micro peritoneal metastasis in these patients[14-17]. In the present study, we used real-time RT-PCR to detect CEA mRNA in peritoneal washes during gastrectomy with its clinical significance evaluated.

MATERIALS AND METHODS

Patients and collection of samples

Sixty-five patients with gastric cancer (aged 35-76 years, mean age 60.6 ± 11.5 years, 50 men and 15 women) undergone curative surgery in our hospital were enrolled

Table 1 Sequences of CEA primers and hybridization probe

Primers	Sense	5′- AACTTCTCCTGGTCTCTCAGCT
	Anti-sense	5′- GCAAATGCTTTAAGGAAGA
Probes	Donor	5′- TGAAATGAAGAAACTACACCAGG-FL
		LC-5′- CTGCTATATCAGAGCAACCCCAA-P
Acceptor		

Table 2 Comparison of clinical pathological factors and CEA mRNA expression

	Positive CEA mRNA [1](%)	*P*-value
Gender:		
Male	48 (24/50)	
Female	47 (7/15)	1.000
Depth of invasion:		
pT3	61 (22/36)	0.024
pT1+pT2	31 (9/29)	
Cancer location		
Cardia	53 (10/19)	0.618
Gastric body	40 (10/25)	
Gastric antrum	52 (11/21)	
Lymph node metastasis		
yes	47 (21/45)	1.000
no	50 (10/20)	
TNM stages		
I + II	33 (10/30)	
III + IV	60 (21/35)	0.046

[1] It is considered as positive If CEA mRNA is detected.

in the present study. No patients received preoperative radiation therapy or pre-chemotherapy prior to their enrollment. Five patients with benign gastric disease undergone gastrectomy and 5 blood samples from health volunteers were included as negative controls. During the operation, peritoneal metastasis was found in 6 patients. At the beginning of operation, 50 mL of saline was poured into the Douglas cavity and aspirated after gentle stirring. After centrifugation, a certain amount of washes was cytopathologically examined. The total RNA was extracted from the rest washes or from blood cells using phenol-chloroform kit according to the manufacturer's instructions(Fermentas Company, Hanover, USA). All experiments were approved by the local ethic committee.

Real-time RT PCR

CEA specific primers and probes were synthesized by the Shengyou Company (Shanghai, China). The donor probe was labeled with fluorescence at the 3' end, while the acceptor probe was labeled with LC Red 640 at 5' end (Table I). The standard CEA mRNA sample was taken from a liver metastasis in a colon cancer patient. All procedures were carried out according to the manufacturer's protocol. In brief, real-time RT-PCR of CEA mRNA was performed in two steps. For reverse transcription, it was carried out in a 40-μL reaction mixture containing 1 μg oligo(dT)18, 2 μg total RNA, 4 μL 10 mmol/L dNTP and 400 units RevertAid M-MuLV reverse transcriptase. For real-time PCR amplification, it was carried out in a 20 μL reaction mixture containing 2 μL cDNA, 22.5 pmol of both forward and reverse primers, 5 pmol probes, 2 μL 10 mmol/L dNTP and 1 unit Tag DNA polymerase. Thermal cycling conditions included at 95 °C for 1 min to activate Taq polymerase. After that, 45 cycles of PCR amplification were performed at 95 °C for 5 s, at 60 °C for 10 s and at 72 °C for 10 s. Samples were amplified in duplicate. All PCRs were performed on LightCycler (Roche Diagnostics).

Table 3 Comparison of CEA mRNA in gastric cancer patients with or without peritoneal metastasis (mean ± SD)

Peritoneal metastasis	CEA mRNA	*P*-value
Yes	16006.2±18242.	$P<0.001$
No	40.5±158.9	

Statistical analysis

The statistical significance of differences in clinical pathological factors and positive rates of CEA mRNA was analyzed with the chi-square test. Comparisons of CEA mRNA values between groups with or without peritoneal metastasis were analyzed by the Mann-Whitney test. $P<0.05$ was considered statistically significant.

RESULTS

Positive CEA mRNA was found in 31 (47.7%) out of the 65 patients by real-time RT-PCR, whereas free cancer cells were found only in 8 patients (12.3%) by cytological method. CEA mRNA in peritoneal washes was significantly correlated to the depth of tumor invasion, peritoneal metastasis and different pathologic classifications (Tables 2 and 3).

DISCUSSION

TNM classification[18, 19] or modified TNM classification[20, 21] has been used to evaluate the prognosis of patients with gastric carcinomas for many years. However, it cannot cover all clinical situations in these patients[21]. The prognosis of gastric cancer is poor mainly due to intraperitoneal relapse. Free cancer cells (FCC) may exfoliate from cancer-invaded serosa contributing to peritoneal dissemination, which is the most frequent pattern of recurrence in patients with gastric cancers[5]. The presence of FCC in the peritoneal cavity is significantly correlated with classical prognostic factors (TNM classification)[22]. Since 1998, Japanese Gastric Cancer Association (JGCA) has suggested that the presence of free cancer cells in the peritoneal cavity should be considered as an independent prognostic marker in patients with gastric cancers[23]. Detecting micro peritoneal metastasis in gastric cancer patients during gastrectomy may determine the prognosis of gastric cancer patients. Many methods can detect free cancer cells in peritoneal washes. Peritoneal lavage cytology (PLC) is an

established routine method for detecting free cancer cells in the peritoneal washes, but the positive rate of PLC for FCC is only 11%[24]. In our study, the positive rate of PLC for FCC was 12.3% (Table 2). More sensitive and specific methods are necessary for the improvement of detecting FCC in peritoneal washes. With the development of PCR technology[25], real-time RT-PCR can detect specific markers of free cancer cells in peritoneal washes. CEA mRNA is one of the most common specific markers of FCC, although Goeminne *et al*[26]reported that mesothelial cells and infiltrating leukocytes also express CEA. This cross-reaction could be avoided by combining different markers. Real-time RT-PCR allows rapid amplification and accurate quantification of CEA mRNA and online data analysis without a post-PCR procedure, making it an applicable method for routine diagnosis[27]. In the present study, no CEA mRNA expression was detected in the control samples collected from patients with benign gastric diseases or from volunteer's blood. Moreover, the positive rate of CEA mRNA was related with membrane invasion, peritoneal metastasis, and stage of gastric cancer, suggesting that free cancer cells in peritoneal washes are closely related to tumor stages. The mean value of CEA mRNA was much higher in patients with peritoneal metastasis than in those without peritoneal metastasis (Table 3), suggesting that CEA mRNA can be used in the evaluation of peritoneal metastasis. Marutsuka *et al*[28] reported that RT-PCR can be used in routine assays. With improvement of skill the sample handling could be shortened and becomes more practicable.

In conclusion, real-time PCR is a sensitive and rapid method for the detection of free cancer cells in peritoneal washes.

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RAPID COMMUNICATION

Pharmacokinetic study of paclitaxel in malignant ascites from advanced gastric cancer patients

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Abstract

AIM: To examine the paclitaxel concentrations in plasma and ascites after its intravenous administration in patients with ascites due to peritonitis carcinomatosa resulting from advanced gastric cancer.

METHODS: Two patients with ascites due to peritonitis carcinomatosa resulting from gastric cancer were included in this study. The paclitaxel concentrations in plasma and ascites were investigated for 72 h in case 1 and 168 h in case 2 after intravenous administration.

RESULTS: The paclitaxel concentration in plasma peaked immediately after administration, followed by rapid decrease below the threshold value of 0.1 μmol (85 ng/mL) within 24 h. In contrast, the paclitaxel concentration in ascites increased gradually for 24 h after administration to a level consistent with the level found in plasma. After 24 h the level of paclitaxel in ascites and plasma became similar, with the optimal level being maintained up to 72 h following administration.

CONCLUSION: The concentration of paclitaxel in ascites is maintained within the optimal level for the treatment of cancer cells for up to 72 h after intravenous administration. Paclitaxel is a promising drug for the treatment of malignant ascites of gastric cancer.

Key words: Paclitaxel; Chemotherapy; Gastric cancer; Peritoneal carcinomatosis; Pharmacokinetic study; Ascites

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INTRODUCTION

Paclitaxel (PTX) is a potent inhibitor of cell replication and was originally isolated from the bark of the tree *Taxus brevifolia*. The anti-mitotic activity of PTX results from its effectively blocking the late G_2 and M phases of the cell cycle. PTX has significant antitumor activity in several human tumors, such as advanced ovarian, lung, and breast cancers[1]. Chang *et al*[2] demonstrated that PTX inhibited the proliferation of gastric carcinoma cell lines at clinically attainable concentrations. Several reports have shown that PTX in combination chemotherapy with 5-fluorouracil and cisplatin is effective in the treatment of advanced gastric cancer[3-8].

We have recently completed two phase I clinical studies of combination chemotherapy involving weekly administration of PXT with either 5-fluorouracil or cisplatin. The recommended dose of PTX was 80 mg/m^2 in both regimens. Some cases with peritoneal carcinomatosis showed a marked decrease in malignant ascites following combination chemotherapy. In the present study, we investigated changes in PTX concentration in plasma and ascites over time to determine the effectiveness of weekly administration of PTX in treating malignant ascites.

MATERIALS AND METHODS

We investigated 2 cases with gastric carcinoma and malignant ascites. Case 1 was recruited in our phase I study of combination chemotherapy involving 5-fluorouracil and weekly administration of 80 mg/m^2 PTX. Case 1 received 60 mg/m^2 of PTX intravenously. Case 2 was involved in our phase II study of combination chemotherapy involving cisplatin and weekly administration of 80 mg/m^2 PTX intravenously. The PTX concentrations in plasma and ascites were measured for both the cases.

PTX was administrated by drip infusion for 1.5 h

Table 1 Changes of paclitaxel concentrations over time in plasma and ascites (ng/mL).

	0	4	8	12	24	36	48	60	72	84 (h)
Case 1 (60 mg/m^2)										
Plasma	1240	-	112	-	55	40	26	20	-	
Ascites	19	30	50	54	62	40	25	20	20	
Case 2 (80 mg/m^2)										
Plasma	468	-	203	-	55	31	27	15	14	
Ascites	11	17	27	30	27	23	19	17	12	ND

ND: Paclitaxel in ascites was not detected after 84 h through to 168 h.

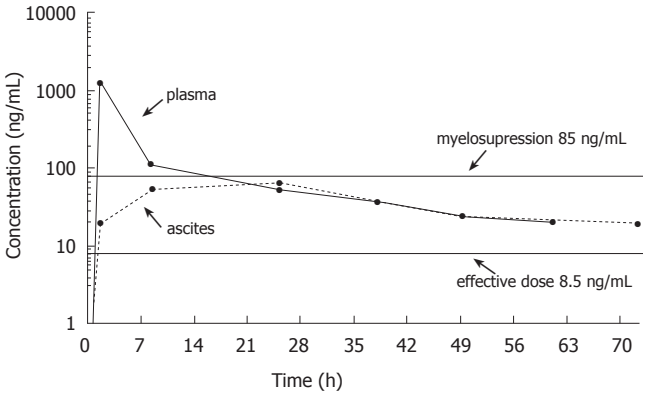

Figure 1 Concentrations of PTX in plasma and ascites in case 1 administrated with 60 mg/m2 PTX intravenously for 1.5 h.

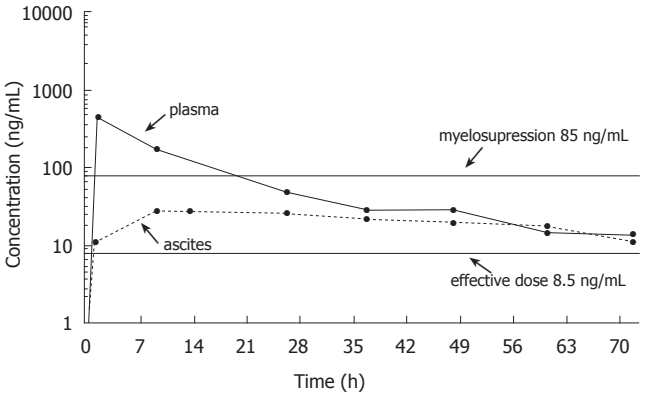

Figure 2 Concentrations of PTX in plasma and ascites in case 2 administrated with 80 mg/m2 PTX intravenously for 1.5 h.

following standard prophylaxis for taxol-associated hypersensitivity reactions with dexamethasone (20 mg iv), diphenhydramine (25 mg *po*), and ranitidine (50 mg iv). After taking informed consent from the patients, samples from blood and ascites were taken prior to PTX administration, immediately after PXT administration, then 4, 8, 12, 24, 36, 48, 60, and 72 h after PXT administration in case 1, and after a further 84, 96, 108, 120, 132, 144, 156, and 168 h in case 2. Ascitic fluid was not drained for the duration of the study and the volume taken for measurement of PTX concentration was limited to 5 mL. Ascites and blood samples were collected in heparinized tubes, centrifuged, and the supernatant was stored at -20℃ until assay. The PTX concentrations in plasma and ascites were analyzed by high-performance liquid chromatography as previously described[9].

RESULTS

The PTX concentrations in plasma and ascites are shown in Table 1. The concentration of PXT in plasma peaked immediately after administration, followed by rapid decrease below the threshold value of 0.1 μmol (85 ng/mL) within 24 h. On contrary, the concentration of PTX in ascites increased gradually to the peak level, as found in plasma, within 24 h of administration. From 24 h, the concentration of PXT in plasma and ascites became almost the same and remained above 0.01 μmol (8.5 ng/mL) up to 72 h after administration. The PTX concentration in ascites after 84 h was below 0.01 μmol in case 2. Figures 1 and 2 show the disappearance curve of PTX in the plasma and ascites in cases 1 and 2, respectively.

Both cases showed marked decrease of ascites after chemotherapy. The CT scan showed that the amount of ascites was markedly decreased after 2 courses of combination chemotherapy of 5-fluorouracil and PTX (Figure 3).

DISCUSSION

Gastric cancer is one of the most common cancers in Japan. Although advances in early diagnosis and surgical technique have improved the outcome of treatment for gastric cancer, optimal chemotherapy regimes have not been determined for advanced or recurrent gastric cancer. The most serious recurrence of gastric cancer is peritoneal carcinomatosis and is usually regarded as the terminal stage of advanced gastric cancer. At present, there are no effective therapeutic regimes for control of malignant ascites due to peritoneal carcinomatosis, which is essential for quality of life. Several reports have demonstrated that intraperitoneal infusion of anti-cancer agents is effective only to a limited shallow area beneath the peritoneum[10,11]. Inadequate exposure of the peritoneum to anti-cancer drugs presents a limitation to their effectiveness. Intraperitoneal administration of PTX has been used to treat ovarian cancer[12-14], but there is limited information on the levels of PTX in ascites following intravenous administration. O'Boyle *et al*[15] investigated the level of PTX in sera and in cerebrospinal, ascitic and pleural

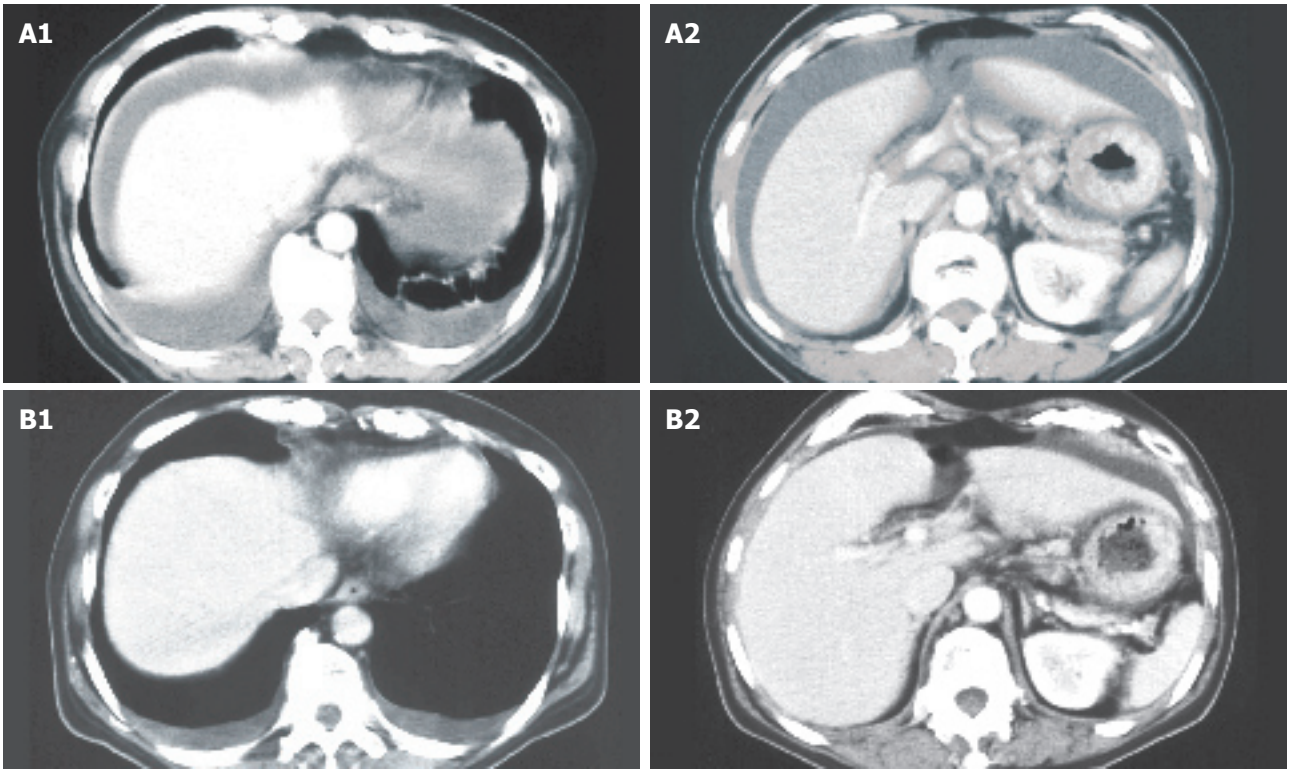

Figure 3 CT scanning of case 1 showing markedly decreased amount of ascites after 2 courses of the combined chemotherapy of 5-fluorouracil and PTX. **A1, A2:** before chemotherapy; **B1, B2:** after chemotherapy.

fluids by immunoradiometric assay and conventional radioimmunoassay and showed the maintenance of high levels of PTX in ascitic fluid in a patient with ovarian carcinoma treated with 135 mg/m^2 PTX.

Several reports have demonstrated that systemic administration of PTX is effective in the control of malignant ascites in gastric cancer patients[16,17]. In the present study, we showed a marked decrease in ascitic fluid volume after combination chemotherapy involving PTX and 5-fluorouracil (case 1), and PTX and cisplatin (case 2).

Chang *et al*[2] demonstrated that exposure of cells to 0.01 μmol/L of PTX for 24 h was cytotoxic. In another study, Gianni *et al*[18] revealed that exposure of plasma to PTX concentrations over 0.05 μmol/L led to myelosuppression and that a concentration of 0.1 μmol/L was clinically relevant. The clinical relevant threshold of 0.1 μmol/l is consistent with findings from other investigators[19-21]. Taken together, the above findings suggest that the clinically effective range for PXT is 0.01 to 0.1 μmol and this range was applied to the present study.

The pharmacokinetic data of the present study revealed that PTX remains within the effective dose range in malignant ascites for at least 72 h following systemic administration. The peak plasma level of PTX varies according to case and peaks immediately after administration. These results are consistent with our previous investigation of 36 cases recruited in two Phase 1 studies (submitted for publication). The observed variation in peak PXT levels according to case may be the result of slight time differences between administration and blood sampling during the period of rapid increase in PTX levels following administration.

PTX has a bulky molecular structure, high molecular weight, and an affinity for binding proteins found at high levels in the peritoneal cavity, especially in malignant effusions. For these reasons, the intraperitoneal clearance of PTX is extraordinarily low, probably the lowest of any known anti-neoplastic agent. As a result of the low clearance rate of PXT from the peritoneal cavity, biologically relevant concentrations can be sustained for long periods allowing maximal exposure to cancer cells. It is well established that the cytotoxic activity of PTX is related to duration of exposure[22-25]. In conclusion, the inherent properties of PXT allowing its sustained exposure to cancer cells in ascites make this drug an effective candidate in the control of malignant ascites. As administration of PXT is systemic, PXT may also be effective for the control of cancer cells in other regions of the body.

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RAPID COMMUNICATION

Magnifying colonoscopy as a non-biopsy technique for differential diagnosis of non-neoplastic and neoplastic lesions

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Abstract

AIM: To clarify whether mucosal crypt patterns observed with magnifying colonoscopy are feasible to distinguish non-neoplastic polyps from neoplastic polyps.

METHODS: From June 1999 through March 2000, 180 consecutive patients with 210 lesions diagnosed with a magnifying colonoscope (CF-200Z, Olympus Optical Co., Ltd., Tokyo, Japan) were enrolled. Magnification and chromoendoscopy with 0.2% indigo-carmine dye was applied to each lesion for mucosal crypt observation. Lesions showing types I and II crypt patterns were considered non-neoplastic and examined histologically by biopsy, whereas lesions showing types III to V crypt patterns were removed endoscopically or surgically. The correlation of endoscopic diagnosis and histologic diagnosis was then investigated.

RESULTS: At endoscopy, 24 lesions showed a type I or II pit pattern, and 186 lesions showed type III to V pit patterns. With histologic examination, 26 lesions were diagnosed as non-neoplastic polyps, and 184 lesions were diagnosed as neoplastic polyps. The overall diagnostic accuracy was 99.1% (208/210). The sensitivity and specificity were 92.3% (24/26) and 99.8% (184/186), respectively.

CONCLUSION: Magnifying colonoscopy could be used as a non-biopsy technique for differentiating neoplastic and non-neoplastic polyps.

Key words: Non-biopsy technique; Magnifying colonoscopy; Indigo-carmine dye spraying; Pit Pattern

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INTRODUCTION

At colonoscopy, the most frequently encountered lesions are polyps, which are either non-neoplastic (hyperplastic, inflammatory, and hamartomatous) or neoplastic (adenoma and carcinoma). Most polyps detected during colonoscopy are hyperplastic or adenomatous. The adenoma-carcinoma sequence suggests that colorectal cancers develop from benign adenomas, and, thus, removal of adenomas but not hyperplastic polyps, could prevent colorectal cancers[1, 2]. Therefore, it is important to distinguish hyperplastic polyps from adenomatous polyps at colonoscopy, as removal or biopsy of hyperplastic polyps wastes time and resources and might increase the incidence of complications, such as bleeding and perforation. We routinely use magnifying colonoscopes, which offer both standard and magnifying views[3]. A magnifying view after chromoendoscopy enables us to observe the lesions' surface structures, the so-called pit patterns, which are reported to be closely related to the histology[4-6]. To investigate whether magnification with chromoendoscopy using indigo-carmine can be used to reliably differentiate non-neoplastic lesions from neoplastic lesions before histological evaluation, we conducted this prospective study.

MATERIALS AND METHODS

From June 1999 through March 2000, all of the patients with lesions detected on colonoscopy without the following exclusion criteria were enrolled in this study, at the National Cancer Center Hospital East. The study protocol was approved by the institutional review board of our hospital. Informed consent was obtained from all patients before examination. Patients with colorectal

Table 1 Patients characteristics and clinicopathological features of resected lesions

Gender (male / female)	124/56
Mean age (range), years	63.0 (37-76)
Histology	
Non-neoplastic (hyperplastic / others)	24 (20/4)
Neoplastic (mild / moderate / severe atypia / mucosal cancer)	186 (21/133/28/4)
Mean size of resected lesions (range), mm	9.1 (3-18)
Location (proximal / distal)	91/119
Meantime to cecum (range), min	8.3 (1-23)
Mean time for diagnosis and treatment (range), min	22.5 (5-45)

Table 2 Correlation of endoscopic diagnosis and histological diagnosis in this study

	Histological diagnosis			
Endoscopic diagnosis	Non-neoplastic	Neoplastic	Total	Diagnostic accuracy
Type I-II	24	0	24	100%
Type III-V	2	184	186	99.8%
Total	26	184	210	

lesions that had been previously evaluated by histologic examination or colonoscopy were excluded from this study. Patients, without informed consents, with inflammatory bowel disease, hereditary nonpolyposis colorectal cancer and familial adenomatous polyposis were also excluded.

A dose of two liters of preparatory solution of electrolytes and polyethylene glycol was administered orally to each patient for preparation before colonoscopy. If there was no contraindication to its use, an anticholinergic agent (buscopan 20 mg) was administered intramuscularly before each examination to prevent persistent colonic spasms. All colonoscopies were carried out using commercially available videocolonoscopes (CF-200Z; Olympus Optical Co., Ltd., Tokyo, Japan) that provide both conventional and magnifying images. All lesions detected at colonoscopy were diagnosed with magnification and chromoendoscopy using 0.2% indigo-carmine dye. The size of a lesion was estimated using open biopsy forceps or a method of removal (i.e., hot biopsy or snare polypectomy). The classification of mucosal crypt patterns was based on Kudo's classification (types I to V). The type I crypt pattern consists of regular round crypts, type II consists of stellar or papillary crypts, type III consists of tubular crypts or roundish crypts smaller than normal crypts, type IV consists of sulcus, branch, or gyrus-like crypts, and type V consists of irregular or severely distorted crypts[4-6]. On the basis of mucosal crypt patterns observed with magnification and chromoendoscopy, detected lesions were divided into a non-neoplastic group (types I and II mucosal crypt patterns) or a neoplastic group (types III to V mucosal crypt patterns). All diagnostic and treatment procedures were performed by a colonoscopist (SK) well trained in magnifying colonoscopy.

For histological evaluation, the lesions diagnosed as non-neoplastic were examined at biopsy and lesions diagnosed as neoplastic were removed endoscopically or surgically without exception. Histological diagnosis was made by a pathologist blinded to the colonoscopic diagnosis. The pathological definition of the lesions was established by the Japanese Research Society for Cancer of the Colon and Rectum[7]. Histologically, adenoma and carcinoma were defined as neoplastic lesions and other non-neoplastic lesions including hyperplastic polyps, were defined as non-neoplastic lesions. The correlation of magnifying colonoscopic diagnosis and final pathological findings was evaluated to calculate the accuracy rate of endoscopic diagnosis.

Chi-square analysis was performed for comparisons. Differences with a p-value less than 0.05 were considered significant. All analyses were performed using Stat View software (Version 5.0 for Windows, SAS Institute Inc., Cary, NC)

RESULTS

The characteristics of the patients and colorectal lesions are shown in Table 1. The 180 patients included 124 males and 56 females, with a mean age of 63.0 years (range, 37 to 76 years). The mean sizes of non-neoplastic lesions and neoplastic lesions were 7.8 mm and 9.2 mm, respectively. Although, neoplastic lesions seemed to be larger than non-neoplastic lesions, they did not differ significantly in size. Two hundred ten colorectal lesions, including 24 non-neoplastic lesions (hyperplastic polyp, 20; others, 4) and 186 neoplastic lesions were detected and evaluated at histologic examination without exception. Ninety-one lesions were located in the proximal colon, and 119 lesions were located in the distal colon. Total colonoscopy and histological evaluation were performed without complications in all cases.

At colonoscopy, 24 lesions showed a type I or II pit pattern, whereas 186 lesions showed types III to V pit patterns. At histologic examination, 26 lesions were diagnosed as non-neoplastic polyps, and 184 lesions were diagnosed as neoplastic polyps. The overall diagnostic accuracy was 99.1% (208/210). The diagnostic accuracy for non-neoplastic pit patterns (negative predictive value) was 100% (24/24). The accuracy of neoplastic pit patterns (positive predictive value) was 99.8% (184/186) (Table 2). The sensitivity and specificity of this endoscopic diagnosis

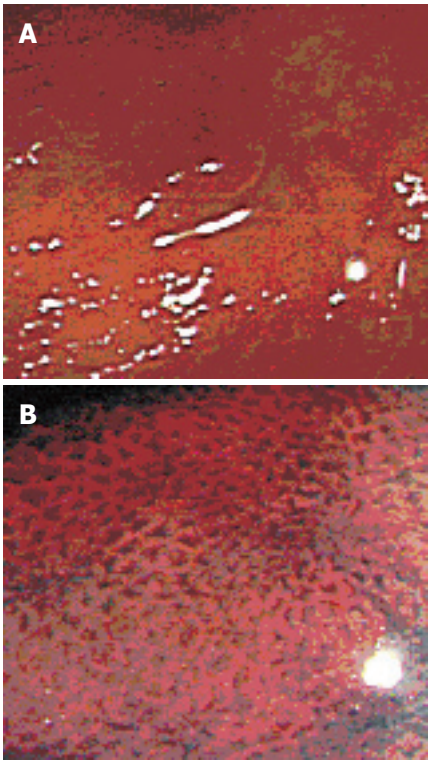

Figure 1 A: Colonoscopy revealed a small reddish polypoid lesion 3 mm in diameter. **B:** Chromoendoscopy with magnification disclosed a type II pit, and the lesion was diagnosed as non-neoplastic and examined at biopsy. The histologic diagnosis was hyperplastic polyp.

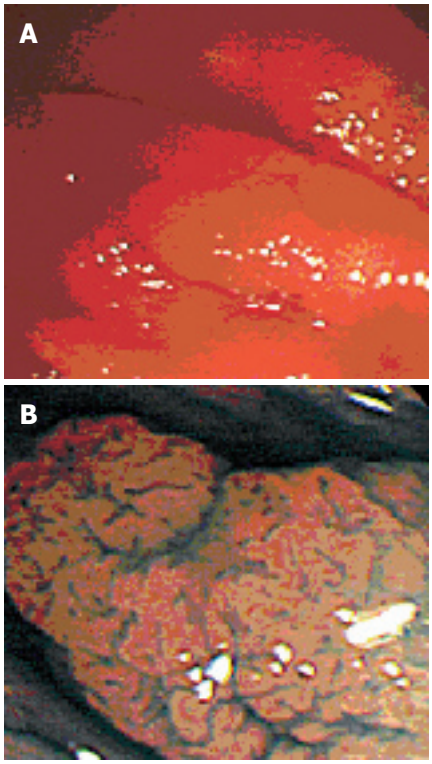

Figure 2 A: Colonoscopy revealed a small flat lesion 5 mm in diameter. **B:** Chromoendoscopy with magnification disclosed a type IIIL pit, and the lesion was diagnosed as neoplastic and resected by snare polypectomy. The histologic diagnosis was adenoma with moderate atypia.

Table 3 Summaries of the previous reports and this study on overall diagnostic accuracy, sensitivity, specificity and predictive values in differentiating non-neoplastic lesions from neoplastic ones

Author	Method	Number of lesions	Overall accuracy (%)	Sensitivity (%)	Specificity (%)	PPV[1] (%)	NPV[2] (%)
Chapius	Ordinary	120	82.5	84.5	77.7	89.8	68.3
Neale	Ordinary	181	80.2	69.2	85.4	69.2	85.5
Konishi	Ordinary	407	68	90	61	94.4	85.1
Fu	Ordinary	206	84.0	88.8	67.4	93.4	63.3
Eisen	Chromoendoscopy	480	82.1	82	82	75	88
Kiesslich	Chromoendoscopy	283	92.6	92.4	93.2	97.5	81
Fu	Chromoendoscopy	206	89.3	93.1	76.1	93.1	76.1
Axelard	Magnifying	55	94.5	92.9	95.1	86.7	97.5
Togashi	Magnifying	923	88.4	92	73.3	94.2	85.2
Tung	Magnifying	175	80.6	93.8	64.6	76.3	89.5
Liu	Magnifying	954	86.1	90.8	72.7	90.4	73.6
Konishi	Magnifying	405	92	97	100	96.3	86.5
Fu	Magnifying	206	95.6	96.3	93.5	98.1	87.8
Hurlstone	Magnifying	1008	95	98	92	95	96
This study	Magnifying	210	99.1	92	100	100	99.8

[1]PPV: positive predictive value, [2]NPV: negative predictive value

are 92.3% (24/26) and 99.8% (184/186), respectively. (Figures 1 and 2).

DISCUSSION

Previous studies in patients undergoing colonoscopy for various reasons were found that small polyps are identified at more than 50% of examinations[8]. More than 50% of these small polyps are adenomas[8-12]. Therefore, a key clinical decision in patients with small polyps may depend on the determination of histology. According to the American Society for Gastrointestinal Endoscopy guidelines issued in 2005, during colonoscopy every effort should be made to obtain a tissue diagnosis when encountering polyps, mass, lesions, or colonic strictures[13]. However, if lesions could be accurately determined to be non-neoplastic or neoplastic at colonoscopy, biopsies or resections would be unnecessary. Various data on the diagnostic abilities of such differentiation by conventional colonoscopy, chromoendoscopy, and magnifying colonoscopy have been reported and are summarized in Table 3[14-24].

Among the methods described above, magnifying colonoscopy with chromoendoscopy seems to provide higher diagnostic accuracy than that by conventional colonoscopy or chromoendoscopy. The efficacy of magnifying colonoscopy is often determined in conjunction with intravital staining techniques, of which indigo-carmine staining is generally preferred. Surface analysis of colorectal lesions by magnifying colonoscopy

in addition to chromoendoscopy has been established by Kudo *et al*[4-6] . They compared the mucosal crypt patterns on stereomicroscopy and colonoscopy with histological sections taken on the horizontal axis and found strong correlations with the histologic features of the lesions. The mucosal crypt patterns were divided into six groups: types I, II, IIIL, IIIs, IV, and V[4-6]. Furthermore, with regard to treatment decision, we re-categorized these patterns into 3 groups as non-neoplastic (types I and II), no treatment or biopsy, non-invasive (types IIIL, IIIs, IV), endoscopic removal; and invasive (type V), surgical resection[25]. The present study was conducted prospectively to show the usefulness of pit patterns for distinguishing non-neoplastic lesions from neoplastic lesions with magnifying colonoscopy.

Our data indicate that magnifying colonoscopy with chromoendoscopy can be used to differentiate almost all lesions detected at colonoscopy before histological evaluation. This method requires a magnifying colonoscope and 0.2% indigo-carmine dye to render the pits clearly visible, but this technique may be slightly troublesome.[26] However, the poor diagnostic reliability of conventional colonoscopy would lead to a significant number of biopsies or resections of non-neoplastic polyps, which would also waste time and resources on unnecessary histopathological examinations. Matsuda *et al* have described the cost-effectiveness of conventional colonoscopy and magnifying colonoscopy[27]. They reported that the percentage of hyperplastic polyps resected after conventional colonoscopy was significantly higher than that after magnifying colonoscopy (8.6% vs 2.9%) and also concluded that routine use of magnifying colonoscopy would reduce unnecessary resections.

Undoubtedly, colonoscopists require training and experience to correctly judge lesions with magnifying colonoscopy. However, there has been little systematic investigation of this issue. Kobayashi *et al*, have investigated the case with which an inexperienced examiner could learn pit pattern diagnosis[28]. According to their results, five nurses, without any prior knowledge of mucosal crypt patterns, could achieve a diagnostic accuracy of up to 85.4% in distinguishing non-neoplastic polyps from neoplastic polyps after hearing short lectures on pit pattern diagnosis. Furthermore, a foreign doctor without prior knowledge of pit patterns required 3 mo of training at our hospital to reach a differential diagnostic ability more than 90%, similar to that of well-trained endoscopists at our hospital[29]. Togashi *et al* have suggested that experience with approximately 200 lesions is needed to learn to correctly diagnose polyps with magnifying colonoscopy[19]. Endoscopic diagnosis based on a conventional view or chromoendoscopy is subjective and unreliable, as it depends on estimations of the size, shape, overlying mucus, and color of the lesions. However, pit patterns are more objective[14-15]. A further well-designed prospective study to investigate the learning curve for endoscopic diagnosis with magnifying colonoscopy compared to that with conventional colonoscopy or with chromoendoscopy is necessary to clarify this issue.

In conclusion, magnification with chromoendoscopy is a reliable tool for predicting histology, especially for differentiation of non-neoplastic and neoplastic lesions. This method could be used as a non-biopsy technique at colonoscopy to limit the need for biopsy or resection, and thus reduce complications, time, and resources required. If possible, an international collaborative prospective study including colonoscopists of eastern and western countries would be desirable for further confirmation of its efficacy.

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RAPID COMMUNICATION

Effects of hyperbaric oxygen and Pgg-glucan on ischemic colon anastomosis

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Abstract

AIM: In colorectal surgery, anastomotic failure is still a problem in ischemia. Here, we analyzed the effects of hyperbaric oxygen and beta-glucan on colon anastomoses in ischemic condition.

METHODS: Colonic resection and anastomosis in rectosigmoid region were done in forty Wistar-Albino rats of four groups of equal number. Colon mesentery was ligated to induce ischemia. The first group was the control group. The subjects of second group were treated with hyperbaric oxygen; the third group with glucan and the forth group were treated with both. At the forth day, rats were sacrificed, anastomotic segment was resected and burst pressures and hydroxyproline levels of anastomotic line were measured.

RESULTS: The burst pressure difference of second and third groups from the control group were meaningful ($P < 0.01$); the forth group differed significantly from the control ($P < 0.001$). There was no difference between the treated groups on burst pressure level ($P > 0.05$). The hydroxyproline levels in all treated groups were different from the control group significantly ($P < 0.001$). Hydroxyproline levels in the forth group were higher than those of the second and the third groups ($P < 0.001$). There were no significant differences between the second and the fourth groups in burst pressure and hydroxyproline levels ($P > 0.05$).

CONCLUSION: Hyperbaric oxygen and glucan improve healing in ischemic colon anastomoses by anti-microbic, immune stimulating properties and seem to act synergistically when combined together.

Key words: Anastomosis; Ischemic colon; Hyperbaric oxygen; PGG-Glucan

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INTRODUCTION

Insufficient anastomosis healing and resulting anastomotic leakage is still a big problem in colorectal surgery[1,2]. Colon is very riskful to leak due to its rich bacterial flora and insufficient collateral circulation[3].

There are systemic and local factors affecting wound healing. Ischemia and local infection are the two most important factors that interrupt healing[4–7]. Hyperbaric oxygen (HBO) and β-1, 3-glucan are two new subjects of search to overcome these problems.

HBO therapy means the inhalation of 100% oxygen in a high pressure-environment. It is adjuvant to surgery. It causes an increase in plasma oxygen level by 20-25%, thus increasing tissue oxygenation. Its current use is in the treatment of air emboli, carbon monoxide intoxication and as an adjuvant to surgery in some diseases[8].

Its effects include an increase in tissue perfusion by both increasing oxygen level in blood and redistributing oxygen from normal tissues to the ischemic ones via vasoconstriction. It also increases angiogenesis and improves inflammatory cell functions, which are necessary for healing[8-10]. β-1, 3-Glucan is a glucose polymer obtained from cereals, yeasts, and fungi. It was found to have some immune-modulatory effects. It stimulates complement system and macrophages[11]. It has anti-bacterial, anti-tumoral, anti-fungal, and wound-healing properties.

In this study, we analyzed the effects of HBO and β-1, 3-glucan on ischemic colon-anastomosis model.

MATERIALS AND METHODS

Table 1 Burst pressure values of rats for each group (mean±SD)

Control	HBO	PGG-Glucan	HBO+PGG-Glucan
80	80	90	110
40	80	120	140
70	130	140	95
80	130	100	100
100	105	80	140
60	80	100	100
55	120	90	120
70	110	90	130
80	100	110	120
60	105	120	110
69.5±16.7	104±19.4[b]	104±18.3[b]	116.5±16.3[d]

[b]$P<0.01$ significant compared to the control group. [d]$P<0.001$ very significant compared to the control group.

This experimental study was held in Cerrahpasa Medical Faculty. Forty Wistar-albino female rats of 180-220 g were used. They were divided into four groups of equal number. All rats were kept in constant environmental conditions. Without making colonic preparation, all subjects were anesthetized with intramuscular ketamine (Ketala® 50 mg/kg) and ether inhalation. Weight of the rats were measured and recorded. Operations were done in a randomized fashion. Abdominal wall was shaved and Povidone–Iodine solution of 10% was used for cleaning. Median laparotomy was performed. Ischemia was formed in a 4 cm-colonic segment, 3 cm proximal to peritoneal reflection, by ligating marginal arteries and vasa recti with 4/0 silk. The ischemic segment was resected at mid-point and reanastomosed end to end with 6/0 polypropylene suture. Abdominal fascia and skin were closed. Oral feeding was started at the 24th h postoperatively. First group was the control group and no further treatment was given following anastomosis. The subjects of the second group were started HBO at the 3rd h after surgery. The rats in the third group were given 20 mg soluble β-1, 3-glucan intraperitoneally following anastomosis. The fourth group was treated with HBO and β-1, 3-Glucan together. All rats were killed on the 4th postoperative day under ether anesthesia. Healing of anastomosis was evaluated by measuring burst pressure and hydroxyproline level.

HBO treatment was started at the 3rd h of operation and continued for 4 d. Before HBO treatment, pressure cabin was washed out with 1 ATA oxygen for 10 min. HBO was given as oxygen of 100% at 2.5 ATA for 60 min. Total treatment time was 80 min, including pressure increase and decrease periods of 10 min for each. The number of HBO treatment was 14 in total, which were four times in a day for the first 2 d and three times in a day for the last 2 d.

At the end of the 4th post-operative day, relaparotomy was done. The colon was clamped at 2 cm distal to anastomosis and resected at 2 cm proximal to it. Burst pressure was measured by an insufflation pump that was sealed intraluminally in the proximal tip of the segment. Insufflation was at a constant value of 6 mL/min under the water and the pressure level where air bubbles were seen on anastomosis line that was accepted as burst pressure value.

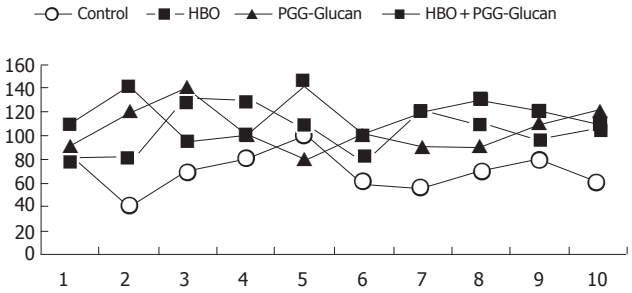

Figure 1 The burst pressure curves of rats for each group (mmHg).

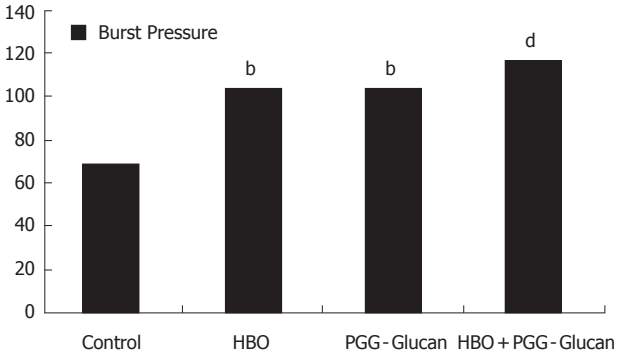

Figure 2 Burst pressure compartment between groups (mm/Hg). [b]$p<0.01$ significant compared to the control group. [d]$p<0.001$ very significant compared to the control group.

The colonic segment 0.5 cm proximal and 0.5 cm distal of anastomosis line was resected, weighed and homogenized in serum physiologic solution to 20% (20% g/mL) homogenisates by using a Potter type glass homogenisator (Heidolphy - RZR 2021, Germany). Homogenisates were centrifuged at 1 500 r/min for 15 min and obtained supernatants were hydrolyzed by adding hydrochloric acid of equal amounts for 16-18 h. Using the hydroxyproline kit (Hipronisticon, Organon, Holland) working on the principles of Stegeman and Stadler, hydroxyprolene amount was calculated in microgram per milligram of wet tissue by reading the absorbance of solution on spectrometry at 560 nm.

statistical analysis

The values were expressed as mean±SD. Differences between groups were evaluated with ANOVA method by using SSPS 10.0 program. Differences within the same group were evaluated with instat test. The values with $P<0.05$ were statistically significant.

RESULTS

There were no deaths during anesthesia or surgical procedure. In all subjects, the burst of the colon was on the anastomotic line. Burst pressure values and standard deviations of groups are shown in Table 1 and Figure 1.

On the comparison of the burst pressure values of the second, third and fourth group to those of control group, results of second and third group showed a significant difference ($P<0.01$); results of fourth group showed a

Table 2 Hydroxyproline levels of rats for each group (mcg/mg wet tissue, mean±SD)

Control	HBO	PGG-Glucan	HBO + PGG-Glucan
0.4416	1.1584	0.8056	2.2144
0.4672	0.9696	0.7264	2.192
0.5592	1.3296	0.7736	2.208
0.4012	1.2384	1.3312	1.6256
0.3904	1.1072	0.9984	2.0352
0.3712	1.3312	0.9664	1.921
0.4096	1.3376	1.221	2.1533
0.4213	1.2672	1.1072	2.2455
0.3517	1.2234	1.2843	2.2612
0.5063	1.2122	1.3152	2.192
0.43±0.06	1.21±0.11[b]	1.05±0.23[b]	2.1±0.19[bdf]

[b]$P<0.001$ very significant compared to the control group. [d]$P<0.001$ very significant compared to HBO group. [f]$P<0.001$ very significant compared to PGG-Glucan group.

very significant difference ($P<0.001$, Figure 2).

There was no significant difference between the second and third groups ($P>0.05$). Also, there was no significant difference when the fourth group was compared to second and third groups ($P>0.05$, Figure 2).

When the groups were compared on the level of tissue hydroxyproline (Table 2), there were significant differences in the results of all treated groups compared to that of the control group ($P<0.001$, Figures 3 and 4).

When the results of the second group and third group were compared, no significant difference was determined ($P>0.05$) but, when the results of fourth group were compared to those of second and third groups respectively, significant differences were found ($P<0.001$, Figure 4).

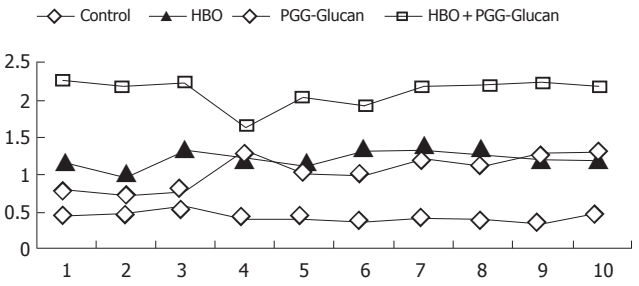

Figure 3 Hydroxyproline level curves of rats for each group (mcg/mg wet tissue).

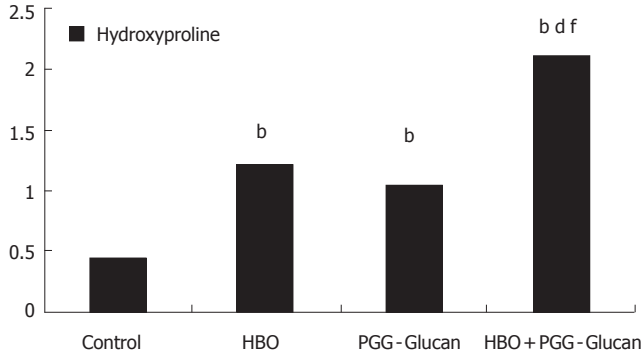

Figure 4 Comparison of Hydroxyproline levels (mcg/mg wet tissue). [b]$P<0.001$ very significant compared to the control group. [d]$P<0.001$ very significant compared to HBO group. [f]$P<0.001$ very significant compared to HBO+PGG-Glucan group.

DISCUSSION

Though there is advancement in techniques, complications in colorectal anastomosis is still a big problem with high mortality and morbidity, especially in ischemic circumstances[12]. Leakage of anastomosis has been reported to be 0-30%. In a series of 1 446 patients, the leakage rate was reported as 13%[2]. Radiological studies revealed that the leakage rate might be up to 51%[13].

There are local factors like infection, perfusion, suture materials, surgical techniques and systemic factors like systemic diseases, septicemia, malnutrition that affect healing[14]. Insufficient oxygenation is one of the most important factor.

Hypoxia interrupts wound healing. If oxygenation of tissue is disrupted due to local damage and anaerobic infection present, HBO therapy may be useful[15]. In experimental studies it was found that oxygen and antibiotics had an additive effect and HBO was more effective under hypoxic conditions where antibiotics were less effective[16,17].

Oxygen is necessary for oxidative function of neutrophils, activation of leukocytes, fibroblast production, angiogenesis, and re-epithelialization, which are of great importance in wound healing[18-21]. Hypoxia also disrupts collagen synthesis and decreases stretching force of the healing wound[22].

Tissue oxygenation is provided by blood volume, tissue perfusion and intra-arterial oxygen saturation[22,23]. HBO increases intra-arterial oxygen saturation thus partial oxygen pressure in tissue, which accelerate wound healing[5,21,24]. HBO was reported to be useful in the treatment of different kinds of problematic wounds; it improved survival of skin grafts, decreased mortality and morbidity in clostridial myonecrosis, accelerated healing of burns, increased re-epithelialization of both normal and ischemic tissues, decreased size of wound and wound complications of diabetes mellitus[25,26].

In a study on the patients who underwent colorectal surgery, a direct relationship between subcutaneous partial oxygen pressure and postoperative wound infection was detected. In this study, although increasing the concentration of oxygen in inhalational air, subcutaneous oxygen concentration did not increase and, 45% of patients were infected postoperatively. On the contrary, when tissue was perfused with a partial oxygen pressure higher than 90 mmHg, there was no infection in any of the patients[27].

The mechanism that HBO improves healing is still uncertain. It is thought that HBO affects in the same way in both ischemic and non-ischemic wounds, because all wounds are ischemic in the first period of healing[18].

A perianastomotic oxygen pressure less than 20 mmHg was shown to result in a major leakage in 100% of subjects and increasing the pressure, rate, and quantity of leakage decreased[12]. On the contrary, Kirk and Irvin reported oxygen had no significant effect on anastomotic and dermal healing. But, these scientists used normobaric oxygen of 50%, and not hyperbaric[28].

Mechanism of HBO may be also on accelerating an-

giogenesis and re-epithelialization process, which are two main phases of wound healing[24,25]. Increase in oxygen was found to increase energy metabolism, thus ADP and ATP in wounds and to result increase in collagen synthesis[23]. In trauma patients, oxygen decreases in wounds, which interrupts healing and increases infection risk[7]. Infection of the mucosa was shown to decrease oxygenation of tissue fibroblasts, spoiling healing. Enteric flora makes colon very prone to postoperative infections.

HBO decreases postischemic edema formation by vasoconstriction in normal tissues, which disrupts wound healing[29]. Intermittent hyperoxygenation makes it more effective; hypoxic periods increases cytokines which increase healing and, cell response to them is also oxygen dependent[30]. This is called oxygen paradox. It has also antibacterial effect[17,27]. Thus, HBO has a double-effect. In our study, statistical significance of increase in burst pressure and hydroxyproline level in second group correlates to the findings that HBO increases healing of anastomosis in ischemic colon.

It has also some complications such as oxygen toxicity, air embolization, and pneumothorax. Its cost, need of frequent application and difficulty in providing medical support are its disadvantages. But, it decreases mortality and morbidity in surgical patients. Its benefits are more than disadvantages. If applied by a specialist, it can be a useful adjuvant in high-risk patients. Moreover, combination with glucan may be more effective.

Immunosuppression is a significant problem in surgical patients. Some causes such as decrease in circulating T-cell number, decrease in lymphoblast transformation, inhibition of leukocyte migration, decrease in cellular immunity and reticulo-endothelial system activity had been proposed. Immunosuppression makes wound more prone to infection, interrupts wound healing and results in failure of anastomosis[31,32].

β-1, 3-Glucans are glucose polymers in the cell walls of yeasts. They have immune-modulating, anti-bacterial, and anti-tumor effects[11,34,35].

β-Glucans activate both humoral and cellular immunity (Wooles and Di Luzio, 1962). They act in a dose-dependent way by binding the receptors on human neutrophils, monocytes, and macrophages. They activate macrophages and increase phagocytotic activity of neutrophils[36]. Macrophages are key cells in wound healing. These cells produce humoral factors, which controls fibroplasia, fibrogenesis, and angiogenesis[37,38]. They also control energy metabolism of wounds. Cytokines like IL-1 from activated macrophages are secreted. These products result in fibroblast activation and proliferation thus increase collagen production and cross-linking between collagen. Macrophages also increase angiogenesis[39]. Although studies analyzing the effects of macrophage activation on colonic anastomosis exist, there is no study that searched its effects in ischemic conditions. Glucans were shown to increase tensile strength of the wound[39].

Having some adverse effects like headache, tiredness, stomatitis, pharyngitis, glucans are nontoxic, nonimmunogenic and apyrogenic substances[40]. Glucans were reported to be safe and effective in decreasing morbidity and cost of a major surgery[41].

In our study, the burst pressure and hydroxyproline levels in glucan group were found to be significantly different from that of the control ($P<0.01$ and $P<0.001$), suggesting that glucan has an increasing effect on early collagen cross-linking, which improves healing. Our results correlate to the previous studies which showed that glucan improved healing.

On burst pressure levels, groups 2 and 3 differed from the control ($P<0.01$). Comparisons between groups 2 and 3 in each other, and between these 2 and 4 groups show no difference ($P>0.05$).

Hydroxyproline is present only in collagen (14%) and elastine (2%) in animals. Thus, it is a good marker in wound healing. In our study, hydroxyproline level of all groups differed from the control very significantly ($P<0.001$). Also, the levels in group 4 were significantly different from groups 2 and 3 ($P<0.001$). There were no differences between groups 2 and 3 ($P>0.05$).

The use of HBO and PGG-glucan together seems to act synergistically to increase wound tensile strength. We think that the reason for this synergistic effect not seen on burst pressure is due to ischemia related ileus, which affects measurements. Also, the finding burst pressures of group 4 differ very significantly from the control supports that HBO plus PGG-glucan is more effective on wound healing.

As a result, we think that this study showed benefical effects of HBO and PGG-glucan on healing of bowel anastomoses experimentally but, we need advanced clinical research in future.

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RAPID COMMUNICATION

Evaluation of *p53* codon 72 polymorphism in adenocarcinomas of the colon and rectum in La Plata, Argentina

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Abstract

AIM: To evaluate the potential association between *p53* codon 72 polymorphism and sporadic colorectal adenocarcinoma development, and human papillomavirus (HPV) infection.

METHODS: One-hundred and nine controls and 53 patients with colon cancer from the city of La Plata, Argentina were analyzed. *p53* codon 72 genotypes and HPV infection were identified using allele-specific polymerase chain reaction and nested polymerase chain reaction, respectively.

RESULTS: The differences in the distribution of *p53* codon 72 polymorphism between the cases and controls were statistically significant. The arginine allele had a prevalence of 0.65 in controls and 0.77 in cases. The corresponding odds ratio for the homozygous arginine genotype was 2.08 (95% *CI*, 1.06-4.05; $P<0.05$). Lack of association was found between *p53* polymorphism and HPV infection in the set of adenocarcinomas.

CONCLUSION: The findings of the present study indicate that *p53* codon 72 arginine homozygous genotype may represent a genetic predisposing factor for colon cancer development. However, further studies are needed in order to elucidate the role of *p53* codon 72 polymorphism in colorectal cancer.

Key words: *p53* codon 72 polymorphism; Human papillomavirus; Colorectal cancer

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INTRODUCTION

Sporadic carcinoma of the colon and rectum is a common cause of cancer deaths in the more developed countries, accounting for 44/100 000 new cases per year in males from the United States, and 33.1/100 000 in women. The incidence of colon cancer is lower in South America, ranging from 16.4/100 000 in males and 14.8/100 000 in females. By contrast, Argentina has increased rates of colon cancer compared with other South American countries, which represents the second and the third cause of cancer deaths in women and men, respectively[1].

Colorectal cancer development is a complex, gradual, multistep process, in which many factors are known to be implicated. The molecular and histological changes involved are also well described, though not well understood. Most of these cancers are adenocarcinomas (95%), showing a high frequency of *p53* mutations, mainly at advanced stages of colorectal cancer progression[2]. Regarding the etiology, epidemiological studies have associated several risk factors with colorectal cancer, including alcohol consumption, low-fiber or high-fat diet intake, hereditary conditions, familial history of colorectal cancer, personal history of colonic polyps and bowel inflammatory diseases. Subjects with the highest risk for colorectal cancer have either a hereditary condition or a bowel inflammatory disease. However, it is worth mentioning that this group comprises only 6% of the general population, while the remaining of colorectal cancer occurs in individuals with no known risk factors[3].

p53 is an important tumor suppressor gene involved in the regulation of cell growth, DNA maintenance and apoptosis. Also, experimental evidence suggests that the p53 protein is related to cell aggressiveness and tumor metastasis[4]. Recent studies have shown that a common polymorphism at codon 72 of the *p53* gene results in two alleles, the arginine (*Arg*) and proline (*Pro*) isoforms, which differ biologically and biochemically[5]. In this sense, it appears that this polymorphism may be associated with differential susceptibility to cancer. Several studies conducted in different countries reported significant associations

Table 1 Genotype frequencies of *p53* codon 72 polymorphism in colorectal controls and tumors according to Dukes staging

	Genotype frequencies			Odds ratio	95% *CI*
	Arg/*Arg* *n* (%)	*Arg*/*Pro* *n* (%)	*Pro*/*Pro* *n* (%)		
Controls	44 (40.3)	53 (48.6)	12 (11.1)	1 (reference)	
Carcinomas	31 (58.5)	20 (37.7)	2 (3.8)	2.08[a]	1.06-4.05
Dukes A-B	13 (52.0)	11 (44.0)	1 (4)	1.6	0.66-3.83
Dukes C-D	18 (64.3)	9 (32.1)	1 (3.6)	2.66[a]	1.12-6.29

[a] $P<0.05$.

between *p53* polymorphism and human cancer. However, the data available for most cancers remain inconclusive, including for colorectal cancer[6-10]. In addition, the *Arg* isoform of the p53 protein was shown to be more susceptible to degradation by the human papillomavirus (HPV) E6 protein than the proline one, and homozygosity for the arginine allele was found at higher frequency in the germlines of individuals affected by HPV-linked cancer[11]. Although the presence of HPV-DNA in colorectal tissues and adenocarcinomas was reported in populations from different regions[12-16], the association of *p53* codon 72 polymorphism with colorectal cancer taking into account HPV infection was investigated only once[17]. In order to elucidate the potential significance of *p53* polymorphism in sporadic colorectal cancer, in association with high-risk HPV infection, a sample of 53 cases and 109 controls from the city of La Plata, Argentina was characterized, using PCR-based methods.

MATERIALS AND METHODS

Fifty-three patients with sporadic colorectal carcinomas and 109 healthy individuals were screened for *p53* codon 72 polymorphism variants. The age range for cases was 51-80 years, and the age range for the controls was 18-67 years. Colorectal adenocarcinomas were obtained from patients during surgical procedures. Liquid cytologies and gastrointestinal biopsies were taken from controls, consisting of subjects with no evidence of neoplastic disorders. Specimens were classified according to the Dukes staging system, 7 as Dukes A, 18 as Dukes B, 19 as Dukes C and 9 as Dukes D.

Paraffin embedded tissues were washed twice with xylene and then with 100% ethanol, air-dried and suspended in 300 μL of digestion buffer (Tris-HCL pH 8, Triton X-100 and Tween 20) with proteinase K (100 μg/mL) for 2 h at 56℃ (Promega, Madison, Wisconsin, USA). Liquid cytologies were suspended and washed twice with 1 mL PBS, resuspended in 400 μL of digestion buffer and digested with proteinase K for 2 h at 56℃. DNA was extracted from the lysates using the salting-out (salt precipitation) procedure [18]. The samples were stored at –20℃ until used.

p53 codon 72 polymorphism analysis

The *p53* codon 72 polymorphism was studied using allele-specific PCR, as previously described[11], with minor modifications[19]. Detection of the amplicons was made by electrophoresis onto a 6% polyacrylamide minigel and ethidium bromide staining. Genotypes were finally determined under UV illumination.

Human papillomavirus detection and genotyping

HPV infection was investigated in colon tissues from 53 patients. HPV DNA was detected by nested PCR, according to the methods previously described[20]. The region L1 of the viral genome was amplified, using MY09/11 as outer primers, and GP05/06 as inner primers. HPV genotyping was performed using the low ionic strength-single strand conformational polymorphism (LIS-SSCP) procedure, as described elsewhere[21]. To determine DNA quality for PCR amplification, a fragment of the human thymidine kinase gene was amplified by PCR in all the samples[22].

Statistical analysis

Association between *p53* codon 72 genotypes, colorectal adenocarcinoma and HPV infection was assessed by the chi-square (χ^2) test. The basic significance level was fixed at $P < 0.05$. The statistical analysis was performed using the statistical package SPSS™.

RESULTS

Histological classification of adenocarcinomas showed that 41.5% of the cases presented high differentiation, meanwhile 30.2% and 24.5% presented moderate and low differentiation, respectively. The remaining cases were unidentified (3.8%). All DNA samples were successfully amplified by PCR for TK gene, demonstrating that the DNA recovered from the paraffin embedded tissues had quality enough to be analyzed for gene polymorphisms and HPV infection. In control samples, the genotype distribution for *p53* polymorphism showed 40.3%, 48.6% and 11.1% for the *Arg*/*Arg*, *Arg*/*Pro* and *Pro*/*Pro* genotypes, respectively. Allelic frequencies corresponded to 0.65 for the arginine allele and 0.35 for the proline allele. The obtained genotype frequencies fitted the Hardy-Weinberg equilibrium ($P > 0.05$). On the other hand, 58.5% of the cases were *Arg*/*Arg*, 37.7% were *Arg*/*Pro* and 3.8% were *Pro*/*Pro*. The corresponding frequencies were 0.77 for the arginine allele and 0.23 for the proline allele. A significant difference between cases and controls was found for the *Arg*/*Arg* genotype compared with (grouped) *Arg*/*Pro* and *Pro*/*Pro* genotypes. Table 1 shows the obtained risk estimation for

colorectal cancer with its corresponding confidence intervals.

HPV-DNA detection and genotyping was carried out using PCR-SSCP on a smaller set of adenocarcinomas (n = 53). HPV 16 was detected in 41.5% (22/53) of the tissues analyzed, HPV 18 in 24.5% (13/53) and HPV 33 in 7.5%. The distribution of the *p53* polymorphism in HPV negative samples was 53.8% (7/13) and 46.1% (6/13) for the *Arg/Arg* and *Arg/Pro* genotypes, respectively. None of the HPV negative samples tested positive for *Pro/Pro*. On the other hand, 60% (24/40) of the HPV positive samples were *Arg/Arg*, 35% (14/40) were *Arg/Pro* and 5% (2/40) were *Pro/Pro*. No significant differences were found between these two groups regarding *Arg* allele and HPV infection (OR, 1.28; 95% CI, 0.36-4.53; P>0.05).

DISCUSSION

In this study, a positive correlation of *p53* polymorphism and colorectal cancer was observed through analysis of a sample of 162 individuals from La Plata, Argentina. This observation is in agreement with the original study of Storey *et al.* (1998) on cervical cancer[11]. However, the distribution of *p53* genotypes according to type-specific HPV infections showed no significant association in this set of adenocarcinomas.

It is well known that the distribution of *p53* codon 72 polymorphism varies in different geographic regions and ethnicity. According to the literature, general populations from Latin America, United States and Europe exhibit high frequencies of the *Arg* allele compared to the *Pro* one, while lower prevalences of *Arg* are found in African and Asian populations [23-26]. In this study, the frequency for the arginine allele was estimated at 0.65. This result is concordant with that obtained for the control group in a previous case-control study on cervical cancer performed in the city of La Plata[19].

The present study provides additional evidence regarding the role of both HPV infection and *p53* codon 72 variants in colon tissues. In agreement with previous studies, HPV-DNA was detected in a high proportion of adenocarcinomas of the colon[12-16]. These findings may suggest a potential role for high-risk HPV in colorectal cancer. Although the present study did not examine HPV DNA in normal colon tissues, its prevalence was reported in a case-control study on colorectal cancer conducted in La Plata[16]. Using PCR-based techniques, the authors found that 33% (10/30) of the normal samples were positive for HPV DNA. Interestingly, eighty percent (8/10) of the positives were single infections of HPV 16.

With respect to the *p53* polymorphism, the frequency of the *Arg* allele in colorectal cancer lesions showed a two-fold increase compared with that in normal samples. The estimated risk (OR) of the *Arg/Arg* genotype for colorectal cancer was 2.08, and subjects with Dukes C and D reported the highest frequency of *Arg/Arg*. This finding suggests that the *Arg* allele may be associated with increased malignancy in colorectal cancer progression. When stratified by HPV infection, the frequency of *Arg/Arg* genotype in HPV positive adenocarcinomas was not statistically different from that in HPV negative samples. However, it should be considered that this finding may represent an artifact, possibly produced as a result of the small sample size.

Case and control studies conducted in Japan and Turkey failed to find an association between the prevalence of *p53* polymorphism and colorectal cancer[6,17]. In the study conducted by Murata *et al.* (1996), the allelic frequencies were concordant between the controls and two sets of colorectal and lung cancer cases, reaching a frequency of approximately 0.6 for the *Arg* allele. The authors only found a significant association between *p53* polymorphism and lung cancer in non-smoking patients[6]. Similar to our study, Sayhan *et al.* (2001) incorporated the analysis of HPV infection in a case-control study on colorectal cancer and *p53* codon 72 polymorphism. However, *p53* genotypes did not correlate with colon cancer, or with the prevalence of high-risk HPV infections[17]. On the other hand, a case-control study conducted in Spain showed a modest association between the *Pro* allele and colorectal cancer (OR, 1.34; P = 0.066). However, such an association was of borderline significance, and it was lost when the analysis was adjusted to another common polymorphism of *p53* examined in that study, *p53*PIN3[10].

The present study has several strengths, including the use of a representative group of controls from the city of La Plata and the careful examination of all stages of colon cancer. Regarding methodology, misclassification and allelic loss was avoided by the use of two separate allele-specific PCR reactions, so that low copies of one allele would not be affected by the presence of several copies of the other. On the other hand, the present study was not controlled for other potential predisposing factors, such as smoking or life-style habits. This is an important issue to be addressed in further studies in order to assess the role of *p53* polymorphism in this tissue.

The mechanisms which lead to the increase of the *Arg* allele in human cancers are not well-established. Another mechanism proposed to explain the epidemiological findings was postulated by Marin *et al.* (2000)[27]. In their study, the authors demonstrated that certain conformational p53 mutants bind and inactivate the p73 protein, a p53 homologue and transcription factor of some p53 target genes[28]. In experimental assays, the binding of the p53 *Arg* isoform to p73 equaled to or exceeded that promoted by the corresponding *Pro* isoform. Thus, it appears that the *p53* codon 72 polymorphism influences the interaction between p53 mutants and p73, and therefore its ability to activate some p53 target genes. In this sense, preferential mutation and retention of the *Arg* allele was found in a set of various cell cancers from *p53* codon 72 germline heterozygotes[27]. However, a recent study did not find that p53-p73 beta interactions were influenced by the *p53* polymorphism[29]. Moreover, no significant differences were found in *p53* mutation prevalence between *Arg/Arg* (40/97) and *Pro/Pro* (7/16) genotypes in a set of colorectal tumors[7]. These contradictory findings implicate that the involvement of *p53* polymorphism in human cancer demands further study. More recently, Schneider-Stock *et al.* (2004) found preferential mutation of the *Arg* allele in a group of colorectal adenocarcinomas. They also reported selective loss of the *Pro* allele in tumors with loss of het-

erozygosity (LOH), resulting in a positive association between *Arg* prevalence and Dukes progression. The authors discarded the possibility of HPV as a potential mechanism for the higher frequency of *Arg* alleles in colorectal tumors and hypothesized that carcinogenic exposure may selectively affect the *p53 Pro* allele in the development of colon cancer[8].

Overall, the findings of the present study indicate that the *p53* codon 72 polymorphism may act as a predisposing factor to colorectal cancer but it is not associated with high-risk HPV infections. Clearly, the data available are still inconsistent and it would be unwise to draw conclusions. Further studies of larger sample sizes, including the analysis of the premalignant lesions and the status of the *p53* gene, are awaited in order to elucidate the magnitude of genetic susceptibility in sporadic colorectal carcinogenesis.

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RAPID COMMUNICATION

Screening for celiac disease in Down's syndrome patients revealed cases of subtotal villous atrophy without typical for celiac disease HLA-DQ and tissue transglutaminase antibodies

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Abstract

AIM: To investigate the prevalence of celiac disease (CD) as well as CD marker antibodies and susceptibility HLA-DQ haplotypes in 134 karyotyped Down's syndrome (DS) patients.

METHODS: Immunoglobulin A (IgA) and G (IgG) type anti-gliadin antibodies (AGA), IgA type anti-tissue transglutaminase (tTG) antibodies (anti-tTG) with antigen of guinea pig and human source were determined by enzyme-linked immunosorbent assay and endomysium antibodies (EMA) by indirect immunofluoresence test. HLA-DQA1*0501/DQB1*0201 (DQ2) was revealed by polymerase chain reaction. Celiac disease was diagnosed by revised ESPGHAN criteria.

RESULTS: 41% of DS patients had AGA, 6.0% IgA anti-tTG with guinea pig antigen, and 3.0 % IgA EMA (all positive for anti-tTG with human tTG). Subtotal villous atrophy was found in 5 out of 9 DS patients who had agreed to small bowel biopsy. One of them had DQA1*0501/DQB1*0201 and anti-tTG and EMA i.e. typical for CD markers (this case also fulfilled the ESPGHAN diagnostic criteria), but other four lacked these markers. Three non-biopsied DS patients had also most probably CD because DQA1*0501/DQB1*0201 and IgA anti-tTG (EMA) were detected. Thus, the prevalence of CD among our DS patients population is 3.0 % (95 % of confidence interval [CI]: 0.1-5.9 %).

CONCLUSION: We confirm the increased frequency of CD among DS patients. In addition, we have revealed a subgroup of patients with subtotal villous atrophy but without characteristic for CD immunological and genetic markers. Whether these cases represent CD (with atypical immunopathogenesis) or some other immune enteropathy, requires further investigations.

Key words: Down's syndrome; Subtotal villous atrophy; Celiac disease; Screening; Autoantibodies; Anti-gliadin antibodies; HLA

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INTRODUCTION

Patients with Down's syndrome (DS), trisomy 21, have a variety of gastrointestinal disorders[1] and immunological disturbances that are related to the gastrointestinal tract[2]. However, the mechanisms underlying the complex phenotype of these associations have remained largely unknown.

Celiac disease (CD), characterized by villous atrophy of the small intestine induced by wheat, rye, and barley in the food [3], is the most common immune disease in patients with DS being detected in 1.6% to 16.9% of cases[4 - 12]. In general, susceptibility to CD is associated with the major histocompatibility complex (MHC) genes from extended HLA haplotypes DR3-DQ2 (*DRB1*03, DQA1*0501, DQB1*0201*) or DR5/DR7-DQ2 *(DRB1*11/DRB1*07 or DRB1*12/DRB1*07, DQA1*0501, DQB1*0201*) and about 95% of CD patients have these haplotypes[13, 14]. However, as many as 25-30 % of the general Caucasian population carry DQ2 molecules, showing that other non-HLA genes are also involved[14]. Systematic genome screenings in CD and affected siblings have revealed several other loci possibly involved in CD susceptibility. However, no CD associated

loci have been revealed in chromosome 21[15, 16]. The reason for the association of CD and DS, as well as variability of CD frequency in different populations of DS patients, is unknown. It seems that at least one cannot ascribe it to the increased number of polymorphic susceptibility genes on chromosome 21[17] and chromosome 21 located autoimmune regulator (*AIRE*) gene[18].

Typically, CD is characterized by chronic diarrhoea, weight loss, and failure to thrive. However, in most cases, the symptoms might be mild and non-specific or even absent, which makes it difficult to diagnose. Early diagnosis is needed because the long-term persistence of untreated CD leads to the development of various complications, including malignancy[3]. The gold standard for the diagnosis of CD is small bowel biopsy. According to the revised criteria of the European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), the diagnosis of CD is based on the results of histological investigations of small bowel mucosa and confirmed by the demonstration of gluten dependence on clinical symptoms[19, 20]. However, in some cases where the small bowel biopsy procedure is not applicable or the investigation results are unequivocal, CD might be exceptionally diagnosed by specific clinical, serological, or HLA data[21]. Patients with DS may be very difficult continent for biopsy due to their mental development retardation, particularly if the the peroral biopsy capsule is used[22].

During the last decades many efforts have been made to find serological markers for CD. Since the 1970s, antigliadin antibodies (AGA) of IgG and IgA types have been used for CD screening, but these antibodies tended to be present also in a number of patients without CD and even in healthy persons[23, 24]. On the other hand, endomysium antibodies (EMA) or antibodies to the EMA's specific target, tissue transglutaminase (tTG), are highly specific for CD[3, 22]. Also, other autoantibodies, including IgA-type anti-smooth muscle (SMA), antiactin and antidesmin antibodies, are frequently detected in patients with CD but revealed in other disease groups as well[25, 26].

The present study aimed to investigate the prevalence of CD, CD marker antibodies and HLA-DQ in DS patients and to compare the results with kariotype and clinical data in these patients.

MATERIALS AND METHODS

Patients

One hundred and thirty-four patients (73 males) with a mean age 11 years (ranging from six months to 45 years) with DS were enrolled in the study. The DS diagnosis was confirmed by chromosome analysis. Regular trisomy was found in 124 patients, translocation in 7 patients (four with 46,XX,der(14;21)(q10;q10),+21 karyotype, one with 46,XY,der(14;21)(q10;q10),+21, and two with 46,XX,der(21;21)(q10;q10),+21), and mosaicism in three cases. One child had translocation between 13;14 chromosomes (46;XY,der(13;14)(q10;q10),+21) with regular trisomy (Table 1). None of the patients had previously been diagnosed with CD and all patients had been on a gluten-containing diet for at least two months. All the studied persons were Caucasians living in Estonia, a country of 45 227 square kilometers and 1.4 million inhabitants. Patients were seen at the Children's Clinic of the Tartu University Clinics. After written informed consent from the patient and his/her parents or guardian, three blood samples were taken – one for antibody analyses, the second for DNA isolation for immunogenetic analysis, and the third for chromosome analysis.

Antibody analysis

In-house enzyme-linked immunosorbent assay (ELISA) was used to detect IgA and IgG AGA using 96-well microtitre plates (Biohit OY, Finland) as described elsewhere [24]. The results were reported in arbitrary units (AU) as a percentage of the optical density of a highly positive serum sample. Values of AU over 59 were considered as a sign for AGA presence. Antiendomysium antibodies of IgA-type were determined by the indirect immunofluorescence test on unfixed frozen sections of the human (blood group 0) umbilical cord using sera from patients and IgA EMA positive and negative controls diluted at 1:10. The serum of a patient was considered positive for IgA EMA if a typical staining pattern was observed around smooth muscle cells of the blood vessels[27]. Smooth muscle antibodies of IgA type were detected using the standard indirect immunofluorescence test with unfixed frozen sections of rat liver, kidney, and mouse stomach in the patients sera by the same procedure as described above. The intracellular staining of smooth muscle cells was designated as IgA SMA[26]. Immunoglobulin A anti-tTG were determined by two assays. The in-house ELISA test[26] was used to determine IgA against the guinea pig tTG (IgA anti-gptTG). The results were reported in arbitrary units (AU) as a percentage of the optical density of a positive serum sample. Test results over 25 AU were considered positive. In order to determine IgA against human tTG (IgA anti-htTG), the Celikey tTG ELISA kit (Pharmacia and Upjohn Diagnostics, Freiburg, Germany) with human recombinant tTG was used according to the manufacturer's instructions. Values of IgA anti-htTG higher than 8 U/ml were considered positive. Antibodies were determined under the external quality control of UK NEQAS (Sheffield, UK). In the disease control group of consecutive untreated CD patients 100.0% had IgA and/or IgG AGA, 89.0% IgA EMA and IgA anti-hutTG (data not shown).

Immunogenetic studies

The HLA-DQA1*0501 and DQB1*0201 alleles, encoding for DQ2 molecule, were determined by PCR-based methodology with allele-specific primers as published elsewhere[28].

Chromosomal analysis

Chromosome preparations were made from peripheral blood lymphocyte cultures. The cytogenetic analysis was performed using GTG banding technique[29].

Diagnosis of celiac disease

Communicating with DS patients and invasive diagnostic

Table 1 Comparative frequency of different karyotypes among 134 Down's syndrome patients and the incidence of EMA, anti-tTG, AGA, and celiac disease in these patients

Karyotype groups	No. of patients	No. with positive AGA	No. with positive anti-tTG (guinea pig antigen)	No. with positive anti-tTG (human antigen) and EMA	No. with celiac disease
Regular trisomy	124	54	8	4	4
Translocation	7	1	0	0	0
Mosaicism	3	0	0	0	0
Total	134	55 (41.0 %)	8 (6.0 %)	4 (3.0 %)	4 (3.0 %)

procedures like endoscopy and intestinal biopsy involves considerable difficulty. Therefore, we did not invite all DS patients with whatever CD marker antibodies for small intestinal biopsy procedure to confirm or deny CD, but only those who had most probably CD, that is, all AGA-positive patients with complaints compatible with CD, all patients with IgA EMA and/or anti-tTG, and all seronegative infants with typical CD symptoms (failure to thrive, chronic diarrhoea). Biopsy specimens were taken from the proximal part of the mucosa of the small intestine (at the level of ligamentum Treiz) under fluoroscopic control using the Watson capsule. The diagnosis of CD was established on the basis of revised ESPGHAN criteria[19].

Ethics

The study was approved by the Ethics Committee for Medical Investigations at the University of Tartu.

Statistical analysis

The SAS/STAT (version 6, 1990, SAS Institute Inc., Cary, NC, USA) statistical package was used for calculations. A *P*-value of less than 0.05 was taken to be significant.

RESULTS

Fifty-five (41.0 %) out of 134 DS patients had a positive test for IgG or/and IgA AGA test (Table 1). Eight (6.0 %) out of 134 DS patients had a positive IgA anti-gptTG test. When the positive sera were retested for IgA anti-htTG, only four that had also IgA EMA, remained positives. No additional anti-htTG positive cases were revealed among 2 DS patients with borderline anti-gptTG values (18-25 AU) and 24 randomly selected anti-gptTG negative DS patients (including 16 with the positive IgA AGA test).

Altogether, 11 DS patients with antibodies and gastrointestinal symptoms compatible with CD were invited to small bowel biopsy. In addition, there was a 11-months-old infant with typical CD symptoms (failure to thrive, chronic diarrhea) but without IgA AGA and IgA anti-htTG (EMA). In all patients cow milk protein allergy, as another possible reason for intestinal villous atrophy, was excluded. Among 9 patients who agreed to the procedure, subtotal villous atrophy (SVA) was revealed in 5 and normal small bowel mucosa in 4 (Table 2). Only one out of these 5 patients had characterisic for CD IgA anti-tTG/EMA (none of them had had total serum IgA below the normal value as evaluated by nephelometry - data not shown) and HLA-DQA1*0501/DQB1*0201, although a clear clinical effect from gluten-free diet-disappearance of chronic diarrhea, abdominal distension and discomfort, and/or failure to thrive with the disappearance of IgA AGA positivity was revealed in four. However, we revealed IgA SMA in 3 out of 5 DS cases with SVA but only in 8 out of 119 DS cases without SVA ($P < 0.001$; Chi-square with Yates correction).

In all 4 IgA anti-htTG positive cases HLA-DQA1*0501/DQB1*0201 haplotype was revealed. This group of DS patients includes aforementioned patient with biopsy verified CD and 3 patients who had not agreed with intestinal biopsy procedure (two had IgA SMA). All patients had positive clinical effect from gluten free diet and therefore CD was confirmed in all four patients. Thus, we had revealed the CD prevalence at least 3.0% (95% CI: 0.1-5.9) among our DS patients.

No significant differences were found in the karyotype characteristics between DS patients with and without antibodies or CD (Table 1).

DISCUSSION

Celiac disease deserves special attention as most common gastrointestinal autoimmune associate of DS[2, 3]. However, the mechanisms underlying the development of CD in DS have remained unknown. We have revealed SVA compatible for CD and/or CD by characeristic IgA anti-tTG and HLA-DQ2 data as well as clinical effect of gluten-free diet in 4 (3.0 %) of studied 134 (95 % confidence interval ([CI]: 0.1-5.9%) DS patients. This finding 3 or more times exceeds the prevalence of CD in general population[3].

Similar CD frequencies in DS patients have been revealed in countries of different regions of the world [4-12, 22]. The only exceptionally high prevalence of CD in DS (16.9%) was revealed in Sweden by Jansson and Johansson [5]. However, the selection of DS patients and screening methods, as well as frequency of CD cases in local background population could significantly affect the results. Aforementioned Swedish authors have screened DS patients by AGA test and diagnosed CD in 9 of 19 biopsied patients. In line of their results we have also detected a set of EMA-negative but igA AGA positive DS patients with SVA (Table 2).

In the present study we used in parallel all the commonly available serological CD screening assays-AGA, EMA and anti-tTG tests – and confirmed three opinions presented in the literature. First, we found a high prevalence (41%) of IgA and/or IgG AGA among DS patients[4-11]. Second, the IgA anti-tTG reactivity is best detected by human tTG[12, 30, 31]. Third, the IgA EMA and anti-htTG highly

Table 2 Profile of 12 Down's syndrome patients (all with regular trisomy) invited for small bowel biopsy

Patient No.	Age (yr) sex	Symptoms	Small bowel biopsy	IgA AGA	IgG AGA	IgA SMA	IgA EMA	IgA anti-gptTG	IgA anti-htTG	HLA-DQB1 *0201	HLA-DQA1 *0501
6	0.9 F	+ #	SVA	-	-	+	-	-	-	-	+
10	13 M	+ ##	SVA	+	+	-	-	-	-	-	-
18	10 M	-	Norm.	+	+	-	-	+	-	-	-
59	1 M	+ ##	SVA	+	+	+	-	-	-	-	-
78	5 M	+ ###	SVA	-	-	-	+	+	+	+	+
91	40 F	+ ####	n. d.	+	+	-	+	+	+	+	+
92	17 M	+ ####	n. d.	+	+	+	+	+	+	+	+
96	18 F	+ ##	SVA	+	+	+	-	-	-	-	-
97	8 M	-	Norm.	-	+	-	-	-	-	n. d.	n. d.
98	7 F	-	Norm.	-	+	-	-	-	-	n. d.	n. d.
105	9 M	+ ####	n. d.	+	+	+	+	+	+	+	+
130	3 F	-	Norm.	-	+	-	-	-	-	n. d.	n. d.

Numbers in bold denote positive values in antibody tests; SVA – subtotal villous atrophy; Norm. – normal mucosa; n. d. – studies not done (biopsy procedure was denied). # - symptoms did not disappear during the four-year gluten-free diet – CD was not diagnosed. ## - symptoms disappeared after introduction of gluten-free diet – CD was not diagnosed due to lack of anti-htTG and HLA-DQ2. ### - symptoms disappeared after introduction of gluten-free diet – CD was diagnosed by morphology data (by ESPGHAN criteria). #### - symptoms disappeared after introduction of gluten-free diet – CD was confirmed without morphology data.

correlates with the presence of DQ2 haplotype[3, 14]. However, as a new observation, we revealed a portion of DS patients with SVA but without characteristic for CD serological and genetical markers. One might ask whether these patients have cow milk protein allergy or B-cell immunodeficiency representing other well-known associates of SVA. This was not a case as revealed by the additional clinical inves-tigations in these patients. However, these patients may have severe imbalances in immune regulation leading to the development of this type of enterpathy. As a support to this view we have detected a substantial decr-ease of peripheral blood regulatory T cells (including $CD4^{+}CD25^{high}$ cells) in one of these SVA patients compared to age-sex mached controls (data not shown). Regulatory T cells play a key role in the maintenance of self-tolerance, thus preventing autoimmune disease, as well as inhibiting harmful inflammatory diseases[32].

Noteworthy, in three of five DS patients with SVA IgA SMA were revealed. Smooth-muscle antibodies group may include different antibodies types, antibodies to actin, tubulin, desmin and others[33], among which antibodies against actin and desmin have been found in untreated CD patients[25, 26]. Also a number of autoimmune enteropathy cases have been described to be associated with SMA (reviewed by Russo and Alvarez[34]). Whether our patients with SVA but without typical immunologic and genetic characteristics of CD represent an entity of autoimmune enteropathy of DS or just a group of atypical CD cases, needs further invstigations. The latter possibility could be easily drawn from the recent studies[11, 12, 35] where immunologically and immunogenetically atypical CD cases were discovered among DS patients.

What is the actual cause of the rised prevalence of CD in DS patients? According to special analysis there is a number of immune response influencing genes in chromosome 21[36]. Thus, the abnormal function of these genes (whatever the mechanism) as the cause of general immune dysfunction, including impaired local immunity and high susceptibility to infections, might contribute to the impairment of the integrity of the small bowel and lead to food antigen leakage through the intestinal mucosa. However, some genes responsible for gut mucosa integrity could be involved as well. As indirect evidence for this suggestion, we have revealed AGA in as many as 41% of DS patients. This supports the earlier studies about the high frequency of AGA[5-8] and other food antibodies in DS[37].

To conclude, the results of our study confirm the earlier reports about an increased prevalence of CD in DS. However, according to our results there are also some DS patients with SVA not fulfilling the typical immunological an genetical criteria for CD. Whether these patients with SVA represent just a subgroup of CD (as judged by the clinical effect of gluten-free diet) but with a deviation in immunopathogenesis, or other types of immune enteropathies (as judged by immunological data), needed to be answered in future studies.

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RAPID COMMUNICATION

Crohn's disease in adults: Observations in a multiracial Asian population

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Abstract

AIM: To determine the demography and clinical presentation of CD and secondly to determine any differences in the prevalence between the different ethnic groups in a multiracial Asian population.

METHODS: Patients with CD who were seen in 2001–2003 in the University of Malaya Medical Centre (UMMC) were enrolled in this study. Prevalence of disease was calculated for the group as a whole and by race with hospital admissions per ethnic group as the denominator.

RESULTS: Thirty-four patients were diagnosed to have CD. Basic demographic data of patients; male:female 17:17; mean age 29.1 years (±13.5 years); ethnic group: Malays 5 (14.7%), Chinese 12 (35.3%) and Indians 17 (50%). Twenty-six (76.5%) were diagnosed under the age of 40 and 8 (23.5%) were diagnosed over the age of 40. Location of the disease was as follows: ileocolonic 13 (38.2%), terminal ileum only 9 (26.5%), colon only 8 (23.5%), and upper gastrointestinal 4 (11.8%). Sixteen (47.1%) had penetrating disease, 9 (26.5%) had stricturing disease and 9 (26.5%) had non-penetrating and non-stricturing disease. The hospital admission prevalence of CD was 26.0 overall, Indians 52.6, Chinese 6.9, and Malays 9.3 per 10^5 admissions per ethnic group. The difference between Indians and Malays: [OR 5.67 (1.97, 17.53) $P < 0.001$] was statistically significant but not between the Indians and the Chinese [OR 1.95 (0.89, 4.35) $P = 0.700$]. The difference between the Chinese and the Malays was also not statistically significant. [OR 2.90 (0.95, 9.42) $P = 0.063$].

CONCLUSION: The clinical presentation of CD is similar to the Western experience. Although the overall prevalence is low, there appears to be a clear racial predominance among the Indians.

INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) have long been recognized in the West, but is thought to be relatively uncommon in the Asia[1]. To illustrate this point, the prevalence rates of CD have been estimated to be 3.6 and 5.8 per 10^5 in Singapore[2] and Japan[3], respectively compared to 144.1 per 10^5 in Olmsted, Minnesota[4] and 147 per 10^5 in North East Scotland[5]. However, the incidence appears to be increasing in this part of the world and is likely to become clinically more important in future. For example, in another Japanese study, the prevalence and the annual incidence were estimated to be approximately 2.9 and 0.6 per 10^5 population, respectively in 1986, but this had increased to 13.5 and 1.2 per 10^5 population in 1998[6]. Leong *et al* found a similar increase in the annual incidence of CD among the Chinese population in Hong Kong[7] and a study by Law *et al* found an increase in the number of admissions for CD among the Chinese population in Singapore[8].

Another interesting observation is that there appears to be ethnic differences in the prevalence of idiopathic inflammatory bowel disease[1,2,9-15]. This is particularly relevant in Malaysia which has a multiracial society mainly made up of three major Asian races: Malay, Chinese, and Indian. In particular, studies in neighboring Singapore which although has a predominantly Chinese population has a similar racial mix; significantly higher prevalence rates among the Indians for UC but not CD compared to the Chinese and the Malays have been found[2,9,10] These racial differences for UC were also seen in our own study in UMMC[15] The aim of this study is to determine the demography and clinical presentation of CD in a hospital setting in Malaysia and to determine any racial differences in prevalence.

MATERIALS AND METHODS

Patients with CD in UMMC as identified by International

Table 1 Demographic characteristics of patients with CD in UMMC

Number	All patients 34
Total males	17 (50%)
Total females	17 (50%)
Age (range)	12-56 years
Mean age	29±13.5 years
Ethinc group	
Malay	5 (14.7%)
Chinese	12 (35.5%)
Indian	17 (50%)
Family history	0 (0%)
Appendicectomy	2 (5.9%)
Smoking history	
Non smoker	27 (79.4%)
Ex smoker	2 (5.9%)
Current smoker	5 (14.7)

Table 2 Prevalence of CD overall and according to ethnicity

	Malays	Chinese	Indians	Overall
CD (*n*)	5	12	17	34
Total admissions	53906	44592	32330	130828
Prevalence per 10^5 admissions	9.2	26.9	52.6	26.0

Classification Coding for Crohn's disease from 2001 to 2003 were retrospectively included into the study. Patients under the age of 12 were excluded. The diagnosis of Crohn's disease was made in accordance with previously accepted criteria based on a combination of clinical presentation as well as typical radiographic, endoscopic, histological, and laboratory findings[16]. Diagnosis was at least of 6 mo duration. Microscopic colitis, collagenous colitis, Behcet's disease and infective enterocolitis were excluded. Stool microscopy and culture, bacterial and amoebic serology, acid-fast staining of biopsies were performed to exclude infectious enterocolitis. Baseline patient characteristics in particular race, date of diagnosis, clinical features, method of diagnosis, location of disease, complications and surgical intervention were documented. Phenotype of the disease was classified according to the Vienna classification[17]. Family history, smoking history and whether or not the patient had a previous appendectomy was also recorded.

The denominator were hospital admissions in UMMC in 2001-2003 as a whole and according to each of the major ethnic groups; Malays, Chinese, and Indians.

Statistical analysis

Data was analyzed using the SPSS 11.5 for Windows. Prevalence of the disease was calculated for the group as a whole with total hospital admissions for the same period of time and by race with hospital admissions per 100 000 ethnic group as the denominator. Prevalence rates were expressed with 95% confidence intervals.

RESULTS

Demographic characteristics

The demographics of the patients with CD are summarized in Table 1. There was no gender difference, 17 males and 17 females with a ratio of 1:1. The mean age was 29.1 years (±13.5 years). Twenty-six (76.5%) were diagnosed under the age of 40 and 8 (23.5%) were diagnosed over the age of 40. The breakdown according to ethnic group was as follows: ethnic group: Malays 5 (14.7%), Chinese 12 (35.3%), and Indians 17 (50%). None of the patients had a family history of IBD and only 5 (14.7%) were smokers whereas 2 (5.9%) were ex-smokers and the majority 27 (79.4%) were lifelong non smokers. Two (5.9%) patients had previous appendectomy.

Clinical features and disease location

In terms of clinical features, the commonest presenting complaints were diarrhea 29 (85.3%), abdominal pain 25 (73.5%), rectal bleeding 17 (50%), and weight loss 15 (44.1%). Other features included fever 8 (23.5%), vomiting 8 (23.5%), and abdominal mass 6 (17.6%). Perianal disease was seen in 10 (26.5%), and extraintestinal manifestations in 7 (20.6%). Thirteen (38.2%) presented with an acute abdomen and diagnosis was made following laparotomy.

Location of the disease was as follows: ileocolonic 13 (38.2%), terminal ileum only (with or without cecal overflow), 9 (26.5%), colon only 8 (23.5%), and upper gastrointestinal 4 (11.8%). Of the patients who had upper gastrointestinal disease (upper GIT), two had coexisting disease in the ileocolon, one in the terminal ileum only and one in the colon only.

Disease behavior and surgical rate

The median duration of follow up from the time of diagnosis till the date of recruitment into this study was 8 years (5-18.25). In terms of disease behavior, 16 (47.1%) had penetrating disease, 9 (26.5%) had stricturing disease and 9 (26.5%) had non-penetrating and non-stricturing disease. Of the 34 patients with CD, 16 (47.1%) of patients had undergone at least one operation. Seven (20.6%) had more than one operation.

Racial prevalence

The ethnic distribution of patients with CD were; Malays 5, Chinese 12, and Indians 17. Over the same period of time as the study, hospital admissions per ethnic group were as follows: Malays 53 906 Chinese 44 592, and Indians 32 330. The hospital admission prevalence of CD was 26.0 overall, Indians 52.6, Chinese 26.9, and Malays 9.3 per 10^5 admissions per ethnic group (Table 2). The difference between Indians and Malays: OR 5.67 (1.97, 17.53) $P<0.001$ was statistically significant but not between the Indians and the Chinese OR 1.95 (0.89, 4.35) $P=0.700$. The difference between the Chinese and the Malays was also not statistically significant [OR 2.90 (0.95, 9.42) $P=0.063$].

DISCUSSION

In terms of gender and typical age of presentation, CD in Malaysia appears similar to other Western studies[12,13] in that there was generally no major gender differences and most patients presented under the age of 40, mainly

between the ages of 16 to 25. However, the majority of patients did not have any of the known risk factors identified from previous studies. For example, 79.4% of them were lifelong non smokers. As the number of patients with CD is less and there are no controls, this may not be a true finding although there have been other studies that have failed to identify smoking as a risk factor of CD[18,19]. None of our patients had a positive family history as with Leong *et al* study from Hong Kong[7]. This is in marked contrast to other published studies from the West , where a positive family history is found in 5-10% of patients with CD[20-22].

The commonest presenting complaints were also typical of CD. One interesting observation is that 38.3% of these patients presented with an acute abdomen requiring laparotomy and the diagnosis was subsequently made on histology. In terms of location of the disease, the distribution is also similar to the Western experience. For example, a study by Freeman looking at 877 patients in a single center in Vancouver, Canada found similar findings; CD was located in the ileocolon in 34.6% followed by colon alone in 27.2% and terminal ileum in 25.3% as divided according the Vienna Classification[21].

It is expected for most Crohn's patients to develop either stricturing or penetrating (fistula, abscess, perforation) complications with time[23] As the median duration of follow up in our patients was fairly long (8 years [5-18.3]), it is not surprising that most of our patients either had stricturing or penetrating disease. Also demonstrated in this study was a high rate of surgery. Almost half the patients had undergone at least one operation.

Therefore, it appears that overall; the clinical presentation of CD in our patient population is similar to that seen in Western countries. Cohort studies looking at the clinical course of the disease in Asian patients suggest that like in the West, most will have chronic, intermittent disease although they may have a more favorable prognosis[24-27].

In terms of prevalence, however, this appears to be low, with CD making up only about 26.0 per 10^5 admissions. The low frequency of diagnosis of CD had been previously reported in our center when only thirteen cases of CD were identified over an eight year period from 1982 to 1989[28]. Our present study was only able to look at the prevalence of hospital admission. The best epidemiological studies should be population based and in countries such as Japan, an IBD registry is available and incidence and prevalence rates are calculated based on the whole population. However, in most countries in Asia such registries are not established and studies are often made based on hospital statistics with the population of the "catchment area" as the denominator[2,7]. Our main limitation is identifying this catchment area to define the population served by our hospital.

Ethnic differences in IBD are intriguing as they point to either differences in host genetic susceptibility or exposure to environmental factors specific to an ethnic group. A recent study on UC carried out in our center showed the highest prevalence among the Indians followed by the Chinese and the Malays[15]. The hospital admission-based prevalence rates were significantly higher in the Indians compared to the Malays but not the Chinese. In Singapore however, there was a statistically significant difference showing a higher prevalence among the Indians compared to the Chinese[2] With respect to CD, the same Singaporean study showed an increased prevalence in CD among the Indians (4.9 per 10^5 compared to 4.0 per 10^5 in the Chinese and 2.9 per 10^5 in the Malays) although this was not statistically significant. In our present study, the highest prevalence of CD was again found in the Indians.

Therefore, in a multiracial Asian country such as Singapore and Malaysia, IBD appears to be more common among the Indians. It is pertinent to note that all the three races have lived together for more than three generations and are exposed to similar environmental factors indicating that perhaps host genetic susceptibility play a more important role in the pathogenesis of IBD in this region.

In conclusion, in our hospital setting, the clinical features of CD are similar to the Western experience. Although the overall prevalence is low, there appears to be ethnic differences in the prevalence of the disease with a probable racial predominance among the Indians. Racial differences in prevalence may provide vital clues to the pathogenesis of the disease, particularly in the Asian setting.

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RAPID COMMUNICATION

Effects of retrorsine on mouse hepatocyte proliferation after liver injury

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Abstract

AIM: To study the effect of retrorsine on mouse hepatocyte proliferation.

METHODS: Mice and rats were treated respectively with two injections of retrorsine (as retrosine-treated group) or saline (as non-treated group) at 2 wk intervals. They received a single injection of carbon tetrachloride (CCl_4) 4 wk later. On d 0, 1, 2, 3, 4, 6, 15 after CCl_4 administration, the animals were killed and their livers were excised. Hematoxylin and eosin (HE) staining and Ki-67 antibody immunohistochemical analysis of liver samples were used to evaluate the pathological changes and hepatocyte proliferation.

RESULTS: In rats treated with retrorsine and CCl_4, the liver displayed obvious megalocytosis, proliferation of mild bile duct, small hepatocyte-forming nodule, which were not found in liver samples from non-treated group. However, in mice treated with retrorsine combined with CCl_4, the liver displayed hepatocyte degeneration and necrosis in perivenous areas. There was no obvious difference between retrorsine-treated group and non-treated group. Ki-67 immunohistochemical analysis showed that in rats treated with retrorsine, the positive hepatocytes mainly found in small hepatocyte nodules, were obviously less than those in non-treated group. The mice treated with retrorsine showed that the number of Ki-67 positive hepatocytes was very high and more than that in non-treated group.

CONCLUSION: Retrorsine has no effect on mouse hepatocyte proliferation.

Key words: Retrorsine; Mouse; Rat; Hepatocyte proliferation

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INTRODUCTION

Hepatocyte transplantation can not only treat liver degenerative disorders and other serious liver injuries, but also replace liver transplantation[1, 2]. Before clinical application, it is necessary to use an animal model to test whether exogenous liver cells can integrate and grow in the recipient liver. Observations in humans and other vertebrates demonstrated that native liver cells have a very high regenerative potential and outgrow the exogenous cells[3]. Therefore, in animal models for hepatocyte transplantation, it is very important to inhibit the proliferation of native hepatocytes. It has been reported that retrorsine, a member of the pyrrolizidine alkaloid (PAs) family can impair the proliferative capacity of mature hepatocytes. Retrorsine-induced blockade is in G1/S, late "S" and /or "G2/M" phase of cell cycle[4-6]. Laconi *et al*.[4] reported that syngeneic transplantation of hepatocytes in liver of dipeptidyl-peptidase type IV-deficient (DPPIV) rats treated with retrorsine could achieve 95% chimerism and restore its normal function. Although retrorsine has been used in rat model, there are very few reports on its use to create a mouse model for hepatocyte transplantation[7]. In this study, we investigated the effect of retrorsine on mouse mature hepatocytes. The results indicate that retrosine cannot inhibit mouse hepatocyte proliferation after liver injury.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice (6 wk) and male F344 rats (5 wk) were purchased from National Rodent Laboratory Animal Resources, Shanghai Branch, China. They were maintained in a 12 h light/dark cycle and fed with standard food and water *ad libitum*. All animals received humane care and study protocols complied with guidelines of Shanghai Second Medical University.

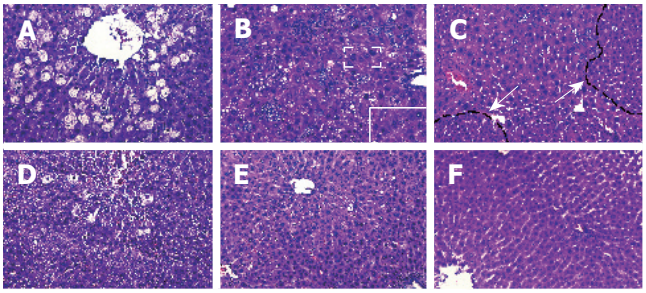

Figure 1 Pathological analysis of rat liver. **A**: Much more severe hepatocyte balloon degeneration and necrosis in perivenous areas of retrorsine-treated group compared with non-treated group (**D**). **B**: Mild bile duct proliferation and megalocytosis (the insert showed the area enclosed in the box at high magnification). **C**: Proliferation of small hepatocytes formed nodules. **E** and **F**: No obvious pathological change was found in non-treated group.

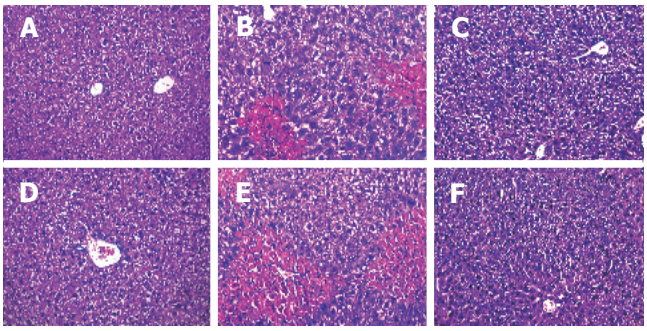

Figure 2 Pathological changes of mouse liver. **A** and **D**: No obvious morphological abnormity. **B** and **E**: Necrosis in perivenous areas, arrow indicates the mitotic figure of hepatocytes. **C** and **F**: Hepatic parenchyma in both groups became normal.

Reagents

Retrorsine (Sigma-Aldrich) was added to distilled water at 10 mg/mL and titrated to pH 2.5 with 1 mol/L HCl to dissolve it completely. The solution was neutralized using 1 mol/L NaOH. Subsequently NaCl was added. The final concentration was 5 mg/mL retrorsine and 0.15 mol/L NaCl, pH 7.0. The working solution was used immediately after preparation.

Carbon tetrachloride (CCl_4) was diluted 1:10 using sterile mineral oil and maintained in a rubber plug-sealed glass tube. Ki-67 (Clone SP6) rabbit monoclonal antibody, a cell proliferation marker[8] was purchased from Lab Vision.

Experimental groups

After one week of acclimatization, the mice and rats were randomly divided into two groups and received two intraperitoneal injections of retrorsine (70 mg/kg for mice[7] and 30 mg/kg for rats[4, 6] (as retrosine-treated group)) or saline (as non-treated group) at 2-week interval. Four weeks after the second injection, diluted CCl_4 was respectively injected into mice and rats, ip 5 mL/kg[7]. Day 0 was set just before CCl_4 injection. On days 1, 2, 3, 4, 6, and 15 after CCl_4 administration, 3-5 animals from each group were killed. The liver of animals was excised and fixed in 40 g/L formaldehyde for the following study.

Pathology and immunohistochemistry

Samples were dehydrated in alcohol and embedded in paraffin. Sections were cut at 5 μm thickness. For pathological analysis, the liver sections were stained with hematoxylin and eosin (HE) according to the standard procedures.

For Ki-67 immunohistochemical staining, antigen retrieval was carried out by incubating slides in antigen retrieval buffer (0.01 mol/L citrated buffer, pH 6.0) at 95 °C for 30 min. The slides were incubated with the primary antibody, Ki-67, at 4°C overnight. The secondary antibody used was anti-rabbit conjugated with horseradish peroxidase (Jackson Immunoresearch). 3,3-diaminobenzidine tetrahydro-chloride containing 0.1g/L hydrogen peroxide was used as a substrate. The proportion of Ki-67 positive hepatocytes was counted from at least 2000 cells from serial fields for each sample under microscope with 20× magnifications.

Statistics analysis

Data were expressed as mean±SE. Sigmaplot 2001 and SAS for windows 6.12 softwares were used for data analysis and plot. The significance of variances was found to be appropriate by Student's *t*- test or χ^2 test.

RESULTS

Pathological analysis

Before CCl_4 administration, liver morphology had no obvious change both in mice and in rats treated with retrorsine. After injecting CCl_4, much more severe hepatocyte balloon degeneration and necrosis in perivenous areas were found on day 1 in retrorsine-treated group than in non-treated group. Some megalocytosis, mild bile duct proliferation and small hepatocyte proliferation-formed nodules occurred in retrosine-treated group but not in non-treated group (Figure 1).

Livers of both retrorsine-treated and non-treated groups showed necrosis in perivenous areas on day 2 after CCl_4 injection. After then hepatocytes in periportal areas began to proliferate, mitotic figures of liver cells could be found. Such a pathological phenomenon might be due to destruction of CCl_4 in liver[9, 10]. There were no megalocytosis and other pathological changes in rats. On the 15th day, hepatic parenchyma in both groups became normal (Figure 2).

Ki-67 immunohistochemical analysis

Before CCl_4 injection, a small number of Ki-67 positive hepatocytes appeared in mice and rats treated with retrorsine. For the rats, the number of positive hepatocytes was increased slowly, reached the peak on day 6 in retrorsine-treated group and most of positive cells were small hepatocytes in nodules. In non-treated group, the maximum number of positive hepatocytes appeared on day 3 (Figures 3 and 5A).

The maximum number of positive hepatocytes was observed on day 4 in retrorsine-treated group of mice and almost all the positive cells were from mature hepatocytes. The maximum number of positive cells appeared on the 2nd day in non-treated group. Both groups showed a similar proliferation pattern (Figures 4 and 5B).

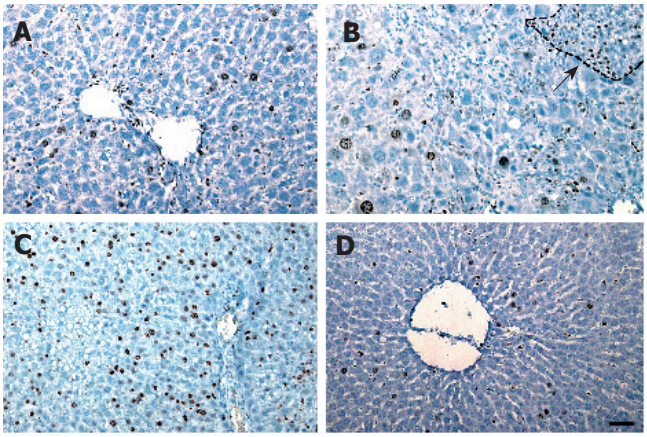

Figure 3 Ki-67 immunohistochemical analysis of rat liver. **A**: A few hepatocytes were Ki-67 positive. **B**: Ki-67 positive hepatocytes were mainly found in small hepatocyte nodules as the arrow indicated. **C**: Abundant Ki-67 positive cells. **D**: Only a few hepatocytes were Ki-67 positive in rat liver.

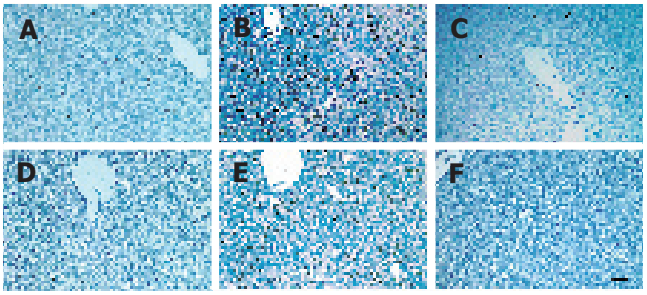

Figure 4 Ki-67 immunohistochemical analysis of mouse liver. **A** and **C**: Some Ki-67 positive cells appeared in retrorsine-treated mice on days 0 and 15. **B** and **D**: The maximum number of Ki-67 positive cells in retrorsine-treated mice on day 4. **E** and **F**: The maximum number of Ki-67 positive cells in non-treated group on day 2.

DISCUSSION

The present study described the comparative pathological changes and kinetic of hepatocyte proliferation in mice and rats treated with retrorsine and CCl_4. Guo *et al* [7] reported that two doses of retrorsine (70 mg/kg at 2 wk interval) could be tolerated by >90% of mice. If the dosage is over 70 mg/kg, the mortality rate of animals would increase. We used this dosage in our experiments and tested ethanol or water as a solvent for retrosine and treated mice with the same dosage (70 mg/kg). The survival rate was 85% (22/26) and 84% (27/32), respectively. No statistically significant difference was displayed between them ($\chi^2=0.001$, $P=0.980$), suggesting that only water can be used as a solvent for retrosine.

The rats treated with retrorsine and CCl_4 showed megalocytosis, mild bile duct proliferation and small hepatocyte proliferation-formed nodules. This phenomenon has been described by many authors[4, 6, 12-15]. But in mice treated with the same protocol, we did not find similar pathological changes. Guo *et al*[7] studied liver repopulation after cell transplantation in mice treated with retrorsine and CCl_4, and found that there are no liver pathological changes.

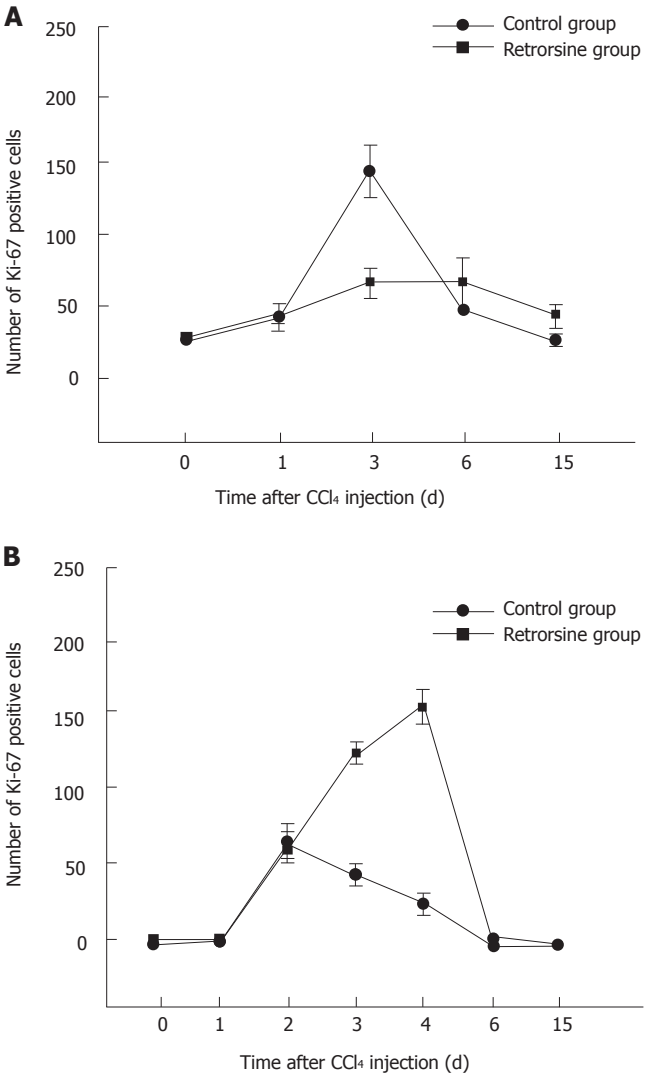

Figure 5 Kinetics of Ki-67 expression in rats (A) and mice (B) after CCl4 injection. **A**: The maximum number of Ki-67 positive hepatocytes on day 6 after injection of CCl4 in retrorsine-treated rats and on day 3 in non-treated group. **B**: The number of Ki-67 positive hepatocytes in retrorsine-treated mice was higher than that in non-treated group delayed. $^aP<0.05$, $^bP<0.01$, $^cP<0.001$ *vs* control group.

Ki-67 immunohistochemical analysis showed that proliferation of hepatocytes in rats treated with retrorsine was blocked. Avril *et al*[16] have reported similar results. Laconi *et al*[6] reported that BrdU labeling index of rats treated with retrorsine combined with partial hepatectomy is also significantly lower than that in non-treated group.

The number of Ki-67 positive hepatocytes in mice treated with the same protocol was higher than that in non-treated group. Guo *et al*[7] reported that the number of proliferating liver cells (detected by proliferating cell nuclear antigen, PCNA) in mice treated with CCl_4 alone is >60%. Our result is consistent with theirs. Since they did not show hepatocyte proliferation after retrorsine treatment combined with CCl_4, we considered that after liver injury mouse hepatocyte proliferation might not inhibited by retrorsine. Since Ki-67 positive hepatocytes in mice treated with retrorsine combined with CCl_4 was higher than that in non-treated group in our study, it is possible that retrorsine might increase the sensitivity of mouse liver to CCl_4 injury because the cell proliferation

response is always dependent on the extent of liver injury.

Retrorsine has long been known for its ability to block hepatocyte division[4-6]. Megalocytosis results from replicating hepatocytes which are blocked after DNA synthesis and prior to mitotic division, thus resulting in a large number of cells with enlarged nuclei (megalocytosis)[4, 6, 17]. Our results demonstrated megalocytosis was only present in livers of rats but not in livers of mice treated with retrorsine, indicating that the effect of retrorsine on mice is different from that on rats. Significant species difference in susceptibility to PAs intoxication has been reported, which is mainly due to the variations in balance between the formation of toxic metabolites and detoxification pathways[18-20]. Although both mice and rats belong to murine, they might have a different process of metabolism or detoxification of PAs in their livers, which may lead to resistance of mice to retrorsine. Moreover, rats receiving CCl_4 2 wk after the second injection of retrorsine displayed higher mortality rate than those receiving CCl_4 4 wk after the second injection of retrorsine (data not shown). There was no obvious difference in the mortality rate and other physiological indices in mice receiving CCl_4 2 wk or 4 wk after the second injection of retrorsine, which proved our hypothesis that retrorsine could seriously injure rat liver but not mouse liver.

As reported by Guo *et al*[7], when hepatocytes are transplanted into mice treated with retrorsine alone, the chimerism rate of exogenous liver cells is less than 1%. It was reported that the chimerism rate of rats treated with retrorsine alone could reach 95%[14]. It is possible that the effect of retrorsine on suppressing proliferation of mouse liver cells is limited in decreasing the chimerism rate. Therefore, retrorsine has no effect on mouse hepatocytes.

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RAPID COMMUNICATION

Ductular proliferation in liver tissues with severe chronic hepatitis B: An immunohistochemical study

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Abstract

AIM: To clarify the pathogenesis of ductular proliferation and its possible association with oval cell activation and hepatocyte regeneration.

METHODS: Immunohistochemical staining and image analysis of the ductular structures in the liver tissues from 11 patients with severe chronic hepatitis B and 2 healthy individuals were performed. The liver specimens were sectioned serially, and then cytokeratin 8 (CK8), CK19, OV6, proliferating cell nuclear antigens (PCNA), glutathione-S-transferase (GST), α-fetal protein (AFP) and albumin were stained immunohistochemically.

RESULTS: Typical and atypical types of ductular proliferation were observed in the portal tracts of the liver tissues in all 11 patients. The proliferating ductular cells were positive for CK8, CK19, OV6 and PCNA staining. Some atypical ductular cells displayed the morphological and immunohistochemical characteristics of hepatic oval cells. Some small hepatocyte-like cells were between hepatic oval cells and mature hepatocytes morphometrically and immunohistochemically.

CONCLUSION: The proliferating ductules in the liver of patients with severe chronic liver disease may have different origins. Some atypical ductular cells are actually activated hepatic oval cells. Atypical ductular proliferation is related to hepatocyte regeneration and small hepatocyte-like cells may be intermediate transient cells between hepatic oval cells and mature hepatocytes.

Key words: Ductular proliferation; Chronic hepatitis B; Hepatocyte regeneration

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INTRODUCTION

Ductular proliferation is often used to describe the appearance of biliary epithelial cells in the portal tracts of diseased livers. The proliferating bile ductules are heterogeneous and are histologically classifiable into typical and atypical types[1]. Atypical and typical ductules have been reported in long-standing biliary diseases such as primary biliary cirrhosis, primary sclerosing cholangitis, extrahepatic biliary obstruction, etc[2-5]. Bile ductular proliferation is also the most commonly observed finding in patients with chronic hepatitis C[6,7]. According to our experience, however, such proliferating ductules are also frequently seen in patients with severe or end-stage chronic liver disease induced by hepatitis B virus (HBV). It is still not clear why these ductular structures are often increased in the liver tissue of such patients. One possibility is that the proliferating ductular cells are human counterparts of rat oval cells or these proliferating ductules may be engaged in hepatocyte regeneration[8-10].

To explore the pathogenesis of ductular proliferation and its possible association with hepatic oval cell activation in patients with severe chronic hepatitis B, we performed immunohistochemical staining and image analysis of the ductular structures in the liver tissues of such patients.

MATERIALS AND METHODS

Liver tissues

A total of 11 liver specimens were obtained from patients (9 male and 2 female) with severe chronic hepatitis B. The average age of the patients was 39.2 years, ranging from 22 to 54. The total serum bilirubin of all patients exceeded 171 μmol/L and the plasma thrombinogen activity of the patients decreased to 40% or less. Among the 11 patients, 10 were infected with HBV and 1 was superinfected with HBV and hepatitis D virus. All of the liver tissue specimens were obtained by needle puncture or by autopsy. For control purposes, archival normal liver tissue was obtained from 2 subjects who had no abnormal liver pathology and

Table 1 Average optical density of typical and atypical ductular cells for CK8, CK19 and OV6 staining

Cell Types	Fields	CK8	CK19	OV6
Typical	8	0.402 ± 0.083	0.902 ± 0.355	0.324 ± 0.121
Atypical	9	0.998 ± 0.238[b]	0.371 ± 0.105[b]	0.752 ± 0.210

[b]$P < 0.001$ *vs* typical

Table 2 Morphometric parameters of hepatic oval cells, hepatocyte-like cells and hepatocytes

Cell types	Cell numbers	At (μm^2)	D_{max} (μm)	D_{max}/D_{min} ratio
Hepatic oval cells	97	19.5 ± 5.9	12.4 ± 7.4	1.8 ± 0.5
Hepatocyte-like cells	42	32.1 ± 6.3[b]	18.9 ± 7.8[b]	1.2 ± 0.1
Hepatocytes	114	41.5 ± 2.3	24.7 ± 5.1	1.1 ± 0.1

[b]$P < 0.001$ *vs* hepatic oval cells and hepatocytes

no biochemical or serological evidence of liver disease. All 10 specimens were fixed in 10% neutral-buffer formalin and embedded in paraffin.

Histology and immunohistochemistry

Serial 4 μm thick sections were prepared and the sections were deparaffinized in xylene and rehydrated through graded alcohol. Part of the sections were stained with hematoxylin and eosin for histological diagnosis. The remaining sections were processed for immunohistochemical staining and a three-step indirect immunoperoxidase procedure was used. The sections were digested with protease (Sigma Chemical Co., St. Louis, MO) for 4 min at 37℃ or boiled in 10 mmol/L citrate buffer, pH6.0, in a microwave for two treatments of 2 min. Endogenous peroxidases were inactivated by immersing the sections in 3% hydrogen peroxide for 10 min. Sections to be used were incubated for 10 min with normal goat serum in Tris-buffered saline to block nonspecific binding. The sections were subsequently incubated overnight at 4℃ with the relevant antibodies. The following day, the sections were incubated with biotinylated anti-mouse IgG (Dako A/S, Glostrup, Denmark) for 45 min at 37℃. The sections were then incubated with peroxidase-conjugated streptavidin (Dako A/S, Glostrup, Denmark) for 45 min at 37℃. The chromogenic reaction was developed with diaminobenzidine and all of the sections were counterstained with hematoxylin. The monoclonal antibodies used were mouse-anti-human cytokeratin 8 (CK8) (Dako A/S, Glostrup, Denmark), CK19 (Serotec Ltd, UK), mouse-anti-human proliferating cell nuclear antigen (PCNA)(Zymed Laboratories, Inc., CA), mouse-anti-human α-fetal protein (AFP) (Zymed Laboratories, Inc., CA), mouse-anti-human glutathione-S-transferase (GST), mouse-anti-human albumin (Zymed Laboratories, Inc., CA) and mouse-anti-rat OV6 (a kind donation from Dr. D. Hixson, Brown University, USA).

Image analysis

Cell image analysis was performed on the liver tissue sections by Tiger® cell image analysis system (Center of Industrial Computer Tomography, Chongqing University, Chongqing, China) to determine total cell area (At), maximum diameter (D_{max}), minimum diameter (D_{min}), D_{max}/D_{min} ratio and average optical density of proliferating ductular structures.

Statistical analysis

Data were analyzed using the t test. $P<0.05$ was considered statistically significant.

RESULTS

Histopathological findings

Massive/submassive hepatocyte necrosis, lymphocyte infiltration, fibrous tissue hyperplasia and cholestasis were observed apparently in the liver tissues of all 11 patients. Hepatocyte proliferation foci and pseudolobules were seen in 6 and 4 patients respectively. In the portal tracts of all the liver specimens from the patients, obviously increased bile ductules were seen, some of which invaded into parenchyma. However, only 1 or 2 bile ductules were observed in the portal tracts of normal liver tissues. The proliferating ductules were also classifiable into typical and atypical types, but cell image analysis showed that the ductular cells from typical and atypical ductules had no significant difference in At, D_{max} and D_{max}/D_{min} ratio.

Immunohistochemical findings

Both types of proliferating ductular cells of patient group were positive for CK8, CK19, OV6 and PCNA staining. However, typical proliferating ductular cells showed strong staining for CK19 and weak staining for CK8 and OV6. In contrast, some atypical proliferating ductular cells showed strongly positive for CK8 and OV6 staining and weakly positive for CK19 staining. There was no significant difference between typical and atypical ductular cells for PCNA staining. In the normal liver tissue taken as control, ductular cells were positive only for CK19 staining. Image analysis demonstrated that typical and atypical ductular cells had significant difference in the intensity and extent of staining (Table 1).

Atypical ductular cells and hepatic oval cells

Morphometrically, the proliferating ductular cells were similar to hepatic oval cells, which were characterized by ovoid nuclei and scanty basophilic cytoplasm. Some atypical ductular cells expressed phenotypes of both ductular cells and fetal hepatocytes, including CK8, CK19, OV6, AFP and GST. These cells were consistent with hepatic oval cells morphologically and immunohistochemically.

Ductular proliferation and liver regeneration

The foci of hepatocyte proliferation were observed in the liver tissues of 6 patients. The regenerating hepatocytes were characterized by different size of cells, anisokaryosis and basophilic cytoplasm. Some hepatocyte-like cells surrounding the proliferative foci were between hepatic oval cells and mature hepatocytes in cell size and morphology (Table 2), and were positive for AFP, GST, OV6 and albumin, demonstrating the features of both hepatocytes and biliary epithelial cells.

DISCUSSION

Bile ductules connect bile canaliculi of periportal hepatocytes with interlobular bile ducts in portal tracts. The bile canaliculi, which form a complicated polyponal network with many anastomotic interconnections, drain into a relatively few bile ductules in periportal area. These bile ductules adjacent to bile canaliculi are the commencement of the biliary tree, and they are composed of ductular cells and hepatocytes. Some may show slight luminal dilatation where several bile canaliculi converge. In normal livers, only a few bile ductules are recognizable in a portal tract, while in various chronic hepatobiliary diseases these ductular structures are often increased. The pathogenesis and significance of this kind of ductular proliferation differ from disease to disease, but generally speaking, typical type of proliferating ductules originate from preexisting ductules and atypical type of proliferating ductules have more complicated origins and may be related to the activation of hepatic stem cells and the transformation of hepatocytes[11].

In ordinary circumstances, liver regeneration is usually achieved by the entry of normal, proliferatively quiescent, differentiated hepatocytes into the cell cycles, but, when hepatocyte regeneration is defective, bile ductular cells can migrate outwards from the portal tracts and then differentiate into hepatocytes. These biliary cells are called hepatic oval cells or oval cells, and their emergence when hepatocyte regeneration is impaired suggests they are the progeny of hepatic stem cells. End-stage chronic liver disease often develops from advanced chronic liver disease and is characterized by massive/submassive hepatocyte necrosis and severe liver failure. The mortality of severe chronic hepatitis B is very high. Our data showed that 72.5% (306/422) of such patients would die of the disease[12]. Theoretically, hepatic stem cells might be activated and proliferated in the liver of those patients. On one hand, massive/submassive necrosis of hepatocytes leads to a sharp decrease in the mass of hepatocytes, and thus the liver has a demand of regeneration. On the other hand, various toxins resulting from hepatocyte necrosis inhibit the proliferation and regeneration of residual hepatocytes. These conditions will activate hepatic stem cells and induce them to proliferate and differentiate toward hepatocytes. However, the above speculation has yet to be confirmed. Investigation of the nature of ductular proliferation will help to understand the mechanism of hepatocytes regeneration in the liver tissues of chronic hepatitis B patients.

Our results showed that two types of ductular proliferation, typical and atypical, existed in the liver of patients with severe chronic hepatitis B, and the phenotypes of the two types of ductular cells differed in some ways. The former were characterized by well-formed lumina and distinct cell borders and the latter characteristically had vague or no visible lumina and rather indistinct cell border. The typical ductular cells and ductules in normal liver were strongly positive for CK19 staining, suggesting that typical ductules originated from preexisting ductules. Some atypical ductular cells displayed the phenotypes of both biliary duct cells and hepatocytes and had the morphometric features of hepatic oval cells, suggesting that at least part of the atypical ductular cells originated from hepatic stem cells. It implied that some atypical ductular cells were actually activated hepatic oval cells. Our results also demonstrated that hepatocyte regeneration in the liver of patients with severe chronic liver disease was related to ductular proliferation. Firstly, some atypical ductular cells were actually activated hepatic oval cells, which could differentiate into hepatocytes ultimately. Secondly, there was histopathological and immunochemical evidence of hepatocyte regeneration in the liver of the patients. And thirdly, some regenerating small hepatocyte-like cells were between hepatic oval cells and mature hepatocytes morphometrically and immunohistochemically, suggesting that these cells were differentiated from hepatic oval cells and were the transient cell type from hepatic oval cells to mature hepatocytes. These findings are consistent with those of *Tan et al*[13]. It is believed that liver stem cells are a potential source of hepatocytes[14-16]. Although hepatic stem cells are activated and can differentiate into hepatic oval cells and transient hepatocyte-like cells ultimately, it is not clear why the diseased liver cannot be regenerated completely. We speculate that the toxins released from hepatocyte necrosis inhibit not only the proliferation and regeneration of residual hepatocytes but also the differentiation and evolution of hepatic oval cells toward hepatocytes.

In conclusion, the proliferating ductules in the liver of patients with severe chronic hepatitis B may have different origins. Some atypical ductular cells are actually activated hepatic oval cells. Atypical ductular proliferation is related to hepatocyte regeneration and small hepatocyte-like cells may be intermediate transient cells between hepatic oval cells and mature hepatocytes. However, why the diseased liver cannot be regenerated completely after hepatic stem cell activation remains to be investigated further.

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RAPID COMMUNICATION

Expression of ICAM-1, HLA-DR, and CD80 on peripheral circulating CD1 α DCs induced *in vivo* by IFN-α in patients with chronic hepatitis B

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Abstract

AIM: To explore the effects of interferon-α (IFN-α) application on peripheral circulating CD1α dendritic cells (DCs) in patients with chronic hepatitis B, and the expression of HLA-DR, CD80, and ICAM-1 on CD1α DCs in order to explore the mechanism of immune modulation of IFN-α.

METHODS: By flow cytometry technique, changes of CD1α DCs were monitored in 22 patients with chronic hepatitis B treated with IFN-α and in 16 such patients not treated with IFN-α within three months. Meanwhile, the expression of HLA-DR, CD80, and ICAM-1 on CD1α DCs was detected.

RESULTS: In the group of IFN-α treatment, the percentage of CD1α DCs in peripheral blood mononuclear cells was increased after three months of therapy. In patients who became negative for HBV-DNA after IFN-α treatment, the increase of DCs was more prominent, while in control, these changes were not observed. Increased expression of HLA-DR, CD80, and ICAM-1 on CD1α DCs was also observed.

CONCLUSION: CD1α DCs can be induced by IFN-α *in vivo*, and the immune related molecules such as HLA-DR, CD80, and ICAM-1 are up-regulated to some degree. This might be an important immune related mechanism of IFN-α treatment for chronic hepatitis B.

Key words: Chronic hepatitis B; DC; Immune costimulatory molecules; Immunotherapy; IFN-α

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INTRODUCTION

Dendritic cells (DCs) play a key role in the process of immune response. They are involved in pathogenesis of tumor and virus infection, and show prospect in immunotherapy[1-3]. Recent studies suggest that DCs are involved in the development of chronic hepatitis B in some extent, and more attentions have been paid to the relation between DCs and hepatitis B[4,5]. Patients with chronic HBV infection have the defective function and immature phenotype of DCs, which may be associated with the inability of efficient presentation of HBV antigens to host immune system for the clearance of HBV[6]. How to increase the levels of DCs and improve their function is important in patients with chronic HBV infection. Patients in the immune active phase are candidates for antiviral therapy.

Interferon-α (IFN-α) is an important therapeutic agent in non-cirrhotic hepatitis patients with mild to moderate disease activity. The primary goal of therapy for chronic hepatitis B is suppression of viral replication, which has been shown to reduce hepatic necroinflammation and retard progression of hepatic fibrosis. Long-term suppression of serum HBV DNA is likely to reduce progression to cirrhosis and hepatic decompensation and may also decrease the risk of hepatocellular carcinoma, but the efficiency of IFN-α is not satisfactory, and further study on its mechanism in the treatment of chronic hepatitis B is necessary.

Many kinds of cytokines such as GM-CSF, IL-4 can induce DCs *in vitro*, and the expression of immune molecules can be up-regulated[7]. Some recent studies suggested that GM-CSF induce DCs *in vivo*[8]. It has been shown that IFN-α could exert the anti-tumour immune modulation action through inducing DCs *in vitro*[9], but the mechanisms of IFN-α to induce DCs *in vivo*, especially to affect immune molecules on the surface of DCs are unclear. CD1α is an important surface marker on DCs[7].

The aim of this study was to evaluate the immune modulation function of IFN-α on DCs besides the direct

Table 1 Effects of IFN-α on CD1 α DC in peripheral blood with chronic hepatitis B

Group	*n*	Percentage of CD1 α DC		*P*
		Pre-therapy	Post-therapy	
IFN-α	22	0.70±0.29%	1.27±0.38%	<0.05
Control	16	0.63±0.17%	0.72±0.22%	>0.05

anti-HBV action of IFN-α. The CD1 α DC and the expression of ICAM-1, HLA-DR, and CD80 on the cells were determined in the peripheral blood of patients with chronic hepatitis B. Whether DCs can be induced *in vivo* in patients with chronic hepatitis B treated with IFN-αwas investigated. Our study will shed new light on the mechanism of enhanced immune response by IFN-α.

MATERIALS AND METHODS

Subjects

Thirty-eight patients with chronic viral hepatitis B were randomly divided into IFN-α group ($n=22$, 16 males, 6 females, aged from 24-48 years, mean 34±6.67) and control group ($n=16$, 11 males, 5 females, aged from 25-45 years, mean 35±5.40). Patients were diagnosed with chronic hepatitis B according to the Programme of Prevention and Treatment for Viral Hepatitis revised by Chinese Society of Infectious Disease and Parasitology, Chinese Society of Hepatology, Chinese Medical Association in 2000. Those patients who were diagnosed with chronic hepatitis B were seropositive for HBV markers (HBsAg, HBeAg/anti-HBe, anti-HBc) for more than 6 months, characterized by an intermittent pattern of disease activity with elevations of alanine aminotransferase (ALT) values preceded, and in most instances, by an increase of HBV-DNA levels. The enrolled patients were diagnosed with chronic hepatitis B, in whom serum markers of HBV (HBsAg, HBeAg, HBcAb, HBV-DNA) were positive, ALT level between two and five times normal limit, total bilirubin below two times normal limit, who could bear treatment with IFN-α. Patients who were infected by other virus and diagnosed with autoimmune disease were excluded.

Treatment procedures and reagents

Patients in IFN-αgroup were treated with 5.0 million units IFN-α daily for 15 d and then every other day for an additional (85) d. The control group was not treated with IFN-α or any other anti-viral or immune modulation drug except routine therapy for protecting liver. Two milliliter blood each time was taken from the enrolled patients with informed consent pre- and post-therapy. The samples were sent for CD1 α DCs assay and the expression of the immune molecules on the cells. Fluorochrome-labeled antibodies were used for flow cytometric analysis such as anti-CD1α-PE, anti-CD80-FITC, anti-ICAM-1-FITC and anti-HLA-DR-FITC (Immunotech, Marseille, France).

Flow cytometric analysis of CD1αDC

CD1 α DCs of peripheral blood was analyzed by flow cytometry, and the expressions of ICAM-1, CD80, and HLA-DR was examined. The ratio of CD1 α DCs/peripheral blood mononuclear cells (PBMC) was counted. One hundred microliter peripheral blood with heparin was mixed with 10 μL homogenic antibody Ig-PE, then were hemolyzed by optilyse C, which served as negative control. One hundred microliter peripheral blood with heparin was mixed with 10 μL anti-CD1α-PE, then hemolyzed with optilyse C. After that, they were analyzed by flow cytometry. Lymphocyte or mononuclear cell subpopulations were distinguished by Forward Scatter (FSC) and Side Scatter (SSC). The percentage of CD1 α DCs in PBMC was investigated.

Analysis of ICAM-1, HLA-DR, and CD80 on surface of CD1αDC

The expression of immune associated molecules(ICAM-1, HLA-DR, CD80) on surface of CD1 α DC was evaluated by flow cytometry by analyzing the percentage in double-stained PBMCs. One hundred microliter peripheral blood with heparin was mixed with 10 ul homogenic antibody Ig-PE, then hemolyzed by formic acid, which served as negative control. One hundred microliter peripheral blood with heparin was hemolyzed by formic acid in each group, next mixed with 10 μL anti-CD1α-PE, and then blended respectively with anti-ICAM-1-FITC, anti-HLA-DR-FITC, and anti-CD80- FITC. Lymphocyte or mononuclear cell subpopulations were distinguished by FSC and SSC. The percentage of $CD1\alpha^+$ $ICAM\text{-}1^+$, $CD1\alpha^+$ $CD80^+$ or $CD1\alpha^+$ HLA-DR in PBMCs was investigated.

Statistical analysis

The results were expressed as mean ± SD and analyzed using the Student's *t* test. $P<0.05$ was taken as statistically significant.

RESULTS

CD1αDC in peripheral blood with chronic hepatitis B and effect of IFN-αon DCs

The results showed that CD1 α DCs existed in peripheral blood of patients with active chronic hepatitis B, and most of them was less than 1% of PBMCs. In IFN-αgroup, after treatment for three months, the percentage of CD1αDCs in PBMC in some patients was increased. There were significant differences between pre- and post-therapy with IFN-α ($P<0.05$). In control group, the percentage of CD1 α DCs pre-therapy was close to that post-therapy ($p>0.05$).In IFN-αgroup, the percentage of CD1 α DCs in the group with decreased HBV-DNA was more than that in the group with HBV-DNA not decreased ($p<0.05$) (Table 1).

Effects of IFN-α on the expression of ICAM-1,HLA-DR,and CD-80 on surface of CD1αDC

It showed that ICAM-1, HLA-DR, and CD-80 on surface of CD1 α DC in peripheral blood of patients with active chronic hepatitis B also existed to some extent. Among them, the expression of ICAM-1, HLA-DR was stronger than that of CD80. After treatment with IFN-α for three months, the expression of ICAM-1, HLA-DR, and CD80

Table 2 Effects of IFN-α on immune related molecules on CD1 α DC in peripheral blood of patients pre- and post-therapy

Group	*n*	ICAM-1		HLA-DR		CD80	
		Pre-	Post-	Pre-	Post-	Pre-	Post-
IFN-α	22	54.97±5.95%	70.61±5.72%a	44.84±9.14%	57.86±7.78%[a]	33.97±8.37%	43.41±8.13%[a]
Control	16	60.17±4.83%	59.90±4.83%	45.01±9.08%	40.50±9.47%	33.42±11.58%	34.80±6.01%

[a]$P<0.05$ *vs* pre-therapy.

on surface of CD1 α DC was increased to some extent. In IFN-α group, the percentage of ICAM-1, CD80, and HLA-DR on CD1αin peripheral blood after treatment with IFN-α for three months was more than that before treatment. There were significant differences between pre- and post-therapy with IFN-α ($P<0.05$). In control group, there were no significant differences between pre- and post-therapy ($P>0.05$) (Table 2).

DISCUSSION

Currently, IFN-α is frequently chosen in treatment of chronic hepatitis B[10], but only 30%-40% of treated patients show response to it. How to improve the efficacy of IFN-α is a challenge. IFN-α is involved in immune modulation besides direct anti-HBV effect. The defect in specific T cell immunity, especially HBV-specific cytotoxic T lymphocyte (CTL) dysfunction has long been assumed to be a central mechanism for hepatitis B virus persistent infection. The cause that effective specific T cell immunity is not induced in patients with chronic hepatitis B is not completely clear. DCs are the most potent antigen-presenting cells that initiate protective T-cell immunity. Recent studies in transgenic mice have suggested, however, that functional deficit of DCs is an underlying cause for T cell dysfunction. Studies showed that HBsAg presentation by cytokine-activated DCs can break tolerance and trigger an anti-viral CTL response in HBV transgenic mice[11-12].In chronic hepatitis B, DCs are present to some degree in peripheral blood and in the liver tissue and might be involved in the immunopathogenesis of chronic liver diseases[13-15]. The current results showed that CD1 α DCs existed in peripheral blood in patients with active chronic hepatitis B, and most of them was less than 1% of PBMC. Whether the immune function of these DCs is effective in patients with chronic hepatitis B needs to be studied.

In literature, the reports on whether IFN-α is involved in immune modulation through DCs in treatment of chronic hepatitis B are few. DCs are most powerful to induce immune response among antigen presenting cells *in vivo*[16]. In recent years, more attentions have been paid to the relation between DCs and hepatitis B. DCs sensitized with HBsAg *in vitro* enhanced the proliferation response of T cell from chronic hepatitis B patients, and successfully induced MHC-I restricted HBV-specific CTLs in mice[4]. These suggest that DCs have powerful ability to present HBsAg. HBV-specific CTLs could be induced in HBV transgenic mice treated with sensitized DCs, therefore,the immune tolerance state of HBV transgenic mice would be broken[11]. It was reported that degree of activation of DC following vaccination would possibly help to predict the outcome of vaccine therapy in HBV carriers[17,18]. All above suggest that DCs play a role in inducing effective immune response to HBV.

However, recent studies have suggested that functional deficit of DCs is an underlying cause for T cell dysfunction. In hepatitis B, not only the numbers of DC subsets were decreased, but also the function of these DCs was impaired in peripheral blood[19-24]. DCs in liver from murine hepatitis B carriers also showed impaired functional capacities[25]. Therefore, to increase the number of and improve the function of DCs is important in patients with chronic HBV infection. DCs could be induced by many kinds of cytokines such as GM-CSF, IL-4 *in vitro*, and the expression of immune costimulatory molecules can be also up-regulated[7,9]. Most of studies on DCs in hepatitis B were done through incubation of PBMCs *in vitro*. In the process, some cytokines were added in order that DCs were induced. So the results observed *in vitro* could not reflect truly the condition of DCs *in vivo*. Whether DCs can be induced *in vivo* in patients with chronic hepatitis B treated with IFN-α was investigated in the current study in an effort to explore the mechanism of enhanced immune response through DCs of IFN-α treatment. In order to observe the changes of peripheral circulating CD1α+DCs and the expression of ICAM-1, HLA-DR, and CD80 on CD1 α DCs *in vivo* by IFN-α application, flow cytometry technique was employed to detect directly CD1α+DCs.

In our study, the effects of IFN-α on CD1α+DCs were investigated. The results showed IFN-α could up-regulate the percentage of CD1α+ DCs in PBMCs besides its direct anti-HBV action. Our findings that the number of CD1α+DCs rose in peripheral blood of patients treated with IFN-α suggested that IFN-α can induce DCs *in vivo*. Previous studies argued that DCs were lack in patients with chronic hepatitis B, and that immune activity was weak. IFN-α could enhance specific immune response to HBV through inducing DCs *in vivo*, consequently, facilitating antigen presentation of HBV and specific T cell triggering.

Previous studies suggested that DCs from patients with chronic hepatitis B showed significantly lower expression of costimulatory molecules B7-1 (CD80), B7-2 (CD86) and impaired allostimulatory mixed lymphocyte reaction, as well as decreased number compared with normal group[3,18]. It indicates that the immune response to HBV is enhanced by up-regulating the immune associated molecules on DCs in patients with chronic hepatitis B. Recent studies showed that IFN-α had similar action *in vitro*. IFN-α is partially involved in immune modulation by inducing DCs *in vitro*[9]. In our study, the expression

of ICAM-1, HLA-DR, and CD80 on surface of $CD1\alpha^+$ DCs differently increased in IFN-α group, but there was no significant change in control group. It suggests that IFN-α played some role in up-regulating the expression of ICAM-1, HLA-DR, and CD80. Many immune molecules are involved in triggering T lymphocyte cells by DCs, especially MHC-II, B7-1 (CD80), B7-2 (CD86), ICAM-1, LFA-3, etc. MHC molecules combined with antigen peptides provide first signal for the activation of T lymphocyte cells. The costimulatory molecules (B7-1, B7-2) are important to trigger T lymphocyte cells. The lowered expression of those immune molecules impaires DCs to trigger T lymphocyte cells. During the treatment of IFN-α for patients with chronic hepatitis B, the ability of DCs presenting HBV antigen was differently improved. Our results suggest that IFN-α improves the presenting ability of DCs to some degree, and strengthens the interaction of DCs and T lymphocyte cells through up-regulating the expression of ICAM-1, HLA-DR, and CD80. IFN-α up-regulating the expression of immune molecules on DCs might be an important mechanism of immune modulation in anti-HBV treatment for patients with chronic hepatitis B.

However, the correlation between the efficacy of IFN-α and DCs is still unclear. Although in patients with chronic hepatitis B, peripheral circulating DCs and their expression of ICAM-1, HLA-DR, CD80 are increased by IFN-αapplication, only a minority of treated patients show response to IFN-α. It indicates that the effects of anti-HBV treatment of IFN-α are affected by many other factors[26,27], for example, CD80-B7 interaction promotes immune response, and CD80-CTLA4 interaction down-regulates the response. The function of DC might be affected by these factors, as well[28,29].

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RAPID COMMUNICATION

High expression level of soluble SARS spike protein mediated by adenovirus in HEK293 cells

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Abstract

AIM: To develop a highly efficacious method for preparation of soluble SARS S-protein using adenovirus vector to meet the requirement for S-protein investigation.

METHODS: The human adenovirus vector was used to express the soluble S-protein (corresponding to 1~1190 amino acids) fused with Myc/His tag using codon-optimized gene construct in HEK239 cells. The recombinant adenovirus bearing S-protein gene was generated by ligation method. The expressed S-protein with Myc/His tag was purified from culture medium with Ni-NTA agarose beads followed by dialysis. The S-protein was detected by Western blot and its biologic activity was analyzed by binding to Vero cells.

RESULTS: Under the conditions of infection dose (MOI of 50) and expression time (48 h), the high-level expression of S-protein was obtained. The expression level was determined to be approximately 75 μg/10^6 cells after purification. Purified soluble S-protein was readily detected by Western blot with anti-Myc antibody and showed the ability to bind to surface of Vero cells, demonstrating that the soluble S-protein could remain the biologic activity in the native molecule.

CONCLUSION: The high-level expression of S-protein in HEK293 cells mediated by adenovirus can be achieved under the optimized expression conditions. The proteins possess the biologic activity, which lays a foundation for further investigation of S-protein biological function.

Key words: SARS-CoV; S-protein; Expression; Adenovirus vector

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INTRODUCTION

Severe acute respiratory syndrome (SARS) is a life-threatening disease. The etiological agent of SARS has been identified as a novel coronavirus, called SARS coronavirus (SARS-CoV)[1-3]. SARS-CoV is a positive-strand RNA virus encoding several structural proteins including spike (S) glycoprotein, nucleocapsid protein (N), membrane protein (M) and envelope protein (E)[4, 5]. S-protein, a 1255 amino-acid type I membrane glycoprotein, is the prominent protein present in viral membrane and consists of a signal peptide sequence (amino acids 1 to 13), an extracellular domain (amino acid 14 to 1190), a transmembrane domain (amino acids 1191 to 1227) and a short intracellular tail (amino acids 1228 to 1255). S-protein plays an important role in SARS coronavirus infection by interaction with cellular receptor, angiotensin-converting enzyme 2 (ACE2) on the host cells[6-8]. S-protein also has efficient antigenicity and could induce neutralization antibodies[9-12]. These characteristics make it a potential candidate for subunit vaccine and anti-SARS drug development and diagnostic application. Large-scale preparation of S protein is desirable for S protein investigation, such as protein-protein interaction, S protein-based vaccination and diagnostic approach. In order to obtain sufficient quantities of S-protein, we used human adenovirus vector to construct the recombinant adenovirus bearing SARS soluble S-protein gene and express it in human embryonic kidney 293 cells (HEK293 cells).

MATERIALS AND METHODS

Materials

HEK293, HEK293T and Vero African green monkey kidney cells were purchased from American Type Culture Collection (ATCC); Manassas VA. HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 u/mL penicillin, 100 μg/mL streptomycin, 2mM L-glutamine and 1×MEM non-essential amino acid solution (DMEM

complete medium) at 37℃ and 50 mL/L CO_2. Vero cells were cultured in medium 199 supplemented with the same components as above except for 1×MEM non-essential amino acid solution (medium-199 complete medium). Adenovirus vectors (pShuttle2 and Adeno-X DNA) based on human adenovirus type 5 (Ad5) were purchased from BD Sciences Clontech (Palo Alto, CA). QuikChange® XL site-directed mutagenesis kit was obtained from Stratagene (La Jolla, CA). Restriction enzymes and T4 DNA ligase were obtained from Promega (Madison, WI).

Plasmid constructions

The full-length codon-optimized SARS-CoV S-protein cDNA, cloned into *Xba*I/*Bam*HI sites in the pcDNA3.1 (-) expression vector, was kindly provided by Dr. Mecheal Farzan (Brigham and Women's Hospital, Harvard University, Boston, USA) [6]. The vector was modified to express soluble SARS S-protein fused with Myc/His tag at C-terminus. Briefly, a *Eco*RI site was introduced in S-protein DNA between extracellular and transmembrane domains (corresponding to residues K1193 and W1194) using QuikChange® XL site-directed mutagenesis kit and the following primers: 5'gtacgagcagtacatcgaattcccttggtatgtg tggctg3' and 5' cagccacacataccaagggaattcgatgtaatgctcgtac3'. The EcoRI-mutated expression vector was then digested with EcoRI and BamHI. The DNA fragment encoding Myc/His with additional EcoRI and BamHI sticky ends at 5' and 3' terminuses was generated by annealing the following primers: 5'aattcgaacaaaaactcatctcagaagaggatctg (myc)accggtcatcatcaccatcaccat(6His) tga(stop codon)g3' / 5'gatcctca(stop codon)atggtgacggtgatgatg(6His)accggtc agatcctc ttctgagatgagtttttgttc(myc)g3'. Then the Myc/His fragment was inserted into the *Eco*RI and *Bam*HI sites of *Eco*RI-mutated vector to generate pcDNA3.1-S/MH containing the soluble S-protein fused with Myc/His gene.

In order to conveniently subclone fused soluble S-protein gene into adenoviral shuttle vector, the multiple cloning sites (MCS) of pShuttle2 were modified with a new MCS sequence. Briefly, the new MCS sequence was amplified by PCR method with a pair of primers: 5'tttggctagcggtaccgcggccgcgggatccaagatatcgggc3' and 5'tttcccgggttcgaatctagagggcccgatatcttggatc3' under the conditions of 94 ℃ for 5 min, 50 ℃ for 1 min and 72 ℃ for 10 min. The amplified 65 bp PCR product (the new MCS sequence) was digested with *Sma*I (blunt end) and *Nhe*I, respectively. The digested products were purified with GFX™ PCR DNA and gel band purification kit (Amersham). The pShuttle2 plasmid was first digested with *Kpn*I, after filled using *Klenow* DNA polymerase to generate blunt end, the linearized plasmid was then digested with *Nhe*I. Finally, the new MCS was inserted into NheI and blunt sites of the pShuttle2 to generate pShuttle3 vector.

The fused soluble S-protein gene in pcDNA3.1-S/MH was cut out with *Xba*I and *Bam*HI and downstream cytomegalovirus immediate early (CMV) promoter of pShuttle3 was subcloned using *Nhe*I and *Bam*HI sites to produce pShuttle3-S/MH.

Construction of recombinant adenoviral plasmid

Recombinant adenovirus vector expressing fused soluble S-protein gene downstream of CMV promoter was constructed using the Adeno-X™ expression system (BD Sciences Clontech), following the manufacturer's instructions by the ligation method (Mizuguchi & Kay 1998)[13]. Briefly, the pShuttle3-S/MH was digested with PI-*Sce*I and I-*Ceu*I to release the expression cassette including the promoter, fused soluble S-protein gene and the SV40 polyadenylation signal (SV40 poly A). This DNA fragment was subsequently ligated to the pAdeno-X DNA (provided with kit) to generate pAdeno-S/MH. After ligation it was digested with *Swa*I restriction enzyme to eliminate non-recombinant plasmids and used to transform *E. coli* DH5α competent cells, clones bearing ampicillin resistance were selected. Positive recombinant plasmids were identified by screening plasmids with *Xho*I digestion.

Generation and identification of recombinant adenovirus

Approximately 1-2×10^6 HEK293 cells were plated in T25 flasks 24h before transfection when they reached 60-80% confluence. About 3 μg of pAdeno-S/MH, digested with *Pac*I, was transfected into HEK293 cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The transfected HEK293 cells were cultured in DMEM complete medium at 37 ℃ and 50mL/L CO_2. After two days the medium was changed to maintaining medium (containing 5% serum). After about two-week maintaining culture, the cytopathic effect (CPE) appeared in the monolayer of HEK293 cells. The cells was harvasted and viral DNA was prepared with Hirt method[14]. The recombinant adenovirus carrying S-protein gene (Ad-S/MH) was identified with PCR method using a pair of primers: 5'agcaacttccgcgtg3'/5'ggtgttgcggtcctg3' (corresponding to Ser_{301} to Thr_{760} residues) and the viral DNA as template. The viral titer was determined by plague assay in HEK 293 cells.

S-protein purification and identification

Eighty percent confluent HEK 293 cells in 4×T75 flasks were infected with 3 mL harvested medium from the initial recombinant adenovirus lysates (~10 MOI) in 20 mL culture medium. One day later, the medium was replaced with serum-free medium (Opti-MEM, 50μg/mL BSA, L-glutamine and antibiotics). After 48h, the medium containing S-protein was harvested and centrifuged at 8 000 r/min for 10 min. The supernatant was mixed with Ni-NTA-agrose beads and 1×binding buffer (20 mmol/L Tris, 150 mmol/L NaCl, 5 mmol/L imidazole, pH 8.0), incubated at 4 ℃ overnight with shaking, and then precipitated. The beads were washed with washing buffer (20 mmol/L Tris, 500 mmol/L NaCl, 10 mmol/L imidazole, pH 8.0). Bound S-protein was eluted with elutting buffer (20 mmol/L Tris, 500 mmol/L NaCl, 250 mmol/L imidazole, pH 8.0).

The eluted samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% (w/v) gels. The separated proteins were either stained with silver staining method or electro-blotted and detected by Western-blotting using a mouse anti-His antibody followed by a goat anti-mouse IgG conjugated with alkaline phosphatase. The blots were developed using the Immun-Star™ substrate pack (Bio-Rad).

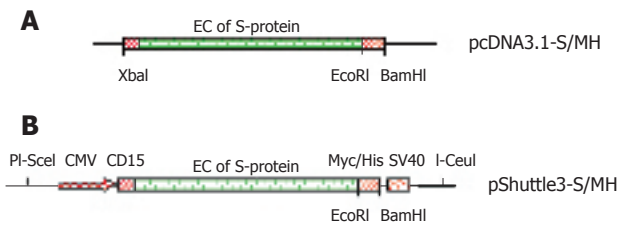

Figure 1 Construct of codon-optimized SARS S-protein genes. The full-length of SARS codon-optimized S-protein gene was modified by inserting Myc/His gene immediately downstream extracellular domain of S-protein gene to generate pcDNA3.1-S/MH (**A**) and the S-protein fused with Myc/His gene was transferred into pShuttle3 vector to produce pShuttle3-S/MH vector (**B**).

The eluted protein was dialysed against PBS (pH 8.0) to elimminate imidazole. The protein concentrations were determined using the Bio-Rad (CA, USA) protein assay kit developed based on the method of Bradford (Bradford, 1976) [15].

S-protein binding to the cell surface

The Vero and HEK293T cells were detached from flask walls with 0.5mM EDTA in PBS, washed and re-suspended in PBS containing 0.5% (w/v) BSA (PBS-BSA) at ~4×10^7 cells/mL. Each cell sample (50 μL) was incubated with 2 μg of S-protein for 3 h at 4℃ with rotation. The cells were washed with PBS-BSA and incubated in 20% (v/v) goat serum in PBS-BSA for 30 min on ice. After washing with cold PBS-BSA, the cells were incubated with a mouse anti-myc antibody (Roche, Indianapolis, OIN) at 10 μg/mL for 30 min on ice. As a control, the cells were not incubated with S-protein. After washing with PBS-BSA, the cells were incubated with goat anti-mouse IgG conjugated with PRE for 40 min on ice, were washed and fixed in 1% (w/v) paraformaldehyde and analyzed by a FACScalibur flow cytometer (BD Biosciences).

RESULTS

Construction of pAdeno--S/MH recombinant adenovirus plasmid

SARS-CoV S-protein, like other coronaviruses, is the prominent surface protein existed in viral lipid envelope, which mediates virus interaction with host cells leading to infection. To investigate the S-protein-mediated interaction between virus and host, soluble S-protein was needed for the investigations.

In order to express the codon-optimized soluble S-protein fused with Myc/His tag using adenovirus vector, the transmembrane and C-terminal domain of full-length S-protein gene were deleted by creating *Eco*RI site immediately downstream the extracellular domain and then the DNA fragment encoding Myc/His with stop codon was inserted into *Eco*RI and *Bam*HI sites of the *Eco*RI-mutated vector to generate pcDNA3.1-S/MH (Figure 1A). The pShuttle3-S/MH (Figure 1B) was constructed by transferring soluble S-protein gene fused with Myc/His tag from pcDNA3.1-S/MH into adenoviral pShuttle3 vector derived from pShuttle2 by modification on MCS. The fusion of Myc/His tag to S-protein could facilitate purification and detection of expressed S-protein.

The entire expression cassette of S-protein, which in pShuttle3-S/MH (Figure 1B) is flanked by unique PI-*Sce*I and I-*Ceu*I sites, was released using these enzymes and ligated into the pAdeno-X DNA vector. The new vector contained a different resistance marker (ampicillin) from pShuttle3-S/MH (kanamycin), preventing pShuttle3-S/MH recovery. The candidate clones were screened by digesting the recombinant plasmids using *Xho*I enzyme. Three positive pAdeno--S/MH plasmids were constructed.

Obtaining Ad-S/MH adenovirus in HEK293 cells

The plasmid pAdeno-S/MH containing two *Pac*I restriction sites, was located in both ends of the viral genome, 3' and 5' of the inverted terminal repeats (ITRs). Thus, to ensure efficient replication and packaging of recombinant adenovirus carrying S-protein gene (Ad-S/MH), the plasmid was digested with *Pac*I and used to transfect HEK293 cells. After 15 d plaques appeard and then a large number of cells were detached from the flask, indicating the presence of adenovirus. To confirm the presence of the S-protein gene in recombinant adenovirus, the viral DNA was extracted and identified by PCR using the viral DNA as template. The specific DNA fragment (1 370 bp) was amplified from recombinant adenoviral DNA .

Expression of soluble SARS-CoV S-protein

Using Ad-S/MH adenoviruses to infect HEK 293 cells cultured in serum-free medium, the medium was harvested. The S-protein was purified using Ni-NTA agarose columns binding to the C-terminal His tag at 4 ℃. Fractions of eluted proteins were analyzed by SDS-PAGE under reducing conditions and identified either by silver staining and assignment using a molecular marker (Figure 2A) or Western-blotting using anti-Myc antibody detection (Figure 2B). The analysis revealed that the size of the purified S-protein under reducing conditions was consistent with previous studies[6,16]. However, it was much larger than the calculated molecular weight of ~132ku (1199 residues: 1181 residues of extracellular domain and 18 residues of myc/His tag (EQKLISEEDLTGHHHHHH)). This difference was considered to be related to glycosylation[16] since the extracellular domain of S-protein was predicted to have 18 potential N-link glycosylation sites, contributing to the observed such bigger molecular weight of S-protein.

Tunicamycin, an inhibitor of N-linked glycosylation, was used to treat the cells (7μg/mL) when they were infected with Ad-S/MH adenoviruses. S-protein secretion from the cells into the media was effectively blocked in the presence of tunicamycin, S-protein could not be detected by Western blotting from the culture media. In contract, without tunicamycin, S-protein could be detected from the media. However, when the cell lysate was detected by Western blotting, S-protein was detected from tunicamycin-treated cells as a size of 130 ku, smaller than the size of ~200 ku detected from non-tunicamycin-treated cells (Figure 3). This indicated that the low molecular weight of S-protein in tunicamycin-treated cells was due to lack of N-liked glycosylation, interfering with S-protein maturation and secretion.

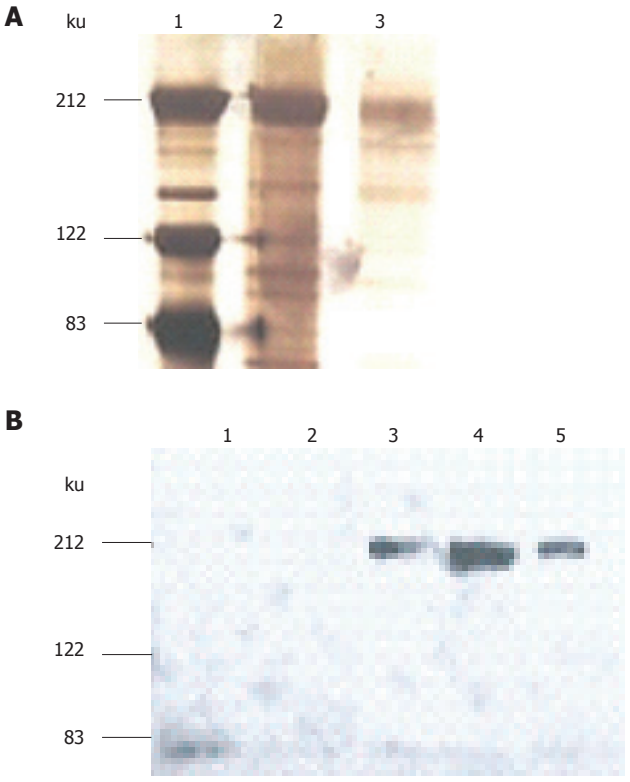

Figure 2 Expression and purification of fused S-protein soluble. S-protein fused with Myc/His was expressed in HEK293 cells mediated by adenovirus and purified on Ni-NTA-agarose column and eluted with imidazole at 6 fractions. The eluted proteins were separated by 7% SDS-PAGE gels and identified by both silver staining (**A**) for elutions 4, 5 and Western-blotting using anti-Myc antibody (**B**) for elutions 1-5.

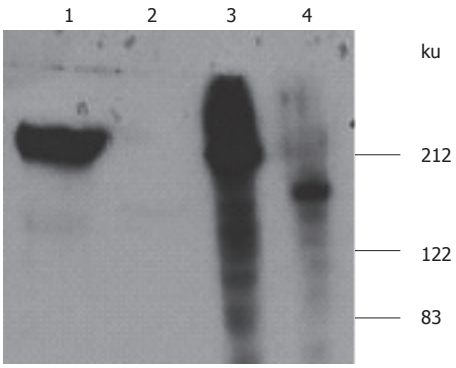

Figure 3 Effects of tunicamycin on expression of S-protein. HEK293 cells were infected with Ad-S/MS virus for 48h in the presence or absence of tunicamycin (7μg/mL). The media were mixed with Ni-NTA-agarose beads and bound protein was eluted with imidazole elution buffer. The pelleted cells were lysed. After centrifugation, the supernatant with eluted protein from the media was separated on SDS-PAGE gels (7%, v/v). Separated proteins were detected by Western blotting using anti-Myc antibody.

To determine the optimal dose (M.O.I.) of adenovirus for cell infection and optimal time for harvesting the culture media for S-protein purification, the HEK293 cells were prepared at the same confluence (about 70%) and infected with different doses of Ad-S/MH viruses and the culture media were harvested at different time points. The purified S-protein levels were evaluated with Bio-Rad protein assay kit. The high-level S-protein (above 75μg/10^6 cells) was obtained using an MOI of 10-100 at 72 h, or

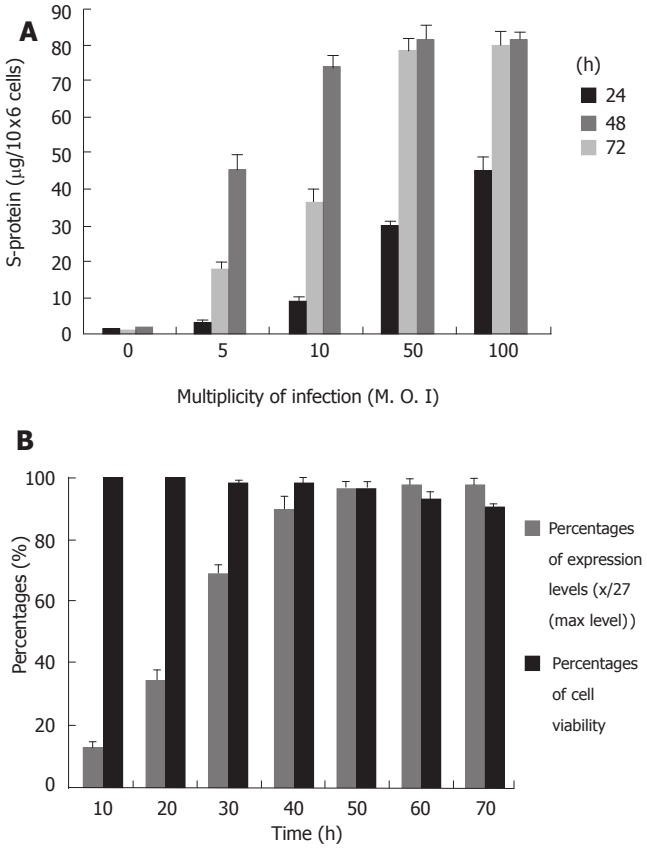

Figure 4 Analysis of expression conditions. **A:** HEK293 cells were infected with Ad-S/MH viruses at different MOI of 5-100. The culture media were harvested at 24, 48 and 72 h after infection. **B:** Cells were infected with same viruses at MOI of 50. The media were harvested at 10, 20, 30, 40, 50, 60 and 70h after infection. The expressed protein was purified with Ni-NTA-agarose and measured with protein assay kit. The dada of expression levels were the mean of three separate experiments.

50-100 at 48 h after infection (Figure 4A). On the basis of these results, we chose an MOI of 50 for viral infection and 48 h for expression time as the suitable conditions because these conditions led to high-level expression and low time-consuming. To confirm these conditions, time-course of expression and cell viabilities were carried out with MOI of 50 for infection. The high expression levels (above 75 μg/10^6 cells) and high cell viabilities (above 96%) were obtained using MOI of 50 between 40 h and 50 h periods (Figure 4B). We repeated three expressions and purifications under these conditions. The average yield of purified S-protein was approximately 75 μg/10^6 cells.

Biological analysis of S-protein

ACE2 of host cells was reported to be a functional receptor for SARS-CoV[6]. S-protein interaction with ACE2 was reported to be related to S-protein extracellular domain, in which amino acids 270 to 510 were identified to be essential for the interaction[17,18]. Vero cells, but not 293T cells could permit replication of SARS-CoV since the receptor ACE2 could be expressed on Vero but not 293T cells[6]. To determine whether the expressed S-protein had the biological activity to bind to Vero cells, Vero cells and 293T cells (as a negative control) were incubated with the purified S-protein and bound S-protein was detected by flow

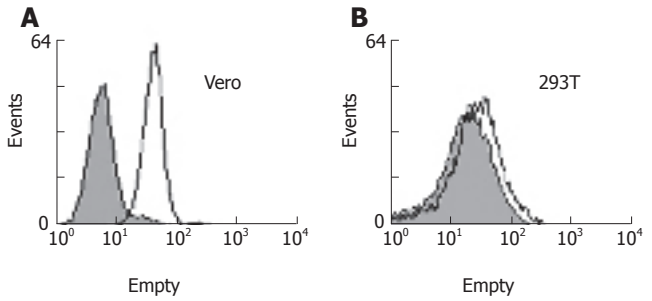

Figure 5 Binding of S-protein to Vero and 293T cells. Vero and 293T cells were detached from culture flask with 0.5 mmol/L EDTA/ PBS, washed and incubated with purified S-protein in 0.5% BSA/ PBS. Binding was measured by flow cytometry using anti-Myc antibody and goat anti-mouse IgG antibody conjugated with RPE (open profiles). As control (filled profiles), the cells were not incubated with S-protein but stained with the same antibodies.

cytometry using a mouse anti-Myc antibody. Binding assay showed that the purified S-protein could bind to Vero cells (Figure 5A) but not 293T cells (Figure 5B). These results demonstrated that the purified S-protein here had the biological activity.

DISCUSSION

Using codon-optimized gene constructs to express the corresponding protein in host cells is a common strategy for increasing expression levels. It was reported that codon-optimized construct can express S-protein in mammalian cells and a reasonably high-level expression is obtained[11,17]. In this paper, we used adenovirus vector to express S-protein with codon-optimized gene and very high-level expression was observed in HEK293 cells. However, we did not successfully express S-protein in 293T cells with wild-type gene construct at a detectable level since the majority of wild-type S-protein codons were deviated from main codons of humans or animals.

Another strategy to increase expression level of S-protein with wild-type S-protein gene in mammalian cells is co-infection of vaccine virus expressing T7 polymerase[16] which facilitates the corresponding mRNA synthesis in cells when the gene clones downstream T7 promoter of the eukaryotic expression vector.

Studies have been conducted to express full-length or truncated S-protein genes in prokaryotic cells[19] and eukaryotic cells[6,16]. Expression of S-protein in *E. coli* leads to the accumulation of recombinant protein in inclusion bodies which requires solubilization followed by *in vitro* refolding. Moreover, the expressed S-proteins tend to lose some biological activities because the proteins are not modified by glycosylation which involves protein-protein interactions and receptor binding. Mammalian cells are widely used to prepare S-protein for the investigation of S-protein interaction with receptors. Although the expressed S-protein is glycosylated and functionally active using mammalian expression system, the expression level of S-protein is relatively lower than expected and the procedure involved in the expression and purification is relatively time consuming and labor intensive. We report here an adenovirus-mediated expression system for SARS S-protein. Once the recombinant adenovirus is made, the high yields of S-protein expression can be obtained in a shorter time period, thus making the procedure more time- and cost-effective.

Many adenovirus expression systems are commercially available and can be used to exploit the different strategies to construct recombinant adenoviruses. The Adeno-X^{TM} expression system we used in this paper could exploit the ligation method to construct recombinant adenovirus, which makes the recombination more efficient. The new MCS sequence of pShuttle vector modified in this paper makes it more flexible for cloning.

Since the replication-deficient recombinant adenovirus grows and propagates extremely fast in HEK293 cells, the conditions for S-protein expression in these cells should be accurately controlled and optimized. In general, the high-titer virus used and the long expression time maintained result in a high level of expression. Nevertheless, exposure of the cells to the high-titer adenovirus for a long time lends to decrease cell viability, in which intracellular components of the dead cells are released into media, interfering with the following S-protein purification.

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RAPID COMMUNICATION

Prognostic factors of young patients with colon cancer after surgery

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Abstract

AIM: To investigate the prognostic factors of 96 young patients with colon cancer within a cancer center by univariate and multivariate analysis.

METHODS: A total of 723 patients with colon cancer were treated surgically during a period of 10 years. Ninty six of them were 40 years old or younger. R0, R1 and R2 operations were performed in 69 (71.9%), 4 (4.1%) and 23 patients (24%), respectively. Left hemicolectomy was performed in 43 patients, right hemicolectomy in 37 patients, transverse colon resection in 9 patients and low anterior resection in 7 patients. Cox multivariate regression analysis was performed to identify predictors of survival.

RESULTS: The operation mortality was 0%, 54 patients died within 111 mo after operation due to occurrence or metastases of the tumor. Liver, lung and bone metastases occurred in 3, 1 and 5 patients, respectively. The mean survival time for all patients was 77.9 ± 5.01 mo and the overall 3-, 5- and 10- year survival rates were 66.68%，58.14% and 46.54%, respectively. In the univariate survival analysis, patient age，type of operation, radical resection, blood transfusion, histological type, diameter of tumor, depth of tumor invasion, lymphatic invasion, distant metastases, liver metastases and TNM stage were found to be predictors of survival in young patients with colon cancer. In the Cox-regression analysis, blood transfusion and lymphatic invasion were determined as independent prognostic factors of survival.

CONCLUSIONS: Age, type of operation, radical resection, blood transfusion, histological type, diameter of tumor, depth of tumor invasion, lymphatic invasion, distant metastasis and TNM stage are the predictors of survival in young patients with colon cancer after surgery.

Key Words: Prognosis; Colon cancer; Young patient; Surgery

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INTRODUCTION

Colorectal cancer is one of the most common malignancies and nearly 600 000 cases are diagnosed annually worldwide [1]. Advances in the management of colon cancer over the past decades have resulted in improvement of the prognosis of this disease. The proportion of early stage (stages I and II) patients has increased from 39.6% to 56.6%, with a corresponding decrease in the proportion of patients with advanced stages, leading to an improvement in five-year relative survival from 33.0% in 1970s to 55.3% in 1990s [2]. The 5-year survival rate is around 60% [3].

Most of the patients with colon cancer are middle-aged or older and the peak of morbidity is around 65 years of age [2]. However, young patients are not rare and the incidence of colon cancer in young patients has increased gradually in recent years. We therefore analyzed retrospectively the clinical and pathological characteristics of a large cohort of colon cancer patients (40 years old or younger) treated in a cancer center over a 10-year period to investigate the prognostic factors.

MATERIALS AND METHODS

Patients

A total of 723 patients with colon cancer were treated between January 1991 and January 2000. All the patients were surgically treated, diagnosed pathologically and included in follow-up procedure. Ninety-six (13.3%) were 40 years old or younger. The cancer was found in the ileocecal junction of 23 patients, in ascending colon of 19 patients, in hepatic flexure of 7 patients, in transverse colon of 11 patients, in splenic flexure of 4 patients, in descending colon

Table 1 Prognostic factors of young patients with colon cancer after surgery

Items		Number of cases	AST(m)	3-YSR (%)	5-YSR (%)	10-YSR (%)	*t*	Degree of freedom	*P* value
Gender	Male	58	81 ± 6.42	69.92	61.35	45.30	0.46	1	0.499
	Female	38	73.62 ± 7.92	62.48	53.69	48.32			
Age	≤30	30	49 ± 5.47	52.50	47.73	47.73	2.4	1	0.0387
	30-40	66	82.74 ± 5.76	73.32	63.12	49.55			
Duration of	≤1	15	34 ± 5.28	45	36	-	5.33	4	0.2553
symptoms	1-3	37	108 ± 30.25	77.04	67.56	40.04			
(m)	3-6	25	69	60	60	50			
	6-12	5	71.80 ± 20.15	53.33	53.33	-			
	≥12	14	80.55 ± 12.34	78.57	56.25	56.25			
Operation	LHC	43	67 ± 1.95	97.14	60.43	10.34	9.43	3	0.0241
	RHC	37	78 ± 9.00	83.20	62.04	15.75			
	TCR	9	52 ± 20.99	85.71	42.86	0			
	LAR	7	37	66.67	0	0			
Radical	R0	69	98.2 ± 4.92	87.92	77.67	63.40	80.72	2	<0.01
resection	R1	4	32 ± 3.50	50	75	75			
	R2	23	24 ± 2.04	6.52	0	0			
Blood	0	60	85 ± 6.3	72.59	64.10	59.17	7.69	2	0.0214
transfusion	400	29	69 ± 8.7	61.29	54.08	38.93			
(mL)	≥800	7	31 ± 9.89	35.71	17.86	-			
Pathological	WDAC	5	92.75 ± 15.80	100	75	-	21.69	6	0.0014
type	MDAC	27	101.89 ± 7.20	88.09	83.68	75.32			
	PDAC	18	31 ± 3.09	37.50	31.25	-			
	PAC	4	58.75 ± 11.47	75	75	-			
	MAC	27	60 ± 24.73	67.73	46.95	31.30			
	SRCC	3	23.33 ± 5.39	0	0	0			
	Others	12	76 ± 15.11	58.33	58.33	58.33			
Diameter of	≤4	16	101.19 ± 9.68	78.97	78.97	78.97	14.16	2	<0.001
tumor	4-8	53	84.02 ± 6.25	76.79	61.93	48.38			
(cm)	≥8	27	31 ± 8.83	39.16	39.16	-			
T	T1	4	-	100	100	100	57.91	3	<0.001
	T2	10	111.5 ± 8.06	90	90	90			
	T3	58	108 ± 29.54	82.34	68.15	38.94			
	T4	24	24 ± 3.13	10.33	-	-			
N	N0	45	111.71 ± 3.93	95	91.72	78.62	51.40	2	<0.001
	N1	30	37 ± 2.42	51.85	34.90	29.08			
	N2	21	23 ± 2.86	28.57	19.05	-			
M	M0	75	94.59 ± 4.95	84.66	73.58	61.45	80.03	1	<0.001
	M1	21	24 ± 2.29	4.76	0	0			

AST: average survival time; 3-YSR: 3-year survival rate; 5-YSR: 5-year survival rate; 10-YSR: 10-year survival rate; LHC: left hemicolectomy; RHC: right hemicolectomy; TCR: transverse colon resection; WDAC: well-differentiated adenocarcinoma; MDAC: moderately-differentiated adenocarcinoma; PDAC: poorly-differentiated adenocarcinoma; PAC: papillary adenocarcinoma; MAC: mucinous adenocarcinoma; SRCC: signet-ring cell carcinoma

of 6 patients and in sigmoid colon of 26 patients. Stage III or IV disease was found in 54.2% of the patients, lymph node metastasis occurred in 53% of the patients, poorly-differentiated mucinous adenocarcinoma and/or signet-ring cell carcinoma were found in 48 patients (Table 1).

Treatment methods

All patients received surgical treatment. Right hemicolectomy was performed in 37 patients, left hemicolectomy in 43 patients, transverse colon resection in 9 patients and low anterior resection in 7 patients. R0 (pathological radical resection of tumor) operation was performed in 69 patients (71.8%), R1 (positive microscopic margins) in 4 (4.2%) patients and R2 (gross tumor was seen at the resection margins) in 23 (24%) patients.

Follow-up

All the patients were followed up through telephone, letter or re-examination in outpatient department annually. The dead line of follow-up was up to January 2002. The longest follow-up time was 120 mo with an average period of 67.6 mo.

Statistical analysis

All data were analyzed by SPSS 10.0 statistical software. Analysis of variance was used to determine significant differences in prognosis of patients. Cox multivariate regression analysis was performed to identify predictors of survival. Quantitative data were expressed as mean ± SD. Survival was analyzed using the Kaplan-Meier survival method. $P<0.05$ was considered statistically significant.

RESULTS

Operative mortality

Operative procedure was carried out in all patients. No pa-

Table 2 Multivariate analysis of prognosis of patients with colon cancer

	B	SD	Wald	Exp (B)	*P*
Gender	0.094	0.455	0.043	1.099	0.836
Age	0.280	0.396	0.502	0.756	0.479
Duration of symptoms	0.017	0.183	0.008	0.983	0.927
Operation	0.285	0.228	1.563	1.329	0.211
Radical resection	0.341	0.602	0.321	1.407	0.571
Blood transfusion	0.683	0.325	4.421	1.980	0.035
Pathological type	0.135	0.109	1.513	1.144	0.219
Diameter of tumor	0.233	0.445	0.274	0.792	0.601
Depth of tumor invasion	0.976	0.537	3.303	2.655	0.069
Lymph node metastasis	0.949	0.390	5.910	2.583	0.015
Distant metastasis	0.211	0.928	0.052	0.810	0.820
TNM	0.666	0.622	1.145	1.946	0.285
Liver metastasis	0.566	0.321	3.106	1.761	0.078

tient died within 30 d after operation, and 2 patients died within 3 mo after operation.

Metastasis

A total of 54 patients died of recurrence and/or metastasis within 111 months after operation. Liver, lung and bone metastases were found during follow-up in 3, 1 and 5 patients, respectively.

Survival

The average survival time of the 96 patients was 77.90 ± 5.01 months. The 3-, 5- and 10-year survival rates were 66.68%, 58.14% and 46.54%, respectively. The average survival time for male and female patients was 81.00 ± 6.41 months and 73.62 ± 7.92 months, respectively. The 3-, 5- and 10-year survival rates were 69.92%, 61.35%, 45.5% for male patients respectively and 62.48%, 53.69%, 48.32% for female patients respectively ($P = 0.499$, Table 1).

Results of univariate analysis

The prognostic factors of young patients with colon cancer are demonstrated in Table 1. Among them, age, operative procedures, perioperative blood transfusion, pathological staging, diameter of tumor, depth of tumor invasion, lymph node metastases, distant metastases and liver metastases had a significant impact on the prognosis of young patients with colon cancer.

Results of multivariate analysis

Perioperative blood transfusion and lymph node metastases were the only independent factors of postoperative survival in young patients with colon cancer (Table 2).

DISCUSSION

Colorectal cancer is a predominant disease of elderly. The risk of developing this disease increases with age, but is not unusual in young patients at the age of 40 years or under [1]. It was reported that the incidence of colon cancer remains stable while rectal cancer incidence is decreased 11% in old patients, while the incidence of colon cancer is increased 17% and rectal cancer incidence is increased 75% in young patients [4]. A total of 723 patients with colon cancer were treated in our hospital in a 10-year period. Among them, 96 patients were under 40 years of age (13.3%). The incidence of colon cancer in young patients is higher than that of gastric cancer (4.9%) [3]. Only 3-4% and 1.6% of colorectal cancer patients in Europe and United States are 40 years old or younger [5-7], but the proportion of young patients is 30% in a domestic report [5]. Hereditary colorectal tumors [hereditary nonpolyposis colorectal cancer (HNPCC), adenomatosis coli, and suspected HNPCC] occur in 38.4% of patients younger than 40 years old and in only 3.5% of individuals older than 55 [8]. Thus, hereditary colorectal tumors are detected more often in young individuals demonstrating hereditary factors rather than dietary and life-style [9].

It is generally believed that young patients with colorectal cancer have a worse survival rate. Reports from Europe demonstrate that the 5-year survival rate for patients (30 years old or younger) is only 25-30% [10, 11]. Young patients are more likely to present with late-stage disease. The young patients also have higher grade tumors [12]. About 60-67% of young patients with colorectal cancer have a later stage (III/IV) disease [11, 13], most of which are poorly-differentiated or mucinous tumors [11-15] indicating a very poor prognosis. Domestic reports demonstrate that the 5-year survival rate of patients 30 years old or younger is 40.1% [16]. The 5-year survival rate of patients at the age of 30-40 years in the present study was 63.12%. Poorly-differentiated and mucinous tumors were present in about 50% of them. On the other hand, patients at the age of 30 years or younger are more likely to present with late-stage disease, lymph node metastasis and distant metastasis compared to patients at the age of 30-40 years, but the difference is not significant. It was reported that patients younger than 40 years old with colorectal cancer display three biological indicators of aggressive and potentially

metastatic tumor biology, signet-ring cell carcinoma, infiltrating tumor edge, and aggressive histologic grade in primary adenocarcinoma[16].

The gender was not a prognostic factor for young patients in the present study. The 3- and 5-year survival rates were better for male patients than for females (69.92% *vs* 61.35% and 62.48% *vs* 53.69%). The prognosis of female colorectal cancer patients is better than that of male colorectal cancer patients, especially those with rectal cancer [17].

Location of tumors is one of the prognostic factors. Patients with colon cancer are considered having a better prognosis than those with rectal cancer [18]. Distal location and advanced stage of tumor are determined as independent prognostic factors for survival of young patients with colorectal cancer [19]. The present study indicated that there was no relationship between tumor location and prognosis. The prognosis of the patients undergone left hemicolectomy (splenic flexure of colon, descending colon and most part of sigma colon) was not different from that of the patients undergone right hemicolectomy (caecum, ascending colon and hepatic flexure of colon).

The diameter of tumors is also a prognostic factor. The prognosis of the patients in different groups with different tumor diameters (<4 cm, 4-8 cm, >8 cm) was significantly different ($P<0.001$).

The current data indicate a significant difference in the 5-year survival rate among patients who did not received blood transfusion but 400 mL and ⩾800 mL blood transfusion. Perioperative blood transfusion produces host immunosuppression and contagious diseases, increasing postoperative infectious complications [20]. Blood transfusion may be associated with the tumor staging. In our study, more advanced diseases (stages III and IV) were found in patients who received 400 mL or ⩾ 800 mL blood transfusion than in patients who did not receive blood transfusion (50%, 58.6% and 71.4%). The 10-year survival rate of patients who received blood transfusion was significantly lower than patients who did not received blood transfusion (38.93%, 59.17%). Therefore, blood transfusion is a prognosis factor for young patients with colon cancer.

Pathological classification is one of the prognostic factors for patients with colorectal cancer. Patients with different papillary adenocarcinoma have the best outcome, patients with moderately-differentiated and mucinous adenocarcinoma have a moderate outcome, patients with signet-ring cell poorly-differentiated adenocarcinoma have a poor prognosis. Carcinoma and data from a national registry including 164 628 colorectal cancer patients [21] indicate that the signet-ring cell subtype has worse outcomes, whereas survival rate for mucinous tumor patients is similar to that of adenocarcinoma patients.

Radical resection is one of the important prognostic factors. In our study, the average survival time for patients who received R2 procedure was 24 moths, the 3-year survival rate was 6.5%. Therefore, it is important to emphasize the radical resection.

Another important factor of prognosis is pathological stage. In our study, the 3-year survival rate for IV stage patients was 8.7%. When distant metastasis (including liver metastasis) occurred, the 3-year survival rate was only 4.76%. The 5-year survival rate decreased from 92% to 35% when lymph node metastasis occurred and was 19% when N2 was positive. Multivariate analysis showed that lymph node metastasis was an independent prognostic factor for young patients with colon cancer.

The prognosis of young patients with colon cancer depends mainly on the clinicopathological characteristics. A high proportion of advanced stage tumors is the main reason for the worse prognosis of patients under 30 years old. The prognostic factors for young patients with colon cancer are age, surgical procedure, radical resection, blood transfusion, pathological type, diameter of tumor, depth of tumor invasion, lymph node metastasis and distant metastasis. The independent prognostic factors are only blood transfusion and lymph node metastasis.

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RAPID COMMUNICATION

Relationship between onset of peptic ulcer and meteorological factors

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Abstract

AIM: To discuss the relationship between onset of peptic ulcer (PU) and meteorological factors (MFs).

METHODS: A total of 24 252 patients were found with active PU in 104 121 samples of gastroscoic examination from 17 hospitals in Nanning from 1992 to 1997. The detectable rate of PU (DRPU) was calculated every month, every ten days and every five days. An analysis of DRPU and MFs was made in the same period of the year. A forecast model based on MFs of the previous month was established. The real and forecast values were tested and verified.

RESULTS: During the 6 years, the DRPU from November to April was 24.4 -28.8%. The peak value (28.8%) was in January. The DRPU from May to October was 20.0 -22.6%, with its low peak (20.0%) in June. The DRPU decreased from winter and spring to summer and autumn ($P < 0.005$). The correlated coefficient between DRPU and average temperature value was -0.8704, -0.6624, -0.5384 for one month, ten days , five days respectively ($P < 0.01$). The correlated coefficient between DRPU and average highest temperature value was -0.8000, -0.6470,-0.5167 respectively ($P < 0.01$). The correlated coefficient between DRPU and average lowest temperature value was -0.8091, -0.6617, -0.5384 respectively ($P < 0.01$). The correlated coefficient between DRPU and average dew point temperature was -0.7812, -0.6246, -0.4936 respectively ($P < 0.01$). The correlated coefficient between DRPU and average air pressure value was 0.7320, 0.5777, 0.4579 respectively ($P < 0.01$). The average temperature, average highest and lowest temperature, average air pressure and average dew point temperature value of the previous month, ten days and five days could forecast the onset of PU, with its real and forecast values corresponding to 71.8%, 67.9% and 66.6% respectively.

CONCLUSION: DRPU is closely related with the average temperature, average highest and lowest temperature, average air pressure and average dew point temperature of each month, every ten days and every five days for the same period. When MFs are changed, the human body produces a series of stress actions. A long-term and median-term based medical meteorological forecast of the onset of PU can be made more accurately according to this.

Key words: Peptic ulcer; Meteorological factors; Temperature

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INTRODUCTION

The onset of peptic ulcer (PU) is characterized by seasons, with a high incidence in winter and spring and a low incidence in summer[1-8]. We made a correlated analysis of the relationship between detectable rate of PU (DRPU) from 1992 to 1997 in Nanning and a regressive analysis of the meteorological factors (MFs) in the same period. We found that DRPU was closely related to the seasons and MFs.

Table 1 The 7 meteorological factors

MF	Symbol	Unit
Average temperature of 1 month, ten days and five days	Tpj	^{0}C
Average highest temperature of 1 month, ten days and five days	Tmax	^{0}C
Average lowest temperature of 1 month, ten days and five days	Tmin	^{0}C
Average air pressure of 1 month, ten days and five days	P	hPa
Average relative humidity of 1 month, ten days and five days	FF	%
Average dew point temperature of 1 month, ten days and five days	Td	^{0}C
Average total amount of precipitation of 1 month, ten days and five days	RR	mm

MATERIALS AND METHODS

Materials

A total of 24 252 patients with active PU were found from 17 hospitals in Nanning from 1992–1997. We then calculated the detectable rate of peptic ulcer in every season, every month, every ten days and every five days. The average value of the 7 MFs was provided by Guangxi Meteorological Observation Station (Table 1).

Methods

We used detectable rate of peptic ulcer (DRPU) to represent the incidence. The detectable rate of a disease was higher than the incidence of its natural multitude, but the variation of both was similar as previous;y described[8]. (DRPU = number of persons of PU at the same period of time /total number of persons of gastroscopic examination at the same period of time × 100%).

Statistical analysis

We made a correlated analysis of the DRPU of 1 month, ten days and five days and the 7 MFs in the same period in Nanning. On the basis of it, we carefully chose the meteorological factors. A regressive effect F value test was made for all the independent variables and factors. A multiple linear regressive mathematical model was established for DRPU forecast equation[9].

RESULTS

Relationship between DRPU and seasons

From 1992 to 1997, the average DRPU in Nanning was 23.29%. The average DRPU from November to April was 24.2-28.8%, with its peak value being 28.8% in January. The average DRPU from May to October was 20.0-22.6%, with its low peak value being 20.0% in June. The DRPU in winter was 26.48%, 24.98% in spring, 21.10% in summer, and 20.91% in autumn. The seasonal DRPU was χ^2 = 343.3004 (P <0.005), indicating that the DRPU differed remarkably in seasons. When the DRPU in winter and spring was compared with that in summer and autumn, χ^2 = 327.4435 (P <0.005). When the DRPU in winter was compared with that in spring, summer and autumn, χ^2 = 194.0589 (P <0.005). When the DRPU in spring was compared with that in summer and autumn, χ^2 = 153.8931 (P <0.005). When the DRPU in summer was compared with that in autumn, χ^2 = 0.2744 (P >0.750). The above values showed that the onset of PU was as follows: winter and spring>summer and autumn, winter >spring >summer and autumn. The difference was significant.

Relationship between DRPU AND MFs

We made a further correlated analysis of the DRPU and the 7 MFs in the same period. The results showed that the DRPU was not evidently related to the relative air humidity, but had a negative relation with the average temperature, average highest and lowest temperature, average dew point temperature (P <0.01) and a positive relation with the AAP (P <0.01, Table 2). We found that when the monthly average temperature ≤21℃, the monthly average highest temperature ≤26℃, the monthly average lowest temperature ≤18℃, the monthly average air pressure ≥ 1007 hPa,the monthly average dew point temperature ≤ 15℃, the average ten-day temperature ≤19℃, the average ten-day highest temperature ≤24℃, the average ten-day lowest temperature ≤17℃, the average ten-day air pressure≥1008 hPa, the average ten-day dew point temperature ≤14℃, the average five-day temperature ≤17℃, the average five-day highest temperature ≤20℃, the average five-day lowest temperature ≤14℃, the average five-day air pressure≥1008hPa,the average five-day dew point temperature ≤12℃. The correlated analysis of the high onset of DRPU of 1 month, ten days, five days and the above single factors corresponded to 75%-78%. The statistical relation between the factors, can be described by the multivariate linear regression equation:

$$\hat{y} = b_0 + b_1 x_{i1} + b_2 x_{i2} + \Lambda\Lambda + b_p x_{ip} \quad \ldots\ldots \quad (i = 1,2,3\Lambda\Lambda n)$$

where b_0, b_1, b_2…bp stand for regression coefficients; n is sample size; x_{i1}, x_{i2}, …, x_{ip} are predictors; $\hat{y}$ is the predicted value. The multiple linear regressive equation was established as follows:

YmonthDRPU=127.89366 + 0.95687Tpj - 0.46658Tmax-0.97166Tmin - .0929P-0.07886Td,

average error = 2.0051, complex relative coefficient = 0.8129, F month test value = 25.7068, F0.01 = 3.60, F month > F_a, thus P<0.01.

Yten-dayDRPU=62.93681 = 0.34811Tpj + 0.06056Tmax-0.18958Tmin-0.02957P + 0.0004Td, average error = 2.981, complex relative coefficient = 0.664,Ften-day test value = 33.1117, F0.01 = 3.40, F ten- day > Fa, thus P <0.01.

Yfive-dayDRPU=63.49121-0.76259Tpj+0.27832Tmax-

Table 2 Correlated analysis of DRPU and 7 MFs

Related DRPU	Group	Related coefficient	*P* value
Monthly average temperature	72	-0.8704	<0.01
Average ten-day temperature	216	-0.6624	<0.01
Average five-day temperature	432	-0.5384	<0.01
Average monthly highest temperature	72	-0.8000	<0.01
Average ten-day highest temperature	216	-0.6470	<0.01
Average five-day highest temperature	432	-0.5167	<0.01
Average monthly lowest temperature	72	-0.8091	<0.01
Average ten-day lowest temperature	216	-0.6617	<0.01
Average five-day lowest temperature	432	-0.5384	<0.01
Average monthly air pressure	72	0.7230	<0.01
Average ten-day air pressure	216	0.5777	<0.01
Average five-day air pressure	432	0.4579	<0.01
Average monthly relative humidity	72	-0.2334	>0.05
Average ten-day relative humidity	216	-0.1364	>0.05
Average five-day relative humidity	432	-0.0657	>0.05
Average monthly dew point temperature	72	-0.7812	<0.01
Average ten-day dew point temperature	216	-0.6246	<0.01
Average five-day dew point temperature	432	-0.4936	<0.01
Monthly precipitation	72	-0.2013	>0.05
Ten-day precipitation	216	-0.0916	>0.05
Five-day precipitation	432	-0,0418	>0.05

The results of regressive effect analysis indicated that the 3 multiple regressive equations based on DRPU and variation of the 5 MFs had remarkable effects.

0.06448Tmin - 0.03043P + 0.0764Td, average error = 4.0499, complex relative coefficient = 0.5434, Ffive - day test value = 35.6956, F0.01 = 3.07, F five - day >Fa, thus *P* <0.01.

Establishment of DRPU forecast equation and test of its significance

The correlated analysis of DRPU of 1 month, ten days, five days and 7 MFs of the previous month, ten days and five days indicated that the average temperature, average highest and lowest temperature, average air pressure and average dew point temperature of the previous month were also closely related to the DRPU of the present month, ten days and five days (Table 3). Thus if we made the 5 MFs of the previous month, ten days and five days and the DRPU of the present month , ten days and five days, the factors of three DRPU forecast equations could be established as follows:

YmonthDRPU=-358.09409-4.75833Tpj+2.08101 Tmax+2.97619Tmin+0.37869P-0.29906Td, average error=2.1138, complex relative coefficient=0.7907, F month test value= 21.6887, F0.01 =3.60, F month >Fa, thus *P*<0.01.

Yten-dayDRPU=-45.84159-0.26677Tpj+0.05189Tmax-0.09075Tmin+0.07605P-0.04132Td, average error=3.0625, complex relative coefficient=0.6383, F ten-day test value= 28.7387, F0.01=3.40, F ten- day>Fa, thus *P*<0.01.

Yfive-dayDRPU=-0.826-0.13995Tpj-0.00568Tmax-0.24365Tmin+0.04104P+0.00342Td, average error=4.0629, complex relative coefficient=0.5362, Ffive-day test value=34.2292, F0.01=3.07, F five -day> Fa, thus *P*<0.01.

DISCUSSION

It is generally believed that the occurrence of PU is due to the effect of unbalance between the attacking factors such as hydrochloric acid in gastric juice and the defending factors of the mucosa. *Helicobacter pylori* (*H pylori*) infection is also an important cause of PU[10-25]. Treatment of hydrochloric acid in gastric juice and anti- pylorus bacteria can protect stomach duodenum mucosa, yet the occurrence of PU still has remarkable seasonal variations [8]. Therefore the influence of variations of meteorological factors on PU must be considered. Research has shown that the occurrence of PU in winter is related to the higher air pressure and temperature [8]. The high occurrence of PU

Table 3 Correlated analysis of DRPU and 5 MFs of the previous month, ten days, and five days

Related DRPU	Group	Related coefficient	*P* value
Average monthly temperature	71	-0.6621	<o.o1
Average ten-day temperature	215	-0.6302	<o.o1
Average five-day temperature	431	-0.5324	<o.o1
Average monthly highest temperature	71	-0.6291	<o.o1
Average ten-day highest temperature	215	-0.6097	<o.o1
Average five-day highest temperature	431	-0.5123	<o.o1
Average monthly lowest temperature	71	-0.6781	<o.o1
Average ten-day lowest temperature	215	-0.6350	<o.o1
Average five-day lowest temperature	431	-0.5348	<o.o1
Average monthly air pressure	71	0.7628	<o.o1
Average ten-day air pressure	215	0.5942	<o.o1
Average five-day air pressure	431	0.4840	<o.o1
Average monthly dew point temperature	71	-0.7220	<o.o1
Average ten-day dew point temperature	215	-0.6171	<o.o1
Average five-day dew point temperature	431	-0.5077	<o.o1

The above analysis showed that the 3 forecast equations had good regressive effect. The real test values of the three forecast equations were 71.8%, 67.9% and 66.6%, respectively.

in summer is likely to be related to the lower air pressure. Our study also confirmed the seasonal onset of PU. Analysis of MFs has shown that special attention should be paid to PU patients in winter and spring [26]. Since increased air pressure and dry air result from the cold air, temperature plays a more important role [27]. When these meteorological factors are changed violently, a series of stress action take place, causing endocrinopathy. It was reported that severe cold and changing temperature result in acute stress actions in human body, causing excitation of sympathetic nerve and adrenal gland marrow and rapid secretion of adrenaline and noradrenalin[28]. Animal tests also showed that mouse hypertensin II is much higher compared with acute stress action, sub-acute stress action and other chronic stress action [28]. When human body is stimulated by acute or slow stress action, hypertensin II in plasma increases remarkably. Cold stimulation and oxygen shortage stimulation may accelerate secretion of endothelin. Adrenaline, hypertensin II and endothelin may cause contraction of duodenal mucosa and blood vessel, leading to mucosa blood flow fall and mucosa damage [29-30]. Stomach blood insufficiency may also stimulate the rise of endothelin [31]. Kou and Li [32] reported that the stomach mucosa blood vessels contract immediately after injection of endothelin. It has also been verified that plasma endothelin density of PU patients is related to the degree of damaged stomach mucosa [33]. Stress action may lessen the stomach mucosa mucus, and lower the stomach mucosa's protective function [28]. Furthermore cold stress action may lessen the secretion of inhibitable growth factors [34, 35]. When the secretion of the inhibitable growth factors becomes less, secretion of hydrochloric acid increases in gastric juice. Meanwhile endothelin can accelerate the secretion of hydrochloric acid in gastric juice. It has been verified that when endothelin is injected, plasma progastrin increases progressively [32]. When blood supply is insufficient in the duodenal mucosa and the protection barrier is damaged due to insufficient oxygen, the increase of hydrochloric acid in gastric juice accelerates the occurrence is of PU. Moreover, cold weather decreases human body's immunity function [28]. Some Japanese experts pointed out that from December to March, the number of human body outer lymph cells and B lymph cells is small [36], indicating lower body cell immunity and body fluid immunity function. Therefore when the temperature stimulates human body, adrenocorticotropin increases its secretion and hypertensin II rises, restraining the body immunity function and causing immunity functional disorder, and making it easy for the duodenal mucosa to be attacked by *H pylori* [37], which destroys the stomach mucosa protection barrier, impairs the stomach mucosa and then restrains secretion of inhibitable growth factors and stimulates secretion of progastrin[12-25].

In conclusion, acute, sub-acute and slow stresses on the human body are likely the cause of PU in winter and spring.

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RAPID COMMUNICATION

Expression of pituitary adenylate cyclase-activating polypeptide 1 and 2 receptor mRNA in gallbladder tissue of patients with gallstone or gallbladder polyps

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Abstract

AIM: To detect the expression of pituitary adenylate cyclase-activating polypeptide receptor 1 (VPCAP$_1$-R) and VPCAP$_2$-R mRNA in gallbladder tissues of patients with gallstone or gallbladder polyps.

METHODS: The expression of VPCAP$_1$-R and VPCAP$_2$-R mRNA in gallbladder tissues was detected in 25 patients with gallstone, 8 patients with gallbladder polyps and 7 donors of liver transplantation by reverse transcription polymerase chain reaction (RT-PCR).

RESULTS: The VPCAP$_2$-R mRNA expression level in the control group (1.09±0.58) was lower than that in the gallbladder polyp group (1.64±0.56) and the gallstone group (1.55±0.45) ($P<0.05$) while the VPCAP$_1$-R mRNA expression level in the control group (1.15±0.23) was not apparently different from that in the gallbladder polyp group (1.28±0.56) and the gallstone group (1.27±0.38).

CONCLUSION: The abnormal expression of VPCAP$_2$-R mRNA in gallbladder tissue may play a role in the formation of gallbladder stone and gallbladder polyps.

Key words: VPCAP$_1$-R; VPCAP$_2$-R; RT-PCR; Gallbladder disease

Zhang ZH, Wu SD, Gao H, Shi G, Jin JZ, Kong J, Tian Z, Su Y. Expression of pituitary adenylate cyclase-activating polypeptide receptor 1 and 2 mRNA in gallbladder tissue of patients with gallstone or gallbladder polyps. *World J Gastroenterol* 2006; 12(9): 1468-1471

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INTRODUCTION

Gallbladder motility and bile delivery to the duodenum involve a complex interplay between neural and hormonal factors. Acetylcholine, cholecystokinin (CCK) and vasoactive intestinal polypeptide (VIP) in the nerve endings function as neurotransmitters, leading to contraction and relaxation of the gallbladder musculature [1-3].

VIP can relax the gallbladder, reduce gallbladder tone and inhibit CCK- stimulated contraction in a dose-dependent manner [4]. VIP exerts its action through receptors on the gallbladder wall and binds to two subtypes of VIP receptors, previously called VIP$_1$ and VIP$_2$ receptors. Because these receptors also have a high affinity for pituitary adenylate cyclase-activating polypeptide (PACAP), they have recently been named VPCAP$_1$ and VPCAP$_2$ receptors. The purpose of this study was to detect the expression of VPCAP$_1$-R and VPCAP$_2$-R mRNA in gallbladder tissue and to define their role in the formation of gallstone and gallbladder polyps.

MATERIALS AND METHODS

Patients

Gallbladder tissue from 25 patients with gallbladder cholesterol stone (12 men, 13 women, mean age 59.6 years, range 34-5 years) and 8 patients with gallbladder cholesterol polyps (2 men, 6 women, mean age 46.8 years, range 26-64 years) was obtained during surgery. Patients who had a history of acute cholecystitis were excluded. Gallbladder tissue from 7 donors of liver transplantation (all men, mean age 41.4 years, range 25-63 years) was used as control. The tissues were frozen in liquid nitrogen and stored at -80 ℃.

Extraction of RNA

Total RNA was extracted from 100 mg gallbladder tissue samples using TRIzol reagent according to the manufacturer's instructions. The concentration and purity of RNA were determined by a spectrophotometer at 260 and 280 nm. All RNA isolates had an OD$_{260}$:OD$_{280}$ value of 1.8:2.0, indicating clean RNA isolates.

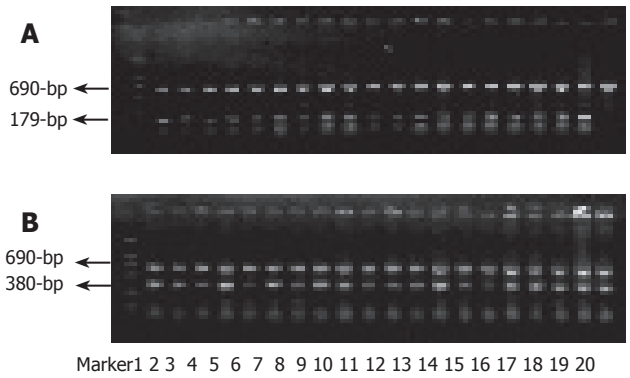

Figure 1 Expression of $VPCAP_1$-R (**A**) and $VPCAP_2$-R (**B**) in gallbladder tissues of patients with gallstones or gallbladder polyps. Lanes 1-15: gallbladder stone group; lanes 16-17: control group; lanes 18-20: gallbladder polyp group.

Reverse transcription-polymerase chain reaction (RT-PCR)

The primers for amplifying $VPCAP_1$-R and $VPCAP_2$-R cDNA were designed by the corresponding software based on the published Homo sapiens $VPCAP_1$-R mRNA (NM 004624) and $VPCAP_2$-R mRNA (NM 003382) sequences. The sequences of primers for $VPCAP_1$-R mRNA were: forward 5'- AGATGCAGCTCACTACCCTAT -3' and reverse 5'- TTCAGAGTCCCTCAGTCCTT-3', which generated a 179-bp amplification product. The sequences of primers for $VPCAP_2$-R mRNA were:forward 5'-TGCTGCAACAAGCTCATCCCT -3' and reverse 5'- GACCCAACACCTTCAGTTACCAC -3', which generated a 380-bp amplification product. The sequences of primers for internal reference gene *β-actin* used to monitor the quality of the RNA samples were:forward 5'- TCTGGATCACCTTCTGCTG G -3' and reverse 5'-GATTGCTCAGGACATTTCTG -3', which generated a 690-bp amplification product.

Two micrograms of total RNA was used as a template for subsequent RT-PCR. The total RNA was mixed with 1 μL oligo$(dT)_{15}$, 1 μL dNTPs and H_20 and preheated at 65 °C for 1 min to denature the secondary structure. The mixture was then cooled rapidly to 30 °C and then 10 μL 2 X RT buffer, 4 μL 25% $MgSO_4$, 1 μL 22 u/μLAMV, 0.5 μL40 u/uL RNase-inhibitor were added. Reverse transcriptase was added for a total volume of 20 μL. The RT mixture was incubated at 65 °C for 30 min and then stopped by heating at 98 °C for 5 min and cooling at 5 °C for 5 min.

RCR was performed on a PTC-200 PCR machine using 3 μL of cDNA, 0.1 μL of each oligonucleotide primer, 2 μL of each dNTP, 0.2 μL Taq polymerase and 10 X Taq polymerase buffer in a total volume of 25 μL. The PCR conditions were denaturation at 94 °C for 3 min, then a 94 °C for 45 s, followed by 35 cycles of annealing of $VPCAP_1$-R mRNA at 52.5 °C for 1 min and $VPCAP_2$-R mRNA at 57.3 °C for 1 min, extension at 72 °C for 1 min, a final extension at 72 °C for 7 min.

The PCR products were analyzed by electrophoresis on 2% agarose gels containing ethidium bromide. The gels were photographed on top of a 280 nm UV light box. The gel images were captured with a digital camera and analyzed with the ID Kodak Imager analysis program. RT-PCR values were presented as a ratio of the receptor mRNA signal divided by the β-actin signal.

Table 1 Expression of $VPCAP_1$-R and $VPCAP_2$-R mRNA in gallbladder tissues of patients with gallstones or gallbladder polyps (mean ± SD)

Group	$VPCAP_1$-R mRNA	$VPCAP_2$-RmRNA
Gallstone (n=25)	1.27±0.38	1.55±0.45[a]
Gallpolyp (n=8)	1.28±0.56	1.64 ±0.56[a]
Control (n=7)	1.15±0.23	1.09±0.58

[a]P <0.05 *vs* control group, n represents the number of patients involved in the study.

Statistical analysis

Data were expressed as mean ± SD. Statistical analyses were performed by the independent two-tailed t test. $P < 0.05$ was considered statistically significant. The SPSS11.5 software was used for statistical analysis.

RESULTS

Total RNA isolated from gallbladder tissues was subjected to reverse transcription-PCR analysis for the expression of $VPCAP_1$-R and $VPCAP_2$-R mRNA. A 179-bp band and a 380-bp band, specific for $VPCAP_1$-R and $VPCAP_2$-RmRNA were found in gallbladder tissue of all the three groups (Figures 1A and 1B). Furthermore, expression of $VPCAP_1$-R and VP C A P $_2$-R mRNA was detected by RT-PCR assay. The levels of PCR amplified $VPCAP_1$-R and RT-PCR amplified $VPCAP_2$-R mRNA and β-actin mRNA in three groups were compared.

Expression of $VPCAP_1$-R mRNA in gallbladder tissue

The $VPCAP_1$-R mRNA level in control group (1.15 ± 0.23) was not significanty different from that in gallbladder polyps group (1.2 8± 0.56) and gallstone group (1.27 ± 0.38) (Table 1).

Expression of $VPCAP_2$-R mRNA in gallbladder tissue

The $VPCAP_2$-R mRNA level in the control group (1.09 ± 0.58) was lower than that in gallbladder polyps group (1.64 ± 0.56) and gallstone group (1.55 ± 0.45) *(P* <0.05) while no difference in the expression of $VPCAP_2$-R mRNA was found between these two groups (Table 1).

DISCUSSION

Vasoactive intestinal peptide (VIP), a 28-amino acid peptide capable of inducing vasodilation, was first isolated from porcine intestine[5]. It has many other actions as a neuroendocrine hormone and neurotransmitter. It may play an important role in the central nervous system (CNS)[6]. VIP can stimulate prolactin secretion from the pituitary[7], regulate noncholinergic trans-synaptic functions of the adrenal medulla[8], and inhibits proliferation of T cells in the immune system[9]. Other functions of VIP include protection against oxidant injury[10], stimulation of

electrolyte secretion[11], relaxation of smooth muscle[12]. Intrinsic neurons modulate gallbladder function. Nitric oxide synthase (NOS) and VIP are present in gall bladder neurons and nitric oxide and VIP modulate its epithelial functions[13]. Intravenous infusion of VIP is associated with the secretion of bicarbonate from the gallbladder mucosa[14]. Relaxation of canine gallbladder depends on nerve stimulation by adrenergic and non-adrenergic as well as non-cholinergic (NANC) nerves. Nitric oxide and VIP contribute to relaxation of NANC nerves in canine gallbladder[15]. The effect of VIP on guinea pig gallbladder *in vitro* suggests that VIP has no effect on basal tone, but produces a $26.7 \pm 6.6\%$ relaxation of CCK-contracted strips[4].

The first recombinant receptor for VIP is isolated from rat lung by Ishihara *et al* [16]. This receptor is originally described as the VIP receptor and subsequently designated as the VIP_1 receptor[17]. Messenger RNA encoding the $VPCAP_1$ receptor is widely distributed in CNS[18], peripheral tissues of liver[19], lung[20] and intestine[20] as well as in T lymphocytes[21]. The second receptor that responds to VIP and PACAP with comparable affinity has been cloned from the rat olfactory bulb by Lutz *et al* [17]. The highest concentration of messenger RNA is found in CNS[18]. The receptor is also present in several peripheral tissues of pancreas, skeletal muscle, heart, kidney, adipose tissue, testis and stomach[22-25].

Researches about the distribution of VIP receptor in the gallbladder tissues are relatively few. Gao *et al* [26] studied VIP receptor expression in patients with gallstones using immunohistochemical technique and found that positive VIP receptor expression level is higher in patients with abnormal fasting gallbladder volume than in patients with normal fasting gallbladder volume. Fu *et al*[27] studied values of the max bind content (Bmax) of VIP receptor in gallbladder wall tissue of guinea pigs by radioligand binding assay and found that the values of Bmax are obviously increased during formation of gallstone. Dupont *et al* [28] found that there are specific binding sites for VIP in isolated epithelial cells of human gallbladder measured by radioimmunoassay. Their results indicate two functionally independent classes of receptor sites and VIP strongly stimulates adenosine 3':5' monophosphate (cyclic AMP) production.

In our study, the$VPCAP_1$ receptor mRNA level in gallstone group was not significantly different from that in control group; the$VPCAP_2$ receptor mRNA level in gallstone group was higher than that in control group; predominant $VPCAP_2$ receptor was found in smooth muscle (in blood vessels and smooth muscle layer of the gastrointestinal and reproductive systems). The main hormonal regulator of gallbladder contraction is CCK. Recent studies suggest that CCK receptor mRNA level is down-regulated in patients with gallstone and animals[29,30]. Previous studies have shown that human gallbladders with cholesterol stone reduce their contractions in response to agonists such as cholecystokinin, acetylcholine and muscle defects responsible for impaired gallbladder muscle contraction in plasma membranes of smooth muscle cells because of excessive incorporation of cholesterol[31,32]. The diffuse membrane defect caused by cholesterol may also affect other transmembrane proteins that mediate muscle relaxation. It was reported that gallbladder relaxation is significantly reduced in gallbladders with cholesterol stones[33]. Up-regulation of $VPCAP_2$ receptor mRNA may compensate for the abnormal receptor function of cholesterol. But the down-regulation of CCK receptor mRNA cannot compensate for the abnormal receptor function of membranes. There fore the contraction function of gallbladder is greatly affected rather than the relaxation function. Since up-regulation of $VPCAP_2$ receptor mRNA in epithelial cells can affect their secreting function, the abnormal expression of $VPCAP_2$ receptor mRNA may play a role in gallstone formation.

Excess cholesterol is the main cause of gallbladder polyps and may reduce the membrane fluidity, which in turn affects receptor function or receptor G-protein interaction. There are two specific binding sites for VIP in isolated epithelial cells of human gallbladder. In our study, $VPCAP_2$ receptor mRNA was over-expressed in patients with gallbladder polyps, which may be due to the abnormal receptor functions of cholesterol. Over-expression of $VPCAP_2$ receptor mRNA may occur in epithelial cells, leading to abnormal secretion and absorption of epithelial cells. This disorder may play a role in formation of gallbladder polyps.

A large number of factors, such as genetics, cholesterol saturation, sphincter of Oddi pressure, bacterial contamination of biliary tree, can induce formation of gallbladder stone and gallbladder polyps. The motility disturbances related to up-regulation of $VPCAP_2$ receptor mRNA may play a role in formation of gallbladder stones and gallbladder polyps. However, what cell membranes does the over-expression of $VPCAP_2$ receptor mRNA occur needs to be further studied.

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CASE REPORT

Primary liposarcoma of gallbladder diagnosed by preoperative imagings: A case report and review of literature

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Abstract

A 49-year-old Japanese woman was referred to our department because of high fever and a huge abdominal mass. Computed tomography (CT) and magnetic resonance (MR) imagings revealed a tumor, about 30 cm in diameter, occupied the right hepatic lobe and the peritoneal cavity. Abdominal angiography showed that the tumor was fed mainly by the cystic artery. We preoperatively diagnosed angiosarcoma of the gallbladder and performed tumor resection with cholecystectomy because the tumor was almost casplated, however the posterior wall of the gallbladder attached to the tumor firmly. Histologically, the tumor was composed of spindle cells including lipoblasts with cellular pleomorphism, which were also detected in the muscular layer of the gallbladder. We finally diagnosed pleomorphic liposarcoma of the gallbladder. At 10 mo and 29 mo after the first operation, she underwent two more operations because of recurrence. Now she has a good quality of life 3 years and 6 mo after the first operation.

Key words: Liposarcoma; Gallbladder; Liver; Pleomorphic type; Recurrence

Hamada T, Yamagiwa K, Okanami Y, Fujii K, Nakamura I, Mizuno S, Yokoi H, Isaji S, Uemoto S. Primary liposarcoma of gallbladder diagnosed by preoperative imagings: A case report and review of literature. *World J Gastroenterol* 2006; 12(9): 1472-1475

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INTRODUCTION

Liposarcoma is a mesenchymal malignant tumor that is usually detected in the extremities and the retroperitoneum[1]. Primary intra-abdominal liposarcomas are rare: most originate from the mesentery and peritoneum[1]. We experienced a case of liposarcoma originated from the gallbladder, which is extremely rare - only one case has been reported[2]. Our case is the longest surviving case treated by surgery in previously reported cases. Herein, we report the case of gallbladder liposarcoma and review the literature.

CASE REPORT

A 49-year-old woman was referred to our department for high fever and a huge abdominal mass in October 2001. At admission, she was emaciated (body height: 150cm, body weight: 42.5 kg, blood pressure: 100/60 mmHg, pulse rate: 78/min, body temperature: 38.4℃); the elastic huge mass, about 30 cm in diameter, was palpated at the right side of the abdomen. Laboratory data revealed severe anemia (hemoglobin: 76 g/L), leukocytosis (white blood cells count: 10 920/mm^3), hypoproteinemia (total protein: 5.8 g/dl, Albumin: 19 g/L) and elevation of serum alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (GTP) levels (ALP: 841 IU/l, γ-GTP: 247 IU/l). Serum levels of tumor markers (carcinoembryonic antigen [CEA], carbohydrate antigen [CA] 19-9, and alpha-fetoprotein [AFP]) were within their normal ranges. By computed tomography (CT), the tumor, which was detected as a low-density area with irregular enhancement, occupied the right hepatic lobe and the peritoneal cavity (Figure 1). As shown by the magnetic resonance imaging (MRI) series, the tumor had homogeneous hypo-intensity on T1-weighted MRI (Figure 2A) and heterogeneous hyper-intensity on fat-suppressed T2-weighted MRI (Figure 2B). Furthermore, MR cholangiography showed that it apparently compressed the right lobe of the liver to the cranial side and the gallbladder to the medial-caudal side (Figure 2C,2D). Stronger enhancement of tumor was revealed by CT during selective cystic arteriography (Figure 3) than that during other arteriographies (i.e., right hepatic artery, middle hepatic artery, left hepatic artery, right subphrenic artery, and right renal artery). Based on those findings, on 23 October 2001, we performed an operation under the preoperative diagnosis of angiosarcoma originated from the liver or the gallbladder.

FIRST OPERATION AND PATHOLOGICAL FINDINGS

At laparotomy, a huge tumor occupied the right side of

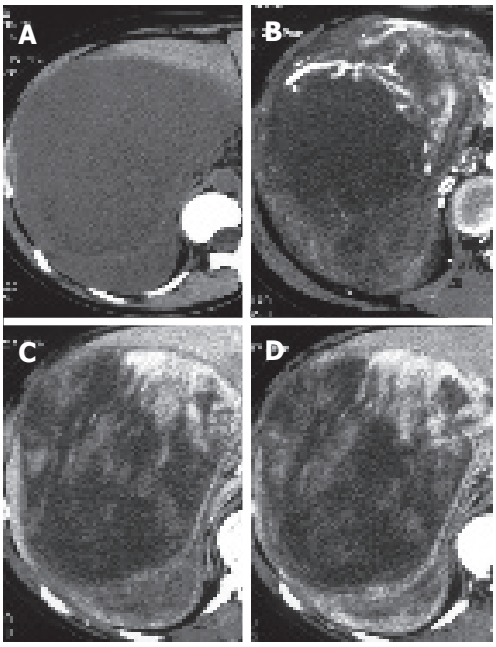

Figure 1 Preoperative dynamic CT images. **A**: plain; **B**: early phase; **C**: delayed phase; **D**: late phase. The tumor detected as a low demsity area and opputied the right hepatic lobe and the peritoneal cavity (**A**). Dynamic study revealed that the tumor enhanced irregularly at any phase (**B**-**D**).

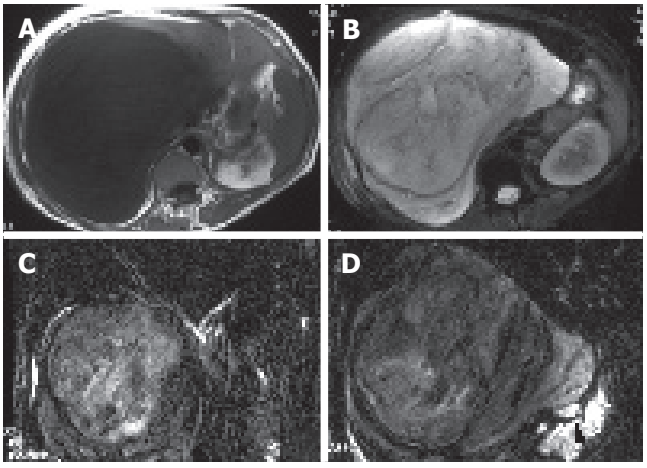

Figure 2 Preoperative MRI images. A: T1-weighted;B:fat-supressed T2-weighted;B and C: MR Cholangiography. The tumor appeared hypointense on T1-weighted images (**A**) and hyperintense on fat-suppressed T2-weighted images (**B**). MR cholangiography revealed that the tumor compressed the right lobe of the liver to the cranial side (**C**) and gallbladder to the medial-caudal side(arrow head: gallbladder) (**D**).

the abdomen; the liver was displaced to the cranial side. The tumor infiltrated tightly into the gallbladder, but not to the liver and the retroperitoneum, thus we performed a resection of the tumor with cholecystectomy. The cystic lymph node enlarged and atypical cells were detected in this node from frozen section. The resected specimen weighed 3 300 g and gross examination revealed a yellowish tumor measuring 25 cm × 23 cm, but the mucosa of the gallbladder was intact with the cholesterosis alone. The cutting section of the tumor appeared whitish solid and included hemorrhage and necrosis in parts (Figure 4A). Histological examination

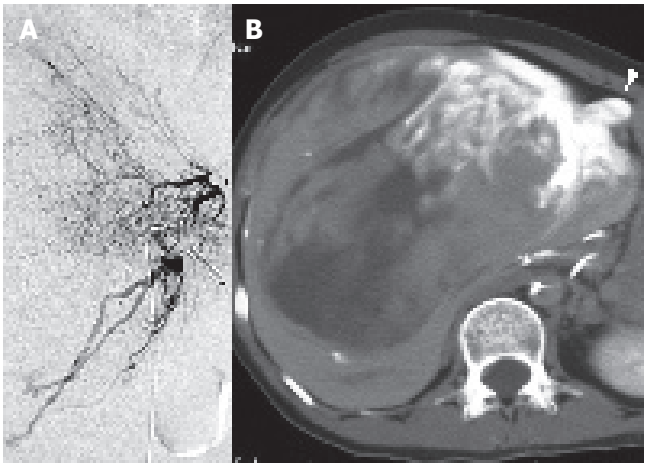

Figure 3 Preoperative angiography. **A**: Hepatic arteriography; **B**: CT during cystic arteriography. Many arteries (i.e., right, middle, left hepatic, and subphrenic arteries fed the tumor (**A**) (arrow head:the root of cystic artery). However, CT during the selective cystic arteriography revealed stronger enhancement of the tumor than during any other arteriographies (**B**)(arrow head:gallbladder).

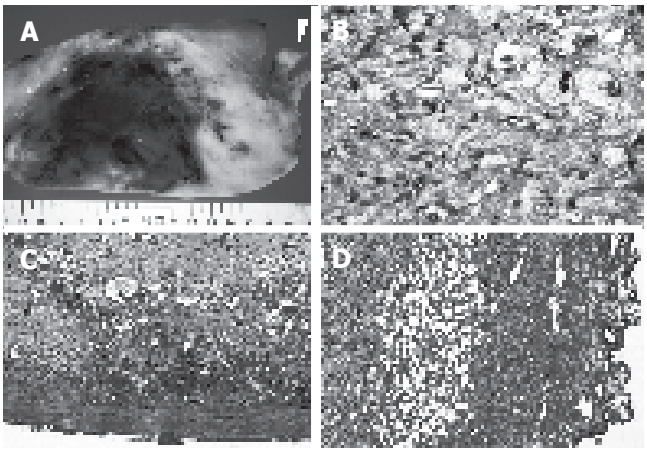

Figure 4 Resected specimen and histological findings. **A**: Cutting section of the tumor; **B**: The main tumor; **C**: The edge of the tumor(hepatic side); **D**: The posterior wall of the gallbladder. The cutting section of the tumor appeared whitish solid and included parts of hemorrhage and necrosis and attached to the gallbladder firmly (**A**) (arrowhead: gallbladder). On histological examination, the tumor was mainly composed of spindle cells with cellular pleomorphism, and included lipoblasts (**B**). The tumor had a capsule (**C**), but the capsule annihilated a border of the gallbladder, where these tumor cells were detected in the muscle layer of the gallbladder (**D**).

revealed that the tumor mainly comprised spindle cells with cellular pleomorphism, and included lipoblasts (Figure 4B). The tumor was almost capsulated (Figure 4C), however the capsule annihilated a border of the posterior wall of the gallbladder, in which these tumor cells were detected in the muscle layer but did not extended to the submucosal layer (Figure 4D). From these findings, we finally diagnosed pleomorphic type liposarcoma originated from the gallbladder with extramural growth (T4N1M0: stage IV).

SECOND AND THIRD OPERATIONS AND FINDINGS

The patient was discharged 24 d after the first operation.

Table 1 Characteristics of previously reported cases of primary liposarcoma of the liver and the gallbladder

Case Number	Source, (yr)	Age Sex	Location	Therapy	Prognosis
Liver					
1	Wolloch Y, et al[3] 1973	22 yr Female	Right lobe	Right hepatic lobectomy	Died on 46 postoperative day because of complications
2	Kim TW, et al[4] 1985	86 yr Male	Right lobe	No therapy	Died
3	Kim YI, et al[5] 1987	30 yr Female	Left lobe	Left lateral subsegmentectomy	Free of recurrence at 10 months after surgery With no further medical and surgical therapy
4	Soares FA, et al[6] 1989	2y 4mo. Male	Hepatic hilum	Chemotherapy	Died
5	Aribal E, et al[7] 1993	48 yr Female	Hepatic hilum	Unresectable Regional chemotherapy	Not described
6	Wright NB, et al[8] 1993	3 yr Male	Hepatic hilum	Surgery Radiation, chemotherapy	Died at 15 y.o. Because of fatal recurrence
7	Khan AN, et al[9] 2001	50 yr Male	Right lobe	Right hepatic lobectomy	Not described
8	Nelson V, et al[10] 2001	54 yr Female	Left and Right lobes	No therapy	Died
Gallbladder					
1	Bader H, et al[2] 1983	79 yr Male	Intramural	Cholecystectomy	Died 2 years after operation because of peritoneal dissemination
2	Our case	49 yr Femal	intramural	tumor resection with cholecystectomy	Alive

Computed tomography (CT) revealed the tumor size as 4.5 cm in Couinaud's segments 4 and 5 of the liver on August 2002. At 10 months after the first operation, she underwent extended right hepatectomy and pancreatoduodenectomy including partial resection of the portal vein according to the intraoperative findings of liposarcoma metastases that were recognized pathologically by frozen sections. Pathological examination of the specimen showed not only liposarcoma metastases in the right lobe of the liver, but also those extending to the surrounding fatty tissue of the hilus and left hepatic bile ducts, the hepatoduodenal ligament, and the pancreas head. She underwent CT scanning every 3 or 4 months at the outpatient clinic. On February 2004, CT revealed tumors of 7 cm and 5 cm in Couinaud's segments 4 and 2 of the liver, respectively, and the recurrent tumor of 1.5 cm in the upper lobe of the left lung. She underwent partial resections of Couinaud's segments 4 and 2 with a partial resection of intrahepatic left portal vein and partial resection of the left lung under a thoracic scope at 29 mo and 30 mo respectively, after the first operation. At more than 3 years 6 mo after the first operation, she is now continuing to enjoy a good quality of life.

DISCUSSION

Liposarcoma is the second-most common soft tissue sarcoma among adults. Its incidence was reported as 10-12% among soft tissue sarcomas. The two major sites of liposarcoma are the lower extremities and retroperitoneum: their respective incidences are 30.1% and 18.5%. Other less common sites have been reported: the inguinal and paratesticular regions, chest wall, breast, and mediastinum[1]. Notwithstanding, few cases of intra-abdominal liposarcoma have been reported. Furthermore, most of those are from the mesentery and peritoneum[1].

In our case, the original sites of liposarcoma were considered to be the posterior wall (hepatic-side surface) of gallbladder, according to operative and pathological findings. At the first operation, the tumor was dissected easily from the liver and the retroperitoneum. The tumor was almost capsulated histologically, except the hepatic surface of the gallbladder. Furthermore, the tumor cells originated from the muscular layer only at the hepatic side of gallbladder, where the capsule disappeared. Generally, liposarcoma takes its origin from primitive mesenchymal cells rather than mature adipose tissue; it often seems to arise from intermuscular fascial planes or richly vascular structures[1]. From these findings, we concluded that the tumor originated from the muscular layer of the gallbladder on the hepatic surface. Thereafter, it might grow rapidly and extramurally.

In this case, we also want to emphasize that we could preoperatively make an accurate diagnosis about the origin of the tumor. It is usually very difficult for many doctors to confirm the origin of such a huge abdominal sarcoma pre- and post-operatively, however, our case was detectable because we could comprehend the relation of the tumor to the surrounding organs (in our case; liver, gallbladder, and bile duct) from MR cholangiography and the main feeding artery of the tumor from selective arteriography with CT imaging (CT during cystic arteriography). These imaging studies might be useful to detect the origin of such a huge abdominal mass as our case.

Among the rare cases of intra-abdominal liposarcoma, the mesentery, omentum, and gastrointestinal organs are its common sites. However, liposarcoma of the liver and the biliary system are exceedingly rare and have been reported in only 10 cases, including our case (Table 1). Those patients were younger than patients with other malignant tumors: two of those cases were infants. No dif-

ferences of sex were apparent in these patients. Seven of the cases received medications, but three cases could not because of complications by the tumors. Only one case of seven medicated patients was unresectable and thus got regional chemotherapy and radiation therapy. Prognoses of liposarcoma of the liver and gallbladder seem to be poor. Previous reports about liposarcoma have described cases among which six of eight patients died, three cases were inoperable because of rapid tumor growth, two cases were recurrent, and one ended in death by complications engendered by a difficult operation. Generally, pleomorphic liposarcoma, as in our case, is defined as a high-grade pleomorphic sarcoma containing multivacuolated lipoblasts and rare form with a poor prognosis [11,12]. It occurs in about 10% of liposarcoma cases and has a 45% local recurrence rate and 42.5% metastatic rate. Five-year overall, metastasis-free, and local recurrence-free survival was shown respectively as 57%, 50%, and 48%[13]. From these reports, she had better postoperative course than many cases of liposarcoma in Table 1, and is now receiving a good quality of life, because the primary and recurrent tumors were located on curatively resectable area luckily and we could detect the recurrences soon by using CT frequently. In the future, it is also important to find recurring tumors using imaging studies and to resect them as soon as possible for her quality of life.

In conclusion, we experienced a case of liposarcoma of the gallbladder that has not yet been reported in detail and for which the patient has survived long after the primary operation because of two operations that were undertaken aggressively.

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CASE REPORT

Congenital tracheoesophageal fistula successfully diagnosed by CT esophagography

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Abstract

Tracheoesophageal fistula (TEF) or bronchoesophageal fistula may be congenital, inflammatory, neoplastic, or secondary to trauma. Congenital TEF or bronchoesophageal fistula is usually associated with esophageal atresia and is readily diagnosed in infancy. But if it is not associated with esophageal atresia, it may persist until adulthood. Some theories have been proposed to explain this delay in diagnosis. We present a case of a 70-year-old man with congenital TEF. The TEF was successfully diagnosed by multidetector-row CT esophagography.

Key words: Tracheoesophageal fistula; Congenital; Virtual endoscopy; Computed tomography; Esophagography

Nagata K, Kamio Y, Ichikawa T, Kadokura M, Kitami A, Endo S, Inoue H, Kudo SE. Congenital tracheoesophageal fistula successfully diagnosed by CT esophagography. *World J Gastroenterol* 2006; 12(9): 1476-1478

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INTRODUCTION

Tracheoesophageal fistula (TEF) or bronchoesophageal fistula may be congenital, inflammatory, neoplastic, or secondary to trauma[1-3]. Congenital TEF or bronchoesophageal fistula is usually associated with esophageal atresia and is readily diagnosed in infancy. But if it is not associated with esophageal atresia, it may persist until adulthood. Some theories have been proposed to explain this delay in diagnosis. The long, silent interval until adulthood and the irregular character of the signs have been attributed to the presence of a membrane that subsequently ruptures, to the proximal fold of esophageal mucosa initially overlapping the orifice but subsequently becoming less mobile, and to the fact that the fistulous tract runs upward and may close during swallowing[1,2,4-6]. The oldest was an 83-year-old man with congenital bronchoesophageal fistula[7].

We present a case of a 70-year-old man with congenital TEF. The TEF was successfully diagnosed by multidetector-row computed tomography (CT) esophagography.

CASE REPORT

A 70-year-old man was admitted to our hospital with the complaint of severe cough and choking after swallowing liquid. The patient had done well until 7 mo prior to the admission. Then increased symptoms of cough and dysphagia were developed. Esophagoscopy showed a depressed lesion that looked as if it was punched without inflammation at a distance of 23 cm from the incisors in the middle intrathoracic esophagus, but the fistula opening was invisible (Figure 1). Histological examination of the esophageal biopsy specimens had only normal findings. A multidetector-row CT scan using Aquilion M8 (Toshiba, Tokyo, Japan) was performed with 3-dimensional reconstruction. A nasogastric tube was inserted into the esophagus during the examination, and the esophagus was inflated with oxygen flow (1L/min). The CT scanning parameters were 120 kVp, 200 mA, 8 × 0.5 mm collimation, a pitch of 10.0, and a 0.3-mm reconstruction interval. CT esophagography revealed the presence of a TEF (Figure 2). Conventional barium esophagography was not performed for the patient.

The patient underwent a right posterior lateral thoracostomy. The fistula was identified between the middle intrathoracic esophagus and the distal trachea. There was no evidence of inflammation or adherent lymph nodes around the fistula. The fistula was divided and closed using endostapler (EndoGIA Universal stapler®; US Surgical Corporation, Norwalk, Conn.), and covered with an intercostal muscle flap to avoid the recurrence of fistula. Intra-

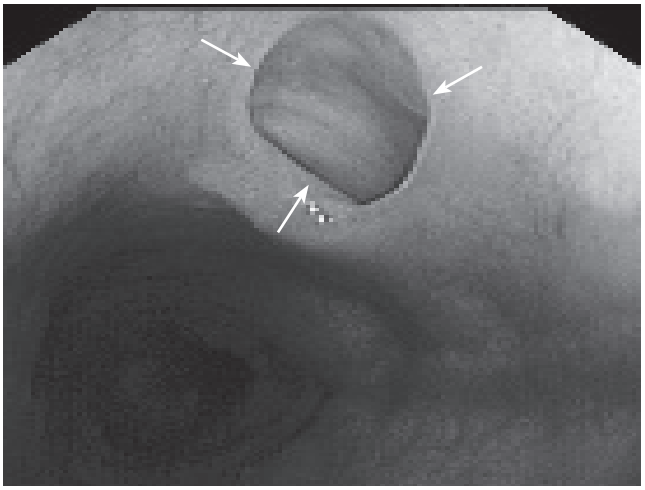

Figure 1 Esophagoscopy reveals a depressed lesion (arrows) that looked as if it was punched at a distance of 23 cm from the incisors in the middle intrathoracic esophagus (E).

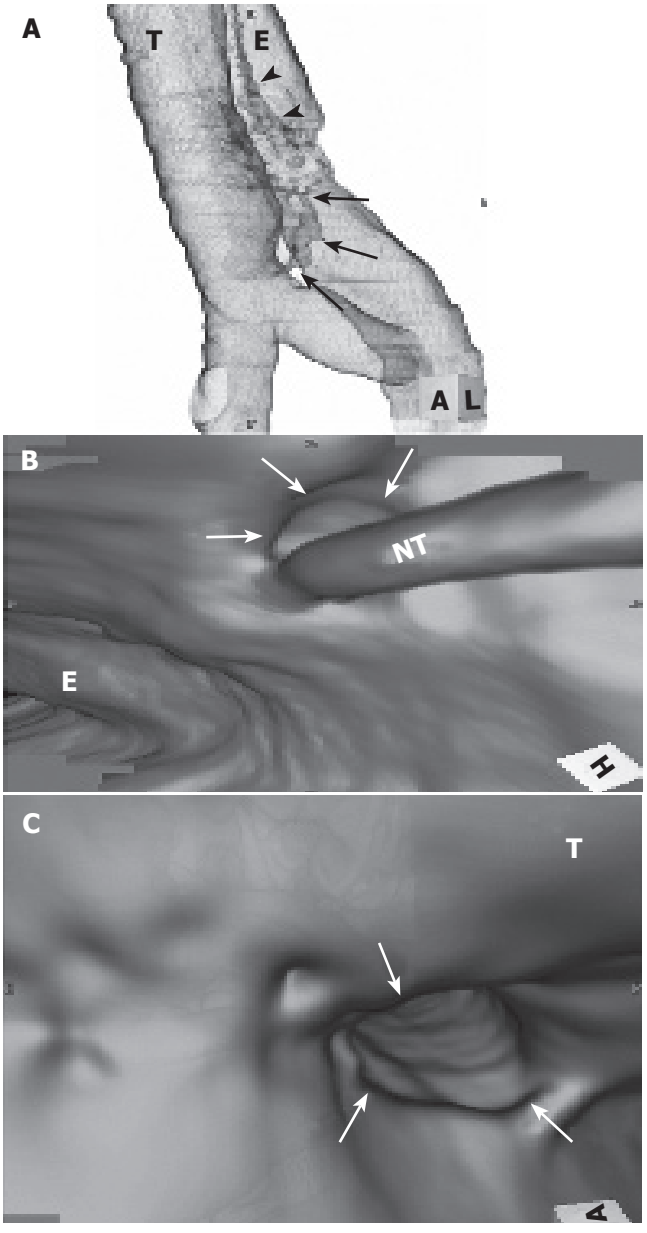

Figure 2 **A:** CT esophagography demonstrates communication between the middle intrathoracic esophagus and the distal trachea just proximal to the carina (arrows). Note a distortion caused by tube inserted into the esophagus (arrowheads). T, trachea; E, esophagus. **B:** Virtual esophagoscopy shows the orifice of the fistula (arrows) and it is similar to that of (conventional) esophagoscopy (Figure 1). NT, naso-gastric tube; E, esophagus. **C:** Virtual bronchoscopy also demonstrates the orifice of the fistula (arrows). T, trachea.

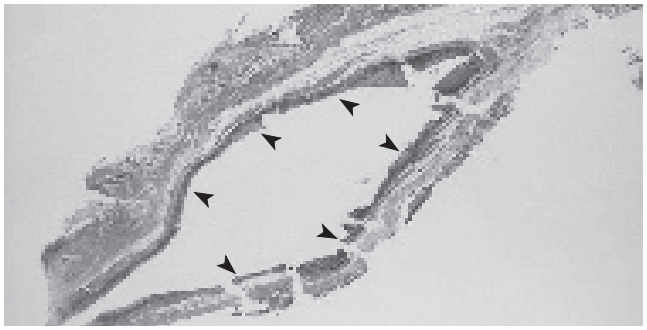

Figure 3 Pathological examination of the resected specimen revealed that the fistula was lined by benign squamous epithelium with the muscularis mucosa (arrowheads).

operative esophagoscopy was found to be useful for the definitive localization and complete excision of the fistula and the avoidance of stenosis of the esophagus. Pathological examination of the resected specimen revealed that the fistula was lined by benign squamous epithelium with the muscularis mucosa, and there was no evidence of malignancy, infection, or chronic inflammation (Figure 3). The postoperative course was uneventful. At 18 mo follow-up, there was no clinical or radiographic evidence of recurrence of the fistula.

DISCUSSION

Congenital TEF or bronchoesophageal fistulas were first reported by Negus in 1929 and Gibson in 1696[8]. These congenital fistulas are still controversial and rare clinical problem in adults. Generally, the criteria for the diagnosis of a congenital fistula in adults has the following features: absence of past or present surrounding inflammation or malignancy, complete recovery after resection, and the presence of normal mucosa and absence of adherent lymph nodes[1,2,7]. We could confirm our case as a congenital TEF by the above mentioned features, even though it was found in a 70-year-old man.

The anatomy of TEF should be shown before surgery because the surgical approach depends on a correct evaluation of TEF. Conventional barium esophagography is considered to be the most sensitive test for diagnosing tracheoesophageal or bronchoesophageal fistula[9,10]. Other examinations that have been used but are less successful include esophagoscopy, bronchoscopy, and bronchography[9,10]. Three-dimensional (3D) displays of many organs and structures using multidetector-row CT have recently become a popular clinical examination tool with significant improvement which was made on quality of image due to a rapid progress in computer technology[11-13]. Multidetector-row CT esophagography correctly showed TEF in our case. Images of TEF could be easily made and provide crucial information for planning surgery. Fitoz *et al* report-

ed that 3D CT imaging are used to assist in the diagnosis of TEF[14].

Fitoz's and our experience indicates CT esophagography might be clinically useful for the accurate diagnosis of tracheoesophageal fistula. CT esophagography without contrast medium is less invasive examination.

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CASE REPORT

Strangulated hernia through a defect of the broad ligament and mobile cecum: A case report

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Abstract

We report a case of 28-year-old woman presenting with small bowel obstruction. She had neither prior surgery nor delivery. An upright abdominal radiograph revealed several air-fluid levels in the small bowel in the midabdomen and the pelvic cavity. Computed tomography demonstrated a dilated small bowel loop in the Douglas's fossa, but no definite diagnosis could be made. Supportive therapy with draining the intestinal fluid by a long intestinal tube did not result in improvement, which suggested the possibility of a strangulated hernia. Exploratory laparotomy revealed mobile cecum and a 20-cm length of the ileum herniated into a defect of the right broad ligament. As a gangrenous change was recognized in the incarcerated bowel, its resection was carried out, followed by end-to-end anastomosis and closure of the defects of the broad ligament. The postoperative course was uneventful. Intestinal obstruction is a very common cause for presentation to an emergency department, while internal hernia is a rare cause of obstruction. Among internal hernias, those through defects of the broad ligament are extremely rare. Defects of the broad ligament can be either congenital or secondary to surgery, pelvic inflammatory disease, and delivery trauma. In conclusion, we emphasize that hernia of the broad ligament should be added to the list of differential diagnosis for female patients presenting with an intestinal obstruction. Early diagnosis and surgical repair reduce morbidity and mortality from strangulation.

Key words: Internal hernia; Broad ligament; Intestinal obstruction; Mobile cecum

Hiraiwa K, Morozumi K, Miyazaki H, Sotome K, Furukawa A, Nakamaru M. Strangulated hernia through a defect of the broad ligament and mobile cecum: A case report. *World J Gastroenterol* 2006; 12(9): 1479-1480

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INTRODUCTION

Intestinal obstruction is a very common cause for presentation to an emergency department, while internal hernia is a rare cause of such obstruction. Even rare is the hernia through defects of the broad ligament. This report describes a rare case of intestinal obstruction from internal hernia through a defect of the right broad ligament. The diagnosis of this condition was not made preoperatively due to its rarity and nonspecific manifestation.

CASE REPORT

A 28-year-old woman, gravida 0 para 0, presented complaining of acute lower abdomen colicky pain, nausea, and vomiting. She had no prior relevant medical history. On physical examination, her right lower quadrant was tender with mild voluntary guarding and increasing bowel sounds. Rectal examination did not show any abnormalities. Laboratory studies showed no definite abnormalities except for an elevated leukocyte count of 13 800/mm^3 with a left shift. An upright abdominal radiograph revealed multiple air-fluid levels in the small bowel in the midabdomen and the pelvic cavity. Computed tomography demonstrated a dilated small bowel loop in the Douglas's fossa (Figure 1), but no definite diagnosis could be made.

Supportive therapy with draining the intestinal fluid by a long intestinal tube did not result in improvement, thereby suggesting the possibility of a strangulated hernia.

The patient underwent an exploratory laparotomy, which revealed two defects of the right broad ligament and mobile cecum. A 20-cm length of the ileum had been herniated into the outer defect. As a gangrenous change was recognized in the incarcerated bowel, it was resected and an end-to-end anastomosis was performed while the defects of the broad ligament were also closed. The postoperative course was uneventful.

DISCUSSION

Internal hernia is responsible for about 0.9% of intestinal obstruction. Hernia of the broad ligament is extremely rare and accounted for less than 7% of all internal hernias.

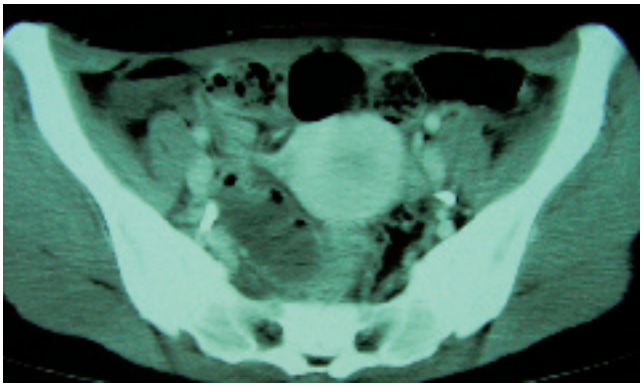

Figure 1 Contrast-enhanced computed tomography demonstrating a dilated small bowel loop in the Douglas's fossa.

The earliest reported case of an incarcerated hernia through a defect of the broad ligament of uterus was in 1861 by Quain, found at autopsy[1]. Two types of hernia of the broad ligament have been classified by Hunt[2]: the fenestra type that involves a complete fenestration through a defect in the broad ligament; and the pouch type that involves hernia into the pouch from an anterior or a posterior aperture. The defects in our case were of the fenestra type.

Preoperative diagnosis of hernia of the broad ligament is quite difficult. The usefulness of computed tomography has been reported[3], but the causal lesion of the intestinal obstruction could not be detected with computed tomography in our case. Although computed tomography suggests an internal hernia, it may be impossible to diagnose the hernia through a defect of the broad ligament[4].

Although the precise pathogenesis of a defect of the broad ligament remains unknown, its causes are considered to include surgery, pelvic inflammatory disease, delivery trauma and congenital anomaly[5]. Our case had neither any relevant medical history nor delivery, thereby suggesting a congenital anomaly in our case. In female embryos, fusion of the paramesonephric ducts forms the broad ligament. The present case showed both incomplete fusion of the broad ligament and incomplete fixation of the ascending colon leading to mobile cecum. These anomalies might have resulted from some abnormalities in a similar embryonic period.

In conclusion, we emphasize that intestinal hernia through a defect of the broad ligament should be added to the list of differential diagnosis for female patients presenting with an intestinal obstruction without any prior history of laparotomy. Early diagnosis and surgical repair reduce morbidity and mortality from strangulation.

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CASE REPORT

Rectal carcinosarcoma: A case report and review of literature

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Abstract

A 60-years old male was admitted to our department for investigation of constipation and hypogastric discomfort intensified during defecation of a few weeks duration. The cause proved to be a rectal carcinosarcoma that was treated by abdominoperineal resection and postoperative chemo-radiotherapy. The patient died 6 months later due to hepatic failure, showing evidence of disseminated disease. In general colonic carcinosarcomas constitute a rare category of malignant neoplasms whose nature is still incompletely understood. No specific treatment guidelines exist. Surgery is the mainstay of treatment and regardless of the addition of adjuvant therapy the prognosis is very poor. Systematic genetic analysis may be the clue for understanding the pathogenesis of these mysterious tumors.

Key words: Carcinosarcoma; Rectum

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INTRODUCTION

The coexistence of carcinomatous and sarcomatous elements within the same tumor is a rare entity, variably labeled as "carcinosarcoma", "metaplastic carcinosarcoma", "sarcomatoid carcinoma", "pseudosarcomatous carcinoma", "carcinoma with mesenchymal stroma", "carcinoma with sarcomatoid change", "spindle cell carcinoma", "pleomorphic anaplastic carcinoma" and "small cell carcinoma"[1]. This confusing, non-standardized terminology reflects the rarity and uncertainty regarding the nature of these tumors.

The term carcinosarcoma implies a mixed malignant tumor that is composed of an epithelial element, typically the common form of carcinoma seen in the tissue harboring the neoplasm, close to or intermixed with a sarcomatous component. The mixed nature of these tumors is confirmed by a positive immunohistochemical staining for both cytokeratins and vimentin. The common denominator of all carcinosarcomas is the lack of staining of the sarcomatous component for epithelial markers. On the contrary in the cases where both the sarcomatous and carcinomatous components are stained positively for epithelial markers the term "sarcomatoid carcinoma" should be addressed[2,3]. For all practical purposes in our review we consider carcinosarcomas as only cases where the sarcomatous component was stained negative for epithelial markers, irrespective of the term an author decided to utilize for describing the mixed tumor encountered.

The earliest report of a possible, due to lack of immunohistochemistry, carcinosarcoma can be tracked back to 1864 by Virchow[4]. These neoplasms usually arise in the female reproductive tract, urinary tract, the head and neck areas, breast and respiratory tract. The gastrointestinal tract is an uncommon location of carcinosarcomas, the esophagus being the most common location within it[1-4]. To our knowledge, only 7 cases of colonic carcinosarcomas have been reported in the English literature, and this case is the 8th. We report this case of rectal carcinosarcoma and review the literature on this rare malignancy.

CASE REPORT

A 60-years old male was admitted to our department for investigation of constipation and hypogastric discomfort intensified during defecation of a few weeks duration. He had a history of acute myocardial infarction and benign prostatic hyperplasia under medical treatment.

Abdominal and chest clinical examinations were unrevealing. However on digital examination a rubbery, fixed polypoid mass could be palpated just proximal to the dentate line. The complete blood count revealed only a slight anemia. Blood biochemistry was unrevealing.

On colonoscopy the mass was confirmed and a smaller polyp of 0.4 cm in diameter was detected on the upper rectum. Biopsies were obtained, which resulted in the

Table 1 Immunohistochemical profile of the tumor

marker	carcinomatous	sarcomatous
Cam 5,2	+	-
MNF 116	+	-
Vimentin	-	+
Actin	-	+
S 100	-	-
Chromogranin	-	-
c Kit	-	-
CD 34	-	-
HER2/neu	-	-
Ki 67	-	-
P53	+	-

diagnosis of adenocarcinoma. A CT scan of the abdomen and pelvis was subsequently performed revealing the lower rectal mass and distortion of adjacent perirectal fat. No metastatic foci to the liver or the abdominal cavity could be detected. The CXR was normal.

An abdominoperineal resection was performed. On intraoperative abdominal inspection and palpation, no abnormal findings could be detected asides from the pelvic mass. The patient had an uneventful postoperative course and was discharged on the 10th d.

The resected specimen, measuring 34 cm in length, consisted of anus, anal canal, rectum and sigmoid colon. The surgical margins were tumor free. The rectal tumor had a diameter of 7 cm, was elastic on palpation and had a whitish-yellow cut surface.

Histological examination revealed an invasive tumor infiltrating the full thickness of the rectal wall, as well as the perirectal fat and external sphincteric muscle fibers at the level of anal canal. The tumor consisted of poorly differentiated carcinomatous and spindle-shaped sarcomatous elements. Extensive areas of necrosis and hyaline degeneration of the stroma were evident. In a few locations the carcinomatous cells were organized into tubular and cribiform structures. 24 lymph nodes were recognized in the resected specimen. Five of them were positive for malignancy. The carcinomatous element was predominant, but sarcomatous cells could also be detected within the involved lymph nodes. The immunohistochemical profile is listed on Table 1. The final histologic diagnosis was rectal carcinosarcoma, TNM stage III.

Postoperatively the patient received 4 cycles of 5-fluorouracil/leucovorin chemotherapy. Follow-up radiological examination revealed a 16cm x 17cm hepatic metastatic focus as well as lung and peritoneal implants, local recurrence in the resection field and inguinal lymph node involvement, evident by the 4th postoperative month. Pelvic and inguinal radiotherapy was added. The patient finally succumbed to its disease at the 6th postoperative month. A postmortem examination was not performed.

DISCUSSION

Carcinosarcomas of the gastrointestinal tract are extremely rare. Most of them occur in the esophagus; about 40 cases have been reported in the gallbladder and less than 30 in the stomach. In a review of the English literature we found only seven immunohistochemicaly confirmed cases of carcinosarcoma of the large intestine, our case being the eighth[1-7]. Four further reports exist regarding colonic sarcomatoid carcinomas that should not be confused with carcinosarcomas, when purely immunohistochemical criteria are employed[8-11]. However the clinical relevance of this distinction is doubtful because the biological behavior of these neoplasms appears to be similar. In three other cases of colonic biphasic tumors the exact nature of the neoplasm is not clear.[12-14] The cases of colonic carcinosarcoma reported in the English literature are listed on Table 2.

Colonic carcinosarcomas present in the age group of 60 to 84 years, averaging 73 years of age at diagnosis. Males and females are equally affected. The tumor can be located anywhere in the large bowel, but a left-sided predominance is evident (six out of eight cases).

Histologically colonic carcinosarcomas are composed of two parts, an epithelial component and a mesenchymal component. The epithelial component appears indistinguishable from colonic adenocarcinoma. The malignant mesenchymal component of the tumor may be differentiated or undifferentiated. The differentiated is described as homologous or heterologous. Homologous elements resemble fibrosarcoma or leiomyosarcoma. If the sarcomatous part contains elements not normally found in the colon (e.g., cartilaginous, osseous, or rhabdomyoblastic), it is then described as heterologous. As stated in carcinosarcomas of other organs, the quality of the sarcomatous component does not appear to affect prognosis[1-3].

The possible histogenesis of colonic carcinosarcomas is a matter of debate. Several theories have been proposed to explain the histologic features of these tumors. These include the "collision tumor theory" suggesting that the two tumor components are derived from separate and distinct malignant cell clones. In favor of this opinion are investigators that observed sharply defined epithelial and sarcomatous components without demonstrable cells showing shared or transitional features on ultrastructural or immunohistochemical examination. Others propose a common origin of both components of carcinosarcomas, since common characteristics, epithelial in nature, between the different cellular populations as well as a transitional population could be observed. This could be either due to a malignant transformation of a pluripotential stem cell capable of both epithelial and mesenchymal differentiation (the combination theory) or due to a sarcomatoid/carcinomatous differentiation of a carcinoma/sarcoma (the conversion theory). Regarding the conversion theory it is believed that the carcinomatous element is the tumor' s "driving force", and the sarcomatous component derives from this as a result of dedifferentiation. Some postulate that this sarcomatoid transition of carcinomatous cells could be related to an infection by a retrovirus[2,10]. Another theory that has been proposed to explain the presence of sarcomatous elements in carcinosarcomas questions the neoplastic nature of this component. According to this theory the sarcomatous component is a benign reparative or granulomatous tissue mass representing a conspicuous

Table 2 Cases of colonic carcinosarcoma reported in the English literature

Case	Author	Age/Sex	Location	Histology	TNM Stage	Recurrence	Adjuvant	Survival (Mo)
1	Weidner (1986)	73/M	Sigmoid	SCC+AC+OS+CS	II	Liver, LN, pelvis	Postop 5-FU, mitomycin C cyclophosphamide, doxorubicin, cisplatin	49, died
2	Chetty (1993)	72/F	Cecum	AC + UDSCS +RMS	IV	?	No	3, lost
3	Roncaroli (1995)	71/F	Anorectum	NEC + RMS	II	Pelvis	Preop radiotherapy	6, died
4	Takeyoshi (2000)	82/M	Anorectum	AC + UDSCS	II	Skin	No	6,died
5	Nakao (1998)	60/F	Transverse	AC + RMS	III	-	Postop 5-FU+cisplatin	14, disease free
6	Ishida (2003)	80/F	rectosigmoid	AC+SCSM-N	III	Pelvis	No	6, died
7	Aramendi (2003)	84/M	Splenic flexure	AC+OS+CS	II	-	No	Died at diagnosis
8	Present case	60/M	Rectum	AC+SCSM	III	Liver, Lung, pelvis, LN, peritoneum	Postop 5-FU, leucovorin and radiotherapy	6, died

SCC: squamous cell carcinoma, AC: adenocarcinoma, OS: osteosarcoma, CS: chondrosarcoma, UDSCS: undifferentiated spindle cell sarcoma, RMS: rhabdomyosarcoma, NEC: neuroendocrine carcinoma, SCSM: spindle cell sarcoma with muscular features, SCSM-N: spindle cell sarcoma exhibiting both muscular and neural features, LN: lymph nodes, postop: postoperative, preop: preoperative

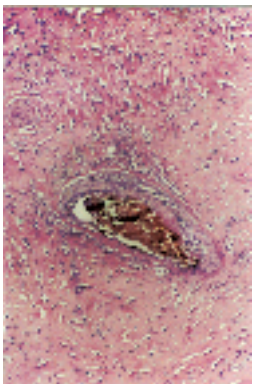

Figure 1 Rectal carcinosarcoma. Infiltration of a vessel wall by carcinomatous cells. The vessel is surrounded by sarcomatous element of carcinosarcoma. (Hematoxylin-eosin, original magnification X100).

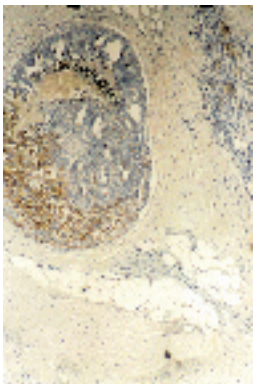

Figure 2 Rectal carcinosarcoma. Carcinomatous elements are positive for cytokeratins (CAM 5.2, original magnification X100).

connective tissue response to the carcinoma, i.e. an epithelial-stromal interaction (the composition theory).

Evidence coming from studies on esophageal, breast, endometrial and uterine carcinosarcomas suggests that the combination or conversion theories, which are not mutually exclusive, are the prime nodes of histogenesis of these tumors, i.e. most carcinosarcomas are monoclonal and should be better considered as metaplastic carcinomas[1-4,15]. It is of interest to note that most lymph node metastases reflect the carcinomatous component. This adds to the hypothesis that the carcinomatous component is the driving force of these tumors, but this is not always the case. In our case one lymph node was infiltrated by both elements.

Over the recent years the increasing use of immunohistochemistry and electron microscopy has added to our understanding of these tumors. However the information gathered was proved to be insufficient. Systematic genetic analysis may be the clue for understanding the pathogenesis of these mysterious tumors. Recently great interest has been shown on p53 status of these tumors. It has been suggested that the transition of carcinoma to sarcoma is associated with progressive accumulation of p53 proteins in this component[10]. However in our case this could not be confirmed since only the carcinomatous element stained positive for p53. Others have demonstrated the same p53 mutations on both sarcomatous and carcinomatous elements. Furthermore reports indicating no p53 mutation detection have been published[16,17](Figures 1 and 2).

The same diagnostic and operative treatment options that apply for colonic adenocarcinoma are also employed in the case of colonic carcinosarcomas, since no carcinosarcoma-specific treatment guidelines exist. All the reported cases of colonic carcinosarcomas underwent resection of the primary tumor and draining lymph nodes in the course of typical colectomies. Adjuvant therapy was added in four cases, consisting of chemotherapy in three and irradiation in two, but the efficacy of this approach remains questionable. However it should be noted that none of the patients who did not receive adjuvant therapy survived more than 6 mo, while the 2 cases that survived more than 6 mo had received postoperative chemotherapy[1-7].

The prognosis of carcinosarcomas is very poor. These tumors are very aggressive neoplasms characterized by rapid growth and a high incidence of both local recurrence and distant spread. Six of the eight reported cases died of the disease within 5 years of diagnosis. Survival ranged from 0 to 49 mo averaging 12 mo. For the remaining two

cases there are no available data concerning their 5 year survival. These patients were reported to be alive at 3 and 14 mo postoperatively respectively, but only the patient that was alive at 14 mo was apparently "disease free". Due to the lack of sufficient amount of cases no safe conclusions regarding prognostic factors can be made. Interestingly the stage of the disease does not appear to provide accurate information about prognosis since all four patients with reportedly stage II disease died within five years. Three of them were dead by the sixth postoperative month[1-7].

In general colonic carcinosarcomas constitute a rare category of malignant neoplasms whose nature is still incompletely understood. No specific treatment guidelines exist. Surgery is the mainstay of treatment and regardless of the addition of adjuvant therapy the prognosis is very poor. However some state that these neoplasms may be commoner than reported. This is due to the fact that the sarcomatous and carcinomatous elements are arranged unevenly at separate areas of the tumor, resulting in common preoperative and possible postoperative misdiagnosis if the specimen is not thoroughly examined. Careful examination of the resected colonic specimens may be the cornerstone of preventing underdiagnosis of these tumors and allow accumulation of knowledge to guide proper therapeutic intervention.

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CASE REPORT

Holmes-Adie syndrome, autoimmune hepatitis and celiac disease: A case report

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Abstract

A 35-year-old female patient presented with the following symptoms of Holmes-Adie syndrome: photophobia, enlargement of the left pupil unresponsive to light, Achilles areflexia. The pilocarpine test was positive. No tumor or other neurological abnormality was found. She had a 19-year history of autoimmune hepatitis. Flares up were observed following each 3 deliveries. At age of 31 she presented with diarrhea and weight loss. Abdominal tumor was detected by ultrasound. The surgically removed tumor was histologically a benign mesenteric multicystic lymphangioma. Simultaneously, celiac disease was diagnosed. Gluten-free diet resulted in a significant improvement of celiac disease, but not of autoimmune hepatitis. Autonomic neuropathy was proven by standard cardiovascular tests. The patient was a homozygous carrier for HLA DQ2 antigen characteristic for celiac disease and heterozygous for HLA DR3 B8 frequent in autoimmune liver diseases. Our novel observation on association of Holmes-Adie syndrome with autoimmune hepatitis and celiac disease is suggestive for a common immunological background for all three entities present in a patient with mesenteric multicystic lymphangioma.

Key words: Holmes-Adie syndrome; Autoimmune hepatitis; Celiac disease; Mesenteric lymphangioma; Autonomic neuropathy

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INTRODUCTION

Holmes-Adie syndrome (HAS) first described in 1931 is characterized by unilateral or bilateral dilatation of the pupil and lack of light reaction because of postganglionic injury of the parasympathetic oculomotor nerve. Ciliary muscle denervation causes accommodation impairment, while sphincter pupillae denervation produces mydriasis. It is often accompanied with peripheral nerve dysfunctions. Deep tendon reflexes (knee- and Achilles-reflex) are absent because of impaired spinal monosynaptic connections[1]. Most cases are caused by viral infection. Trauma, tumors, vascular lesion with ischemia, giant cell arteritis, diabetes mellitus or autonomic neuropathies can also damage the ciliary ganglion[2]. This is the first report on association of Holmes-Adie syndrome with autoimmune hepatitis (AIH) and celiac disease (CD) raising the probability of common autoimmune background in a patient with multicystic mesenterial lymphangioma.

CASE REPORT

A 35-year old female patient presented with symptoms of Holmes-Adie syndrome: photophobia and enlargement of the left pupil with absent reaction to light, accompanied with knee- and Achilles-areflexia (Figure 1). The positivity of pilocarpine test confirmed the diagnosis of HAS. Tumor, actual infection, ischemic disease, trauma and mechanical compression were excluded.

She had a 19-year history of AIH proven by liver biopsies, autoantibody positivity (antinuclear antibody/ANA and smooth muscle antibody/SMA) and exclusion of other causes. The liver disease initially responded well to prednisone and azathioprine treatment. Flares up were observed following each 3 deliveries.

At age of 31, after the third delivery, she presented with anorexia, severe diarrhea, rapid loss of weight (18 kg/6 mo), amenorrhea and sideropenic anemia. Abdominal tumor was found by ultrasound and CT. The surgically removed tumor was 10 cm in diameter, histologically proven as a benign mesenteric multicystic lymphangioma. Simultaneously, in the background of malabsorption syndrome, celiac disease (CD) was diagnosed. Biopsy proved villous atrophy, anti-endomysium and transglutaminase antibody test was positive. Gluten-free diet resulted in a significant improvement and disappearance of symptoms of celiac disease. The secondary lactose intolerance ceased and the severe calcium deficient osteopenia (osteodensitometric Z-score L2-L4: -4,4) improved. The family history

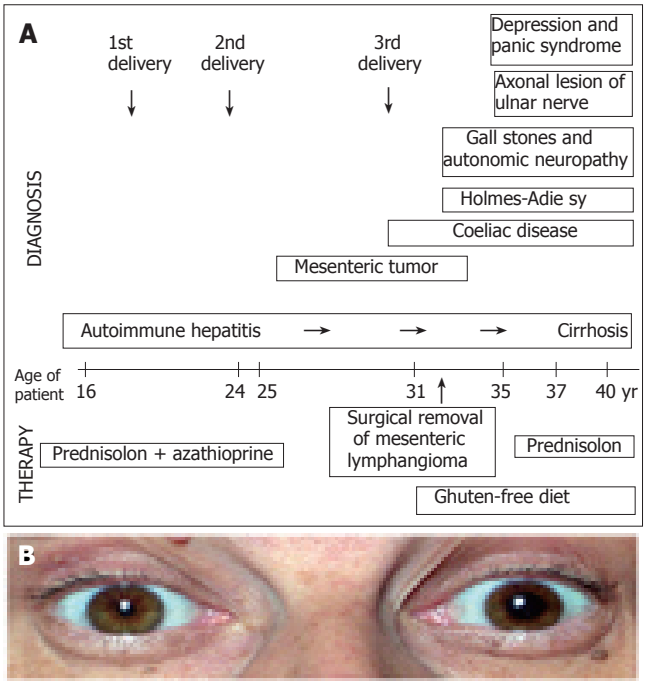

Figure 1 A: Sequence of events, appearance of symptoms and applied treatment. B: Dilated and light insensitive left pupil as symptom of Holmes-Adie syndrome.

was negative for any autoimmune disease.

Holmes-Adie syndrome developed 4 years later and still persisted at the time when she was admitted. The progression of AIH was observed after immunosuppressive treatment and the strict gluten free diet. Now, at her age of 40 the liver biopsy showed cirrhosis. Axonal lesion of ulnar nerve was detected by electroneurography-electromyography (ENG-EMG) and autonomic neuropathy was proven by standard cardiovascular tests. The patient had gallstones in the enlarged gall bladder with reduced motility. In the last few years panic syndrome and severe depression developed. Alprazolam and fluoxetin were applied. She had polyarthralgia with the involvement of lumbar spine, knee and proximal interphalangeal and metacarpophalangeal joints. She complained of morning stiffness of 1 h duration. However, the X-ray examination was not characteristic for rheumatoid arthritis and the rheuma factor was negative. The sequence of events, appearance of symptoms and applied treatment are summarized on Figure 1.

The patient was a homozygous carrier for HLA DQ2 antigen characteristic for celiac disease and heterozygous for HLA DR3 B8 frequent in autoimmune liver diseases.

DISCUSSION

Association of autoimmune hepatitis and celiac disease has been reported, but not with Holmes-Adie syndrome. Initial reports in the late 1970s revealed that hepatic aminotransferase enzymes are frequently elevated at the time of diagnosis of CD[3]. The scale of the liver dysfunction in CD is wide: from raised transaminases with mild hepatic steatosis to chronic hepatitis and cirrhosis caused mainly by primary biliary cirrhosis (PBC), autoimmune hepatitis (AIH) or primary sclerosing cholangitis (PSC). Prevalence of CD in patients with primary biliary cirrhosis (2-7%) and AIH (3-5%) is higher than that in the normal population[4]. Liver dysfunction should therefore be examined in patients with CD at the time of diagnosis. On the other hand, CD should be searched in the background of cryptogenic liver diseases. Two mechanisms must be considered in the pathogenesis of liver dysfunction associated with CD: impaired gut mucosal integrity, malabsorption and increased permeability; and a common inherited predisposition for AIH and CD[4]. The HLA pattern in our patient supports the latter theory. She was a homozygous carrier for HLA DQ2 antigen characteristic for celiac disease and heterozygous for HLA DR3 B8 frequent in autoimmune liver diseases. HLA DR3 B8 is in close linkage disequilibrium with HLA DQ[4, 5].

This is the first report on the association of Holmes-Adie syndrome with these two autoimmune diseases. The exclusion of other etiologies of Holmes-Adie syndrome suggests the possibility of common autoimmune background. Tumors, actual infection, ischaemic disease, diabetes mellitus, trauma or mechanical compression which can damage the ciliary ganglion[2], were excluded in our patient. Autonomic neuropathy is well known in Holmes-Adie syndrome. Evidence of autonomic dysfunction has been proven in 40% of the patients with Holmes-Adie syndrome[5]. In recent years it has been suspected that autonomic failure has an autoimmune pathogenesis and autoantibodies against ganglional nicotinic acetylcholine receptor can induce autonomic neuropathy similar to the pathophysiology of myasthenia gravis[7, 8]. However, there are only three case reports in the literature in which autoimmunity is hypothesized in the background of Holmes-Adie syndrome[9,10]. There are no data about the HLA pattern in Holmes-Adie syndrome. Since no other cause of HAS has been found, it is a logical hypothesis that the neurological abnormalities including damage of postganglionic parasympathetic nerves, are connected with autoimmune pathomechanism characteristic features in celiac disease and autoimmune hepatitis.

The presented case is a further example for manifestation of celiac disease in adulthood[5]. The immunosuppressive treatment for AIH might have delayed the manifestation of CD at age of 31. Several extrahepatic manifestations of CD could be the first presenting symptoms, making difficult the diagnosis. Known associated diseases of CD are autoimmune liver disease, thyroid gland disease, arthritis, Sjörgen syndrome, neurological and psychiatric symptoms, diabetes mellitus, cardiomyopathy, osteoporosis, tumors, infertility and dermatitis herpetiformis[5]. An increased occurrence of axonal neuropathy has been observed in well treated CD[11]. Usai *et al*[12] reported that autonomic nervous system (ANS) abnormalities are found in 45% of patients with CD. In a patient reported by Giorgetti *et al*[13]. ANS dysfunction does not improve after gluten-free diet. They speculated that ANS dysfunction may be a two step process: a gluten-related, reversible first phase and a gluten-independent, probably autoimmune-related second phase, which does not improve on gluten-free diet. Our case supports this hypothesis, because both the axonal lesion of ulnar nerve and the autonomic neuropathy proven by standard cardiovascular tests manifested during a strict gluten-free diet. Probably CD initiates cer-

tain autoimmune processes which enhance and perpetuate the subclinical neuropathy having existed since at least the manifestation of CD or perhaps of AIH.

Patients with CD have a 2.0-fold higher risk of death of gastrointestinal malignancies, mainly lymphomas[14]. However, there are no data on the coincidence of mesenteric multicystic lymphangioma and celiac disease. Mesenteric multicystic lymphangioma is often misdiagnosed as a malignant disease[15]. Retrospectively, rapid weight-loss is the consequence of CD but not of the tumor. Therefore, in the background of rapid loss of body weight, CD should be considered even in patients with large abdominal tumors.

Autonomic neuropathy common in chronic liver diseases might be a cofactor for gallstone formation via gall bladder hypomotility[16, 17]. Decreased gall bladder motility has been described in celiac disease as a risk factor for gallstone formation[18]. In our patient, autonomic neuropathy was proven by standard cardiovascular tests and gall bladder hypomotility by ultrasound as the contributing factors to the development of gallstones.

In conclusion, association of Holmes-Adie syndrome with autoimmune hepatitis and celiac disease is suggestive for a common immunological background for all three entities presented in a patient with mesenteric multicystic lymphangioma. One of the messages of this report is that by observing anisocoria, Holmes-Adie syndrome should be considered, and in case of Holmes-Adie syndrome of unknown etiology, autoimmune diseases as AIH or CD should be searched.

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59	w	W	watt
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61	p	Pa	pascal
62	ev	EV	volt (electronic unit)
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EDITORIAL

Gut flora and bacterial translocation in chronic liver disease

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Abstract

Increasing evidence suggests that derangement of gut flora is of substantial clinical relevance to patients with cirrhosis. Intestinal bacterial overgrowth and increased bacterial translocation of gut flora from the intestinal lumen, in particular, predispose to an increased potential for bacterial infection in this group. Recent studies suggest that, in addition to their role in the pathogenesis of overt infective episodes and the clinical consequences of sepsis, gut flora contributes to the pro-inflammatory state of cirrhosis even in the absence of overt infection. Furthermore, manipulation of gut flora to augment the intestinal content of lactic acid-type bacteria at the expense of other gut flora species with more pathogenic potential may favourably influence liver function in cirrhotic patients. Here we review current concepts of the various inter-relationships between gut flora, bacterial translocation, bacterial infection, pro-inflammatory cytokine production and liver function in this group.

Key words: Gut flora; Bacterial translocation; Cirrhosis

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INTRODUCTION

Disturbances of the microecology of the indigenous gut flora are prevalent in patients with chronic liver disease. Significantly increased viable counts of both Gram-positive and negative bacterial species have been recovered from faeces of cirrhotic patients[1]. A high prevalence of small intestinal bacterial overgrowth, in particular, has been demonstrated in this group[2-5]. This has been attributed, at least in part, to a decrease in small intestinal motility associated with increased adrenergic activity and portal hypertension[4-8]. Studies performed in experimental animals demonstrating important effects of gut flora on intestinal motility[9,10] suggest that the development of small intestinal bacterial overgrowth in the setting of intestinal stasis may further impair intestinal motility, thereby creating a self-perpetuating vicious cycle.

In addition to the range of symptoms and consequences of bacterial overgrowth *per se*[11], increasing evidences suggest that derangement of gut flora is of substantial clinical relevance to patients with cirrhosis. Intestinal bacterial overgrowth and increased bacterial translocation of gut flora from the intestinal lumen, in combination with failure of immune defence mechanisms to efficiently remove these translocating microorganisms, predispose to an increased potential for bacterial infection in this group[12]. Impaired immune defences include reduced opsonic activity due to low hepatic synthesis of complement, deranged function of macrophage Fc gamma receptors and reduced phagocytic and killing capacity of neutrophils[13-16]. Impairment of mucosal immunity may also be important. The high prevalence of associated malnutrition in cirrhotic patients[17] exacerbates the potential for infection to occur.

Recent studies suggest that, in addition to their role in the pathogenesis of overt infective episodes and the clinical consequences of sepsis, gut flora contributes to the pro-inflammatory state of cirrhosis even in the absence of overt infection[18]. Furthermore, manipulation of gut-flora using either a Gram-positive synbiotic (probiotic and fermentable fibre) regimen or fermentable fibre alone to augment the intestinal content of lactic acid-type bacteria at the expense of other gut flora species with more pathogenic potential may favourably influence liver function in cirrhotic patients[19]. Here we review current concepts of the various inter-relationships between gut flora, bacterial translocation, bacterial infection, pro-inflammatory cytokine production and liver function in this group.

BACTERIAL TRANSLOCATION IN CIRRHOSIS

Bacterial translocation is defined as the migration of bacteria from the intestinal lumen to mesenteric lymph nodes or other extra-intestinal sites[20]. Gram-negative

members of the Enterobactereaceae family (such as *Escherichia coli* and *Klebsiella spp*), enterococci and other streptococci species are the most effective at bacterial translocation to mesenteric lymph nodes[21]. These bacterial flora, the organisms most commonly implicated in community-acquired infective episodes in patients with cirrhosis[22], can translocate across even histologically normal intestinal mucosa[21,23]. Certain strains of *Escherichia coli* are especially efficient at translocation, possibly as a result of a greater ability to adhere to the intestinal mucosal surface[24]. While obligate anaerobic bacterial flora outnumber aerobic species by more than 100-fold, these flora only rarely translocate from the intestinal lumen[21]. Conversely, anaerobic strains limit the growth of other species with higher translocation potential and their selective elimination has been shown to promote translocation of such aerobic flora[25].

Most data in support of the occurrence of increased bacterial translocation in cirrhosis come from studies performed in experimental animals, in which bacterial translocation was defined by the presence of positive bacteriological cultures for gut flora in surgically-removed mesenteric lymph nodes[26]. These studies demonstrate that the prevalence of bacterial translocation to mesenteric lymph nodes is around 40% in cirrhotic rats with ascites and around 80% in such animals with spontaneous bacterial peritonitis (SBP)[27-31]. The concept of bacterial translocation predisposing to infection in experimental cirrhosis is further supported by data showing that bacteria isolated from mesenteric lymph nodes are genetically identical to strains causing SBP in the same animal[32].

Clinical studies of bacterial translocation in cirrhosis have been limited by the lack of non-invasive methods to detect its presence. Nonetheless, available evidence suggests that increased translocation of gut flora does occur in cirrhotic patients. A high rate of positive mesenteric lymph node cultures for enteric bacteria (30.8%) has been reported in patients with Child-Pugh class C cirrhosis undergoing liver transplantation or hepatic resection, with the incidence in the order of five times higher in this group than in Child-Pugh class A or B patients[33]. In another study, almost 20% of cirrhotic patients were found to have positive mesenteric lymph node cultures following partial hepatectomy, with bacteria responsible for instances of post-operative infection the same as those recovered from mesenteric lymph nodes in most cases[34].

The presence of bacterial DNA in serum and ascitic fluid has recently been proposed as a relatively non-invasive surrogate marker of bacterial translocation in the clinical setting[35]. Using such molecular techniques, bacterial translocation may be present in as many as one third of cirrhotic patients with portal hypertension and culture-negative ascites, with *Escherichia coli* the most frequently identified bacterial species[35]. The clinical relevance of the presence of bacterial DNA in culture-negative ascites has been suggested by the finding of increased local levels of pro-inflammatory cytokines[36], although the validity of this approach and the full clinical relevance of a positive result remain to be firmly established. Increased serum levels of lipopolysaccharide-binding protein, a phenomenon attributed to translocation of bacteria from the gut to the circulation, have been documented in a proportion of cirrhotic patients without overt infection and to predict the later development of severe bacterial infection in this group[37].

Pathogenesis of bacterial translocation in cirrhosis

Intestinal bacterial overgrowth Intestinal bacterial overgrowth is a major factor promoting bacterial translocation[38-41] and available evidence suggests that factors that promote intestinal bacterial overgrowth in particular, such as impaired intestinal motility, are important in facilitating bacterial translocation in cirrhosis. In experimental animals with cirrhosis, strategies to increase the intestinal transit rate, such as treatment with propranolol or cisapride, have been shown to reduce bacterial overgrowth and bacterial translocation[38,40,41]. In the clinical setting, cisapride has been shown to increase small intestinal motility and reduce bacterial overgrowth in patients with cirrhosis, with a trend towards a lower incidence of infection in treated patients[3,42].

Structural changes in intestinal mucosa Most clinical studies performed to investigate structural changes of intestinal mucosa in patients with cirrhosis have focussed on the small intestine. Shorter and thicker microvilli have been described. Morphologically intact tight junctions, which join together the apical poles of enterocytes and represent the first line of mucosal defence against paracellular absorption, have been reported in a small cohort of clinically stable cirrhotic patients with no prior history of infection with gut-derived bacteria[43]. Whether tight junctions are also intact in cirrhotic patients with a history of such infection and those with elevated nitric oxide levels may be more relevant, since nitric oxide, which is discussed in more detail below in relation to the clinical course of SBP, has been shown to reversibly dilate tight junctions in cultured intestinal epithelial cells[44]. Notably, dilatation of the intercellular space below tight junctions, the second line of defence against paracellular absorption, has been documented in patients with cirrhosis[43]. Mucosal alterations attributed to oxidative stress, including disturbed enterocyte mitochondrial function and increased lipid peroxidation of brush border membranes, have been reported in experimental animal models of cirrhosis[45], although the relevance of these changes to the pathogenesis of bacterial translocation is uncertain.

Thick-walled, dilated capillaries along with oedema of the lamina propria, fibromuscular proliferation, a reduced villus/crypt ratio and thickened muscularis mucosa in the small bowel have been found in cirrhotic patients with portal hypertension[46] and it has been proposed that an increased potential for bacterial translocation may exist in this setting[47]. However, in a study of cirrhotic patients with an elevated mean portal venous pressure in the order of 25 mm Hg undergoing liver transplantation, portal venous pressure was not significantly different in 8 cirrhotic patients with bacterial translocation and 71 with negative mesenteric lymph node culture results, implying that additional factors are required for bacterial translocation to occur in this group[33].

Luminal factors contributing to intestinal barrier function Other factors that contribute to the normal intestinal barrier against bacterial translocation (BT), including luminal factors, such as levels of bile acids, secretory immunoglobulin A, mucins, defensins, lysozyme and phopholipase A2, have been little studied in cirrhotic patients. Bile acids exert a trophic effect on intestinal mucosa[48] and inhibit intestinal bacterial overgrowth, especially of Gram-positive species[49]. An increased incidence of bacterial translocation in patients with obstructive jaundice has been reported[50]. However, whether the reduced intestinal luminal levels of bile acids that may be found in cirrhotic patients, as a consequence of reduced hepatic secretion and luminal deconjugation by overgrowth bacteria[12], contribute importantly to the potential for increased bacterial translocation in this group has not been established.

Intestinal immunity Little is known concerning the functional capacity of intestinal immunity in patients with cirrhosis and whether any disturbance of intestinal immune mechanisms contributes importantly to bacterial translocation, especially in the clinical setting. An increased number of intraepithelial lymphocytes with markedly impaired proliferative activity and capacity for production of interferon-γ have been reported in a murine model, with these changes correlating with increased bacterial translocation[51]. Whether this mechanism is important in the clinical situation is unknown. Notably, modest increases in small intestinal intraepithelial lymphocyte counts are found in patients with small intestinal bacterial overgrowth[52], raising the possibility that the increased levels found in cirrhotic animals may be explained on this basis. Whether the number or function of dendritic cells and other mononuclear cells in intestinal mucosa are deranged and contribute to the pathogenesis of bacterial translocation in patients with cirrhosis remains to be defined.

Anatomical site of bacterial translocation in cirrhosis

The anatomical site(s) from which bacterial translocation in cirrhosis occurs remains to be determined. Data in the non-cirrhotic setting suggest that the small intestine rather than the colon may be the major site of bacterial translocation. In particular, experimental studies involving inoculation of various regions of the gastrointestinal tract with equal quantities of *Escherichia coli* suggest that translocation occurs preferentially from the small bowel rather than the colon[53]. Similarly, in experimental animals with impaired intestinal motility, treatment with the prokinetic agent, cisapride, is associated with a reduced rate of bacterial translocation that parallels a reduction in jejunal but not caecal bacterial counts[3].

Intestinal permeability and its relation to bacterial translocation in cirrhosis

Functional studies have demonstrated that increased intestinal permeability, as reflected by dual sugar absorption tests or absorption of other test substances, occurs in patients with cirrhosis, especially in those with advanced liver disease[54-58]. Factors predisposing to this increased permeability in cirrhosis remain to be defined. In particular, any relationships with the structural changes of intestinal mucosa described above are uncertain. Enhanced intestinal permeability, likely via the paracellular route, has been reported in association with small intestinal bacterial overgrowth with Gram-negative gut flora in the non-cirrhotic setting[52], raising the possibility that this entity may at least contribute to the increased intestinal permeability in patients with cirrhosis. The relationship between intestinal permeability and the potential for bacterial translocation is uncertain, as increased permeability results have been found to correlate with a history of septic complications in some, but not all, reports.

GUT FLORA AND BACTERIAL INFECTION IN CIRRHOSIS

Bacterial infection, especially with intestinal-type flora, is a common complication in patients with cirrhosis[59-62]. Spontaneous bacterial peritonitis (SBP), urinary tract infection, respiratory tract sepsis (pneumonia and spontaneous bacterial empyema) and bacteraemia are the most frequent infective complications occurring in this group. The incidence of infection with Gram-positive cocci, in particular, has increased in recent years, with such flora now the most frequent isolates in hospitalised cirrhotic patients with nosocomial infection, especially those admitted to intensive care units, presumably due to the high rate of invasive procedures, including placement of indwelling vascular and bladder catheters, performed in this group[62]. Recent data suggest that between 15% and 35% of cirrhotic patients admitted to hospital develop nosocomial bacterial infection, substantially higher than the rate of hospital-acquired infection in the order of 5% to 7% in the general hospital setting[63].

Complicating infection may have severe adverse clinical consequences in cirrhotic patients. The associated pro-inflammatory response exacerbates hepatic dysfunction, encephalopathy and the haemodynamic disturbances that underlie the development of portal hypertension and hepatorenal syndrome[60,64,65]. Increasing evidence suggests that bacterial infection is a trigger for variceal haemorrhage in patients with cirrhosis[66,67], possibly as a consequence of both activation of hepatic stellate cells, leading to increased intrahepatic vascular resistance, and prostacyclin-related inhibition of platelet aggregation[66]. Conversely, variceal haemorrhage predisposes cirrhotic patients to bacterial infection with gut-derived flora[68-73], setting up a vicious cycle between gastrointestinal bleeding and infection in this group. This is a phenomenon of substantial clinical importance, as complicating infection is independently associated with early mortality in bleeding cirrhotic patients[69,70,72]. Worsening coagulopathy, due to the consumption of clotting factors by the extrinsic coagulation pathway[74] and the production of endogenous heparinoids[75], occurs in patients with cirrhosis and complicating bacterial infection. Sepsis is a common cause of death in cirrhotic patients[60,61,76-78]. The mortality rate associated with bacterial infection in this group is more than twenty times higher than in the general population[79].

SPONTANEOUS BACTERIAL PERITONITIS(SBP)

SBP, an infection of ascitic fluid typically with a single bacterial species in the absence of any other primary intra-abdominal source, is the most characteristic and serious infection occurring in patients with cirrhosis. Prospective studies suggest that SBP, including culture-negative and non-neutrocytic cases, is present in up to 23% of all cirrhotic patients with ascites admitted to hospital[80]. Gram-negative gut flora, especially *Escherichia coli* and *Klebsiella* species, are isolated in approximately 70% of culture-positive cases of community-acquired SBP. Aerobic Gram-positive bacteria belonging to *Streptococcus* and *Staphylococcus* spp constitute most of the remaining isolates. Pathogens belonging to *Aeromonas, Plesimonas, Listeria, Salmonella* and *Neisseria* spp are occasionally responsible. In keeping with their reduced potential for translocation from the intestine discussed above, obligate anaerobes are isolated in fewer than 5% of cases[81]. In hospitalised cirrhotic patients with nosocomial SBP, Gram-positive pathogens are predominant, accounting for over 70% of isolates, with methicillin-resistant *Staphylococcus aureus* accounting for nearly 25% of cases in one recent series[82].

The overall likelihood of a cirrhotic patient with ascites developing SBP at one year is in the order of 10%[80]. Clinical studies have identified several sub-groups of patients with cirrhotic ascites at particularly high risk for SBP. Over 70% of cases occur in those classified as Child-Pugh C[83]. An increased prevalence of SBP has been reported in patients with small intestinal bacterial overgrowth compared to their non-overgrowth counterparts, presumably due to the increased propensity for bacterial translocation[84]. Gastrointestinal haemorrhage is another important precipitant of SBP in this group. Bacteraemia and/or SBP, typically with Gram-negative enteric-type flora, develop within 48 hours of gastrointestinal haemorrhage in nearly 50% of Child-Pugh C patients with ascites[85]. The likelihood of SBP is related to the functional activity of Kupffer cells, which is impaired in patients with advanced liver disease[86]. However, the most powerful predictive factor identified in several series is an ascitic fluid total protein level $\leqslant$10 g/L, which reflects a low complement protein C3 concentration and, hence, opsonisation capacity. Patients with such low levels are at a six- to ten-fold increased risk of a first episode of SBP compared to cirrhotic counterparts with ascitic fluid total protein levels > 10 g/L[87,88].

CLINICAL COURSE

Despite a trend towards earlier diagnosis and commencement of antibiotic treatment, along with overall improvements in the general medical care of cirrhotic patients, the in-hospital mortality rate associated with SBP remains 30-50%[80]. This is largely related to underlying hepatic decompensation and a high prevalence of complicating hepatorenal syndrome. The latter occurs in approximately 30% of patients with SBP, predominantly in those with pre-existing renal impairment, and is progressive despite cure of infection in half of these cases[89]. Ascitic levels of nitric oxide, which are significantly increased in cirrhotic patients with SBP and persist for over two weeks despite appropriate antibiotic treatment[90,91], independently predict renal impairment in this setting[92]. Use of nephrotoxic antibiotics adds to the risk of renal failure in this setting[93]. The development of renal failure is the most important predictor of in-hospital mortality associated with SBP. This is in the order of 50% in those with complicating renal failure compared to only 6% in those without[89]. Albumin infusion improves systemic haemodynamics, prevents renal failure and improves survival compared to antibiotic treatment alone in patients with SBP. Beneficial effects are related to reduction in arterial vasodilatation and improved cardiac function[94]. Other correlates of poor outcome include increased ascitic levels of the pro-inflammatory cytokines, interleukin-6 and tumor necrosis factor-α (TNF-α)[95]. A recent report suggests a higher mortality rate in patients with nosocomial infection with staphylococcal species compared to that in patients with community-acquired SBP[82]. A trend towards a higher mortality rate when infection is with encapsulated strains of *Escherichia coli* associated with tissue invasiveness has also been reported[96].

The medium term prognosis for cirrhotic patients who have recovered from an episode of SBP is similarly poor, with a mortality rate at one year in the order of 30-80%[80]. Approximately 20% of patients who die within one year of an episode of SBP succumb to a further episode of spontaneous peritoneal infection, the remainder dying of causes, such as variceal haemorrhage, hepatorenal syndrome or hepatocellular carcinoma[97-99]. The risk of SBP recurrence within one year ranges from 40-70% and, as for an initial episode, is influenced mainly by the degree of underlying liver dysfunction and the ascitic fluid total protein level[83,98,99]. Survivors of an episode of SBP should be evaluated for liver transplantation in view of the high risk of recurrence and poor overall prognosis.

PROPHYLAXIS

Antibiotic prophylaxis against SBP may be beneficial in three high risk groups of cirrhotic patients, namely those who have survived a previous episode, those with low ascitic fluid total protein levels and those presenting with gastrointestinal haemorrhage[67-71,73,100-103]. Most prophylaxis studies have aimed to reduce or eradicate aerobic Gram-negative bacilli from the intestine using norfloxacin, a poorly absorbed quinolone with activity against these flora. The importance of antibiotic prophylaxis in patients with cirrhosis presenting with variceal haemorrhage is increasingly recognised. Treatment with norfloxacin, 400 mg twice daily either orally or via a nasogastric tube for seven days commencing immediately after emergency endoscopy, was associated with a significantly reduced in-hospital incidence of bacteraemia and/or SBP, especially with aerobic Gram-negative flora, compared to patients receiving no prophylactic antibiotics (3% *vs* 17%)[68]. Ciprofloxacin, 500 mg twice daily either orally or via nasogastric tube for 7 d immediately following endoscopy, has similarly been shown to significantly reduce the incidences of bacteraemia, SBP and urinary tract infection compared to placebo (0% *vs* 23%, 3% *vs*

13% and 5% *vs* 18%, respectively)[103]. Implementation of treatment protocols that incorporate antibiotic prophylaxis led to a fall in post-variceal bacterial infection rates from 38% to 14% over the past two decades in a large French centre[69]. Antibiotic prophylaxis has been shown to reduce the incidence of not only bacterial infection, but also early re-bleeding following variceal haemorrhage, especially in Child-Pugh class C patients, those requiring ventilatory support and those initially treated with balloon tamponade[67,70,71,73].

Several cost analysis studies have shown that prophylactic treatment with norfloxacin in each of these high risk groups is cost effective, as a consequence of the reduced incidence of SBP and its associated resource utilisation[67,104,105]. Short-term prophylaxis with trimethoprim-sulphamethoxazole is also cost-effective, especially in those at high risk for SBP[105]. Most studies report a reduction in overall mortality associated with prophylactic antibiotic treatment, although a statistically significant survival benefit has been more difficult to demonstrate. This is not surprising, as the likelihood of dying as a result of progressive liver failure, variceal haemorrhage, hepatocellular carcinoma or other causes is unaffected by the use of prophylactic antibiotics. More recently, a meta-analysis of 13 randomized controlled trials has suggested that antibiotic prophylaxis of hospitalized cirrhotic in-patients is efficacious in reducing the relative risk of in-hospital death (relative risk of dying 0.70), irrespective of underlying risk factors[107]. In addition, a meta-analysis of 534 cirrhotic patients with gastrointestinal bleeding has shown that short-term antibiotic prophylaxis significantly increases short-term survival rates by a mean 9% in this setting[107].

A particular concern, especially with long-term antibiotic prophylaxis, is the potential for the development of infection with antibiotic-resistant bacteria. Initial studies of norfloxacin prophylaxis against SBP reported a low incidence of infection with quinolone-resistant Gram-negative bacterial species. More recent studies, however, suggest an increased prevalence of infection with such microorganisms. Over 20% of cases of SBP due to *Escherichia coli* were with a norfloxacin-resistant strain in a recent series from Spain[108], while 50% of cases of culture-positive SBP in patients receiving long-term norfloxacin prophylaxis and 16% of instances in patients not treated with this drug were caused by norfloxacin-resistant Gram-negative bacteria in another Spanish report[62]. A high rate of SBP due to trimethoprim-sulphamethoxazole-resistant Gram-negative flora in excess of 40% was also documented in norfloxacin-treated patients. An increased proportion of infections with Gram-positive bacterial species has also been reported in this setting, including instances of severe hospital-acquired staphylococcal infections[109]. An increased incidence of carriage of methicillin-resistant *Staphylococcus aureus* has also been documented in cirrhotic patients treated with norfloxacin prophylaxis[110].

Such experiences highlight the need for non-antibiotic-based strategies to prevent intestinal bacterial overgrowth, bacterial translocation and SBP in patients with cirrhosis. As alluded to earlier, a six-month trial of the pro-motility agent, cisapride, was associated with improved small intestinal motility, reversal of bacterial overgrowth and a tendency towards a reduced incidence of infection with gut-derived flora in a cohort of cirrhotic patients[3,42]. Unfortunately, cisapride has been withdrawn from use in some countries because of potential for cardiac arrhythmia. Alternatively, treatment with either symbiotics (in the form of four species of lactic acid bacteria and fermentable fibre) or fermentable fibre alone has recently been shown to significantly reduce viable counts of potentially pathogenic Gram-positive and Gram-negative gut flora in patients with cirrhosis[19]. In another study, a lower rate of post-operative bacterial infection was documented in liver transplant recipients treated with an early enteral supply of a synbiotic regimen that included *Lactobacillus plantarum* and fermentable fibre than in patients receiving selective intestinal decontamination[111]. A follow-up randomised double-blind trial in liver transplant recipients showed that early enteral nutrition supplemented with a mixture of lactic acid bacteria and fermentable fibre significantly reduced the incidence of post-operative bacterial infection compared to supplementation with fermentable fibre alone[112]. Based on these reports, the possible use of synbiotics for prophylaxis against SBP in cirrhotic patients warrants investigation. Conversely, probiotic treatment with a lactobacillus strain without additional fermentable fibre was ineffective in preventing bacterial translocation and SBP in rats with experimental cirrhosis, despite successful intestinal colonisation[113].

GUT FLORA AND PRO-INFLAMMATORY STATE OF CIRRHOSIS IN ABSENCE OF OVERT INFECTION

Pro-inflammatory cytokines, such as TNF-α, have been shown to be critically involved in the development and/or exacerbation of liver injury in animal models[115,116]. Activation of macrophages by endotoxin, a cell wall component of Gram-negative bacteria, plays a key role in the over-production of TNF- α and liver injury in these settings[117,118]. Increased translocation from the gut lumen and reduced hepatic clearance have each been proposed to predispose to endotoxaemia in this situation[119,120]. In the clinical setting, increased circulating levels of both endotoxin and pro-inflammatory cytokines have been documented in patients with chronic liver disease, even in the absence of overt infection[120-125]. Based on experiences in experimental animals, it has, until recently, been assumed that endotoxaemia causes the raised pro-inflammatory cytokine levels in this group[121,126-128]. However, a significant correlation between circulating endotoxin and pro-inflammatory cytokine levels has generally not been show n[18,120,121,124,126], raising the possibility that, unlike in animal models, stimuli other than endotoxin may be important.

Recent analyses of the expression of Toll-like receptors (TLRs) in patients with cirrhosis have helped to clarify this issue. These receptors are the human homologues of the Drosophila Toll protein and comprise a family of at least ten transmembrane receptor proteins, the intracellular domains of which show distinct sequence homology with the interleukin-1 receptor. TLRs play a critical role in the

induction of innate immunity to microbial pathogens via recognition of conserved molecular patterns. In particular, it is known that TLR4, in association with CD14 and MD-2, is responsible for signal transduction leading to TNF-α production in response to endotoxin. In contrast, TLR2 is required for signaling in response to a number of Gram-positive microbial stimuli, including whole bacteria and cell wall components, such as peptidoglycan and lipoteichoic acid[129-131]. Indeed, significantly increased expression on peripheral blood mononuclear cells (PBMCs) of TLR2 but not of TLR4 has recently been demonstrated in patients with cirrhosis but no overt infection[18]. Furthermore, PBMC expression of TLR2 but not that of TLR4 was shown to correlate significantly with circulating levels of both TNF-α and anti-inflammatory soluble TNF receptors[18]. These novel findings suggest that signaling via TLR2 but not TLR4 contributes to the increased circulating levels of TNF-α levels found in cirrhosis and imply, contrary to previous assumptions, an important role for Gram-positive microbial stimuli rather than endotoxin in this process. This contention is supported by *in vitro* PBMC stimulation data consistent with presensitisation by Gram-positive microbial stimuli but not endotoxin *in vivo*. In addition, supplementation of cirrhotic patients with a synbiotic regimen including four Gram-positive gut flora and fermentable fibre led to further increases in PBMC expression of TLR2 and circulating TNF-α levels in most cases, suggesting that Gram-positive stimuli derived from the gut, in particular, may be important in promoting the increased circulating levels of both pro-inflammatory and anti-inflammatory TNF-related molecules found in patients with cirrhosis[18].

GUT FLORA AND HEPATIC FUNCTION IN CIRRHOSIS

Reversal of minimal hepatic encephalopathy has been reported in 50% of predominantly Child-Pugh class B or C cirrhotic patients treated with a lactic acid bacteria-based synbiotic regimen for 30 d, an efficacy comparable to that of treatment with lactulose[19]. In addition, the Child-Pugh class improves in nearly 50% of initially Child-Pugh class B or C synbiotic-treated patients, a proportion significantly higher than that in placebo-treated counterparts (8%). Treatment is associated with a significant increase in the faecal content of lactobacilli at the expense of potentially pathogenic Gram-positive and negative bacterial species. Bacterial overgrowth is also reversed and improvements in minimal hepatic encephalopathy and the Child-Pugh class occurs in 50% and 29%, respectively, of cirrhotic patients treated with fermentable fibre alone. Both in synbiotic- and fermentable fibre alone-treated patients, improvement in the Child-Pugh class occurs as a result of significant improvements in the serum bilirubin and albumin levels and in prothrombin activity. Significantly reduced hepatic necro-inflammatory activity, as reflected by serial ALT levels, is documented in both groups. Improvement in clearance of indocyanine green has also been reported in cirrhotic patients treated with synbiotics[132]. These findings are in keeping with reported experiences in experimental animals' suggesting that oral supplementation with Gram-positive probiotics can protect against hepatocellular damage. In particular, rats fed lactobacilli have been shown to be protected against alcohol-induced liver damage[133], while studies of probiotic use in a murine model of non-alcoholic fatty liver disease have found reductions in the intra-hepatic expression of various molecular markers of inflammation, including nuclear factor kappa-B and TNF-α[134,135].

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EDITORIAL

Hepatobiliary and pancreatic disorders in celiac disease

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Abstract

A variety of hepatic and biliary tract disorders may complicate the clinical course of celiac disease. Some of these have been hypothesized to share common genetic factors or have a common immunopathogenesis, such as primary biliary cirrhosis, primary sclerosing cholangitis and autoimmune forms of hepatitis or cholangitis. Other hepatic changes in celiac disease may be associated with malnutrition resulting from impaired nutrient absorption, including hepatic steatosis. In addition, celiac disease may be associated with rare hepatic complications, such as hepatic T-cell lymphoma. Finally, pancreatic exocrine function may be impaired in celiac disease and represent a cause of treatment failure.

Key words: Celiac disease; Liver disease; Cholangitis; Autoimmune hepatitis; Hepatic vein obstruction; Primary biliary cirrhosis

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INTRODUCTION

A number of hepatobiliary and pancreatic disorders occur in celiac disease, a genetically-based small intestinal disorder that resolves with the complete restriction of dietary gluten[1]. Almost 3 decades ago, liver changes in celiac disease were first recognized by Hagander *et al*[2]. Later, Dickey *et al*[3] have confirmed these findings in a prospective evaluation of celiac disease patients and extended observations to results of gluten-free diet therapy. In some, these liver test changes are entirely reversible following administration of a gluten-free diet, while in others, clinically significant liver disease is not amenable to diet treatment alone[3]. Now, almost a decade after this report, recognition of celiac disease has been substantively improved, in part, a result of more modern serological assays for screening[4], the detection of tissue transglutaminase (tTG) as an autoantigen in celiac disease[5] and the increasingly widespread serological use of tTG ELISA to screen for celiac disease[6]. As a result of improved recognition of celiac disease, even more precise estimates of the overall disease burden related to hepatobiliary tract and pancreatic disorders will emerge.

In patients with unexplained elevations of liver enzymes, several studies using serological screening methods have estimated that almost 10% will prove to have celiac disease[7,8]. For example, Volta *et al*[7] examined endomysial and gliadin antibodies in 55 patients with elevations of liver chemistry tests in the absence of a known cause. Five patients had positive serological studies and small intestinal biopsies showed changes of celiac disease that responded to a gluten-free diet. Liver biopsies done in some patients showed a nonspecific inflammatory process and liver chemistry tests normalized with a gluten-free diet. Bardella *et al*[8] screened 140 patients with chronically elevated transaminase values for gliadin and endomysial antibodies; of these, 13 were seropositive. After 1 year on a gluten-free diet, 12 patients had normalization of liver enzyme tests.

In patients with known celiac disease, abnormal liver enzyme tests also occur[2,9-11]. Hagander *et al*[2] described elevated liver enzymes in 30 of 75 (40%) patients, while Bonamico *et al*[9] showed increased levels in 39 of 65 (60%) children, and Jacobsen *et al*[10] documented almost 50% with increased liver enzymes. In some, liver biopsy showed a nonspecific inflammatory process, although a more specific "chronic active hepatitis" was detected in 5 of 37 (13.5%) patients. Bardella *et al*[11] evaluated 158 consecutive adults with celiac disease and showed that 42% had abnormal liver enzyme levels. A gluten-free diet for 1 to 10 years resulted in complete normalization of liver chemistry tests in 95% patients.

In celiac disease, persistently abnormal liver chemistry tests may reflect the presence of a clinically occult hepatobiliary tract disorder with a possibly common immunopathogenesis. Specific examples of immune-mediated disorders include primary biliary cirrhosis, primary (lymphocytic, autoimmune) sclerosing cholangitis or autoimmune hepatitis. Alternatively, in some, a common genetically-based disorder, including altered control of small intestinal iron absorption resulting in a concomitant iron overload disorder, may be present, such as hemochromatosis. In addition, chronic changes

in liver chemistry tests may reflect a direct effect of the celiac disease. For example, impaired absorption and resultant malnutrition may lead to deposition of fat in the liver, related, in part, to reduced fat mobilization from hepatocytes. Indeed, massive hepatic steatosis has occasionally been reported in celiac disease. Finally, but very rarely, patients may develop a specific complication of celiac disease that involves the liver, such as a T-cell form of lymphoma.

HEPATOBILIARY TRACT DISEASES

Primary biliary cirrhosis

In 1978, Logan *et al*[12] described the first cases of primary biliary cirrhosis with celiac disease. Later, numerous additional cases have been reported[13-16]. In both disorders, other conditions having an immunological basis have been described, including diabetes and thyroiditis[16-19]. In addition, co-existence of primary biliary cirrhosis and celiac disease has not only been reported in Europe and the Americas, but also in migrants from South Asia[20] and the Coast Salish, an aboriginal population inhabiting the west coast of Canada thought to be of Asian descent[16]. To date, however, a definitive genetic predisposition or specific immunological alteration has not been clearly identified. Loss of weight, malabsorption, osteopenic bone disease, steatorrhea and elevated alkaline phosphatase activities are common features of both diseases, so that early in their coexistence, celiac disease or primary biliary cirrhosis may not be easily appreciated. In patients reported with both disorders, regardless of geographical origin or race, restriction of dietary gluten may have improved the diarrhea, but abnormal liver chemistry tests were usually not significantly altered with a gluten-free diet.

Some more recent studies have explored serological testing in primary biliary cirrhosis or celiac disease. Kingham and Parker[21] used a patient registry in the United Kingdom and defined the prevalence of primary biliary cirrhosis in 143 celiac patients as 3%, while the prevalence of celiac disease in 67 primary biliary cirrhosis patients was 6%. As a result, screening with antimitochrondrial antibodies in celiac disease was recommended, while in primary biliary cirrhosis, serological screening with gliadin antibodies or small intestinal biopsy was suggested. Dickey *et al*[22] found similar findings of 7% (4/57) primary biliary cirrhosis patients based on initial evaluation using endomysial antibodies (11% positive), followed by later duodenal biopsy confirmation. Despite 12 to 24 mo on gluten-free diets, however, improvement in liver chemistry tests was not detected even though endomysial antibodies disappeared. Using Danish and Swedish registry data based on over 8 000 patients with celiac disease, Sorensen *et al*[23] also suggested an increased risk of primary biliary cirrhosis. Using stored sera from 378 Canadian patients with primary biliary cirrhosis, Gillett *et al*[24] found that screening for IgA antibodies to endomysium and primary biliary cirrhosis were both positive in 10 (2.6%) patients and 5 patients had small intestinal biopsies confirming celiac disease. Intertestingly, however, another 44 primary biliary cirrhosis patients had raised IgA tissue transglutaminase antibodies but were negative for IgA endomysium antibody. In 255 patients with autoimmune cholestatic liver disorders, including 173 with primary biliary cirrhosis, Volta *et al*[25] found 9 with celiac disease (including 7 in those with primary biliary cirrhosis, 4%). In some recent studies, however, the importance of biopsy confirmation in patients with primary biliary cirrhosis has been demonstrated in sero-positive patients as false-positive IgA or IgG tTG antibodies may occur in primary biliary cirrhosis[26,27].

In a recent study using a general practice longitudinal database from the United Kingdom[28], an overall 3-fold risk of primary biliary cirrhosis was demonstrated in 4 732 patients diagnosed with celiac disease as compared with 23 620 age- and sex-matched controls.

Primary sclerosing cholangitis

Primary sclerosing cholangitis was first found to be associated with celiac disease in 1988 in 3 patients with diarrhea and steatorrhea[29]. Two also had concomitant "ulcerative colitis" (one with "inactive" quiescent disease and one with "mild" or "minimal change" colonic disease), a disorder known to be associated with primary sclerosing cholangitis. Although hepatobiliary tract changes were defined by cholangiography and liver biopsy, these did not respond to a gluten-free diet. Later, other cases were reported[10,25,30]. In one, the predominant lymphocytic nature of the portal inflammatory process was emphasized with increased intra-epithelial lymphocytes in biliary ductal epithelium[30], an observation also noted in gastric and colonic epithelium of celiac patients[31,33]. To date, despite some case report data[33], it has been difficult to show good evidence for a response of the hepatobiliary tract disease to a gluten-free diet. This may, in part, reflect sampling difficulties associated with liver biopsy as well as the response or lack of response of relatively non-specific liver chemistry test markers of cholestasis (e.g., serum alkaline phosphatase). Indeed, the origin of alkaline phosphatase activities measured in serum include the hepatobiliary tract and other tissues that may be substantially altered in celiac disease (i.e., bone and the intestine); conceivably all might be improved with a gluten-free diet.

Autoimmune hepatitis and cholangitis

This has been evaluated in only a limited numbers of case reports and survey studies. Unfortunately, many appeared before hepatitis C testing[13,35]. Jacobsen *et al*[10] performed liver biopsies in 37 of 171 celiac patients and found changes of "chronic active hepatitis" in 5 (2.3%) patients. Using antibodies to endomysium and gliadin, Volta *et al*[36] surveyed 157 patients with type 1 autoimmune hepatitis and 24 with type 2 autoimmune hepatitis for celiac disease. They found that 8 of these 181 (4%) patients were positive for endomysial antibodies, including 6 (4%) with type 1 disease and 2 (8%) with type 2 disease. Five of the 8 patients had a duodenal biopsy, most being asymptomatic, and all showed changes of subtotal villous atrophy, consistent with untreated celiac disease. The effects of steroid with or without azothioprine treatment on the underlying small intestinal histological changes were considered and also may have masked intestinal symptoms. Unfortunately, in this study, the effects of gluten-free

diet administration on the hepatic and intestinal changes were not reported. Recently, Villalta *et al*[37] evaluated 47 consecutive patients with autoimmune hepatitis, including 39 with type 1 disease and 8 with type 2 disease. Anti-IgA tissue transglutaminase and endomysial antibodies were positive in 3 (6.4%) patients and small intestinal biopsies confirmed the presence of the celiac disease histological changes[37].

Finally, celiac disease and other types of autoimmune liver and biliary tract disease may coexist. A case report of autoimmune cholangitis[38], a cholestatic liver disorder with biochemical evidence of cholestasis, histological evidence of inflammatory bile duct damage and an absence of anti-mitochondrial antibodies, was has been described in a patient with celiac disease. Interestingly, this patient's small intestinal biopsies were reported to be normal without a gluten-free diet while being treated with steroids and azathioprine. In an another case, Sedlack *et al*[39] reported an improvement in hepatic biochemistries without use of immunosuppressive agents.

HEMOCHROMATOSIS OR IRON OVERLOAD LIVER DISEASE

Celiac disease has been associated with hemochromatosis, which is not surprising, since both are relatively common disorders based on a common Celtic ancestry, so any association could be coincidental[40-42]. Iron absorption largely occurs in the proximal duodenum, the site most often histologically altered in celiac disease. Indeed, "isolated" iron deficiency with anemia may be the initial clinical manifestation of clinically occult celiac disease. In contrast, in iron overload liver disease, inappropriate iron absorption from the proximal small intestine occurs as body iron stores are markedly increased. In one of these early case reports, treatment of celiac disease and improvement in the pathological small intestinal changes led to worsening liver chemistry test values and recognition of occult iron overload liver disease (C282Y-negative), presumably related to improved intestinal uptake of dietary iron[41]. Another similar case of C282Y-positive hemochromatosis presented with diarrhea, positive anti-gliadin and endomysial antibodies. Subsequent small bowel biopsies showed villous atrophy[42]. Interestingly, in this latter case, phlebotomy therapy had to be terminated early because of an unexpectedly rapid fall in the serum ferritin measurement. A genetically-based linkage was also suggested since both diseases are associated with the HLA-region on chromosome 6. Later investigations have sought to resolve this possible relationship. Butterworth *et al*[43] observed that HFE (hemochromatosis susceptibility gene) locus mutations are common in celiac disease patients from the United Kingdom and may be important in protecting the celiac from iron deficiency, while others suggested that the significance of these observations may be controversial[44]. More recent studies in an Italian population with untreated celiac disease found that HFE mutations failed to protect against the development of iron deficiency[45]. Interestingly, in a recent case study of a patient with homozygous C282Y and celiac disease[46], reduced expression of the divalent metal transporter 1 (DMT1) was observed, but not ferroportin 1 (FP1) or transferrin receptor 1 (TfR1).

OTHER LIVER DISORDERS IN CELIAC DISEASE

Hepatic steatosis

Common causes of hepatic steatosis include alcohol-induced steatosis, diabetes mellitus, NASH syndromes and some forms of drug therapy, including corticosteroids. In some countries, dietary protein deficiency and kwashiorkor are important causes. Intestinal malabsorption is often associated with hepatic steatosis in patients with a prior jejunoileal bypass procedure for morbid obesity[47,48] and, sometimes, in those with inflammatory bowel disease, particularly after extensive intestinal resections[49]. Because celiac disease is now frequently recognized in a clinically occult form before manifestations of marked nutrient depletion are detected, hepatic steatosis is probably less common than in other intestinal diseases.

Several cases of fatty infiltration of the liver, often massive, have been described in adults with celiac disease[50-53]. Presumably, lesser degrees of hepatic fat deposition may occur. Most often if massive steatosis is evident, elevated transaminase and alkaline phosphatase activities have been documented along with alterations in coagulation. However, in most, clinical and biochemical changes attributed to the hepatic steatosis were improved with a gluten-free diet. In a patient with massive hepatic steatosis[52], a gluten-free diet for about 1 year also resulted in histological improvement in the fatty changes detected in the liver.

The mechanisms involved in fat deposition in the liver are not defined. Interestingly, after jejunoileal bypass, reduced serum levels of some essential and nonessential amino acids may be observed[47,48]. In addition, changes in serum amino acids have been recorded in patients with starvation-associated kwashiorkor[54,55]. Based on these nutritional disorders, it has been suggested that malabsorption in celiac disease might lead to chronic deficiency of a lipotropic factor (e.g., choline), with an associated pyridoxine deficiency, hepatic steatosis might occur[52]. Further studies are needed to define the precise pathogenetic mechanism or mechanisms for fatty liver in celiac disease.

Gallstone disease

Several studies have focused on gallbladder function in celiac patients. In some studies, slow emptying of the gallbladder has been documented[56,57], along with impaired contraction response to fat[56]. Studies of enteric endocrine cells showed significant quantitative changes in celiac patients, including complete absence of mucosal secretin cells[59]. In addition, studies with test meals have suggested impaired secretion of cholecystokinin in patients with celiac disease[59] or, possibly, impaired gallbladder responsiveness to cholecystokinin[56].

In spite of these physiological alterations, there does not appear to be a significant predisposition to gallstones in celiac disease. Only 9 of 350 patients had a cholecystectomy for gallstone disease[60]. However, in a

survey of elderly celiacs initially diagnosed after the age of 60 years, 6 of 30 (20%) had gallstone disease[61].

Hepatic vein obstruction

Although mesenteric vascular ischemia[62] and vasculitis[63-66] have been described in celiac disease, there are also reports of a unusual Budd-Chiari-like syndrome among celiac children from North Africa, particularly Tunisian and Algeria[67,68]. Hepatic vein obstruction has also been documented in 3 adults[69]. Deficiencies in protein C and antithrombin III are detected, and malabsorption of vitamin K in celiac disease has been proposed to cause transient protein C or protein S deficiencies. Further studies are needed to identify possible factors, either dietary or environmental agents, that may be important. More recently, a celiac patient with a Budd-Chiari syndrome associated with membranous obstruction of the inferior vena cava treated successfully with percutaneous balloon angioplasty has been reported[70].

Hepatic malignancies

While hepatocellular cancer has been reported in 1 patient, cirrhosis was also present[71]. Occasionally, the liver may be involved with lymphoma, the most frequently detected malignant disorder in celiac disease patients[72]. In some patients with celiac disease, lymphomatous deposits have been detected in the liver, presumably as metastatic lesions[71]. For example, lymphoma in the liver is apparently secondary to jejunal lymphoma, complicating celiac disease[71]. In general, involvement of the liver in celiac disease patients with lymphoma is limited and overshadowed by the clinical course of the intestinal disease. However, a fulminant cholestatic syndrome has been described in a celiac disease patient, resulting in hepatic failure[73]. Later investigations have shown widespread hepatic involvement with an unusual lymphoid neoplasm classified as a hepatosplenic lymphoma, a rare type of peripheral T-cell lymphoma with rearrangement of the gamma-delta T-cell receptor[74,75].

Liver failure

In patients with severe liver failure from a variety of causes in celiac disease, dietary treatment reverses hepatic dysfunction, even in patients with consideration for possible liver transplantation[76].

PANCREATIC DISEASE

While celiac disease is associated with insulin-dependent diabetes[77], pancreatic exocrine insufficiency and celiac disease have only occasionally been recorded[78-84]. Pancreatic calcification is most often associated with chronic or persisting pancreatic inflammation which is usually due to excessive consumption of alcoholic beverages. Atrophy, fibrosis and altered pancreatic function have been observed in experimental animals treated with diets deficient in protein, in adults with protein-energy malnutrition, in children with kwashiorkor and in some early autopsy studies of patients with celiac disease. In addition, pancreatic calcification has been reported with chronic protein malnutrition in the Indian subcontinent and in some African countries. Finally, a patient with celiac disease and pancreatitis with calcification has been described[83].

Although the frequency of pancreatic disease in celiac patients is not known, impaired pancreatic function occurs and may be a cause of persistently impaired nutrient assimilation and malnutrition. It has been estimated that over 20% of children with celiac disease have defective exocrine pancreatic function[85]. This may be related to several factors. Impaired secretion and/or release of pancreatic stimulating hormones from the diseased proximal small intestine may be important[60]. Immunohistochemical studies have demonstrated alterations in enteric endocrine cells, and in biopsies from patients with untreated celiac disease, an absence of secretin cells has been reported[59]. Studies with test meals in celiac patients have suggested impaired secretion of cholecystokinin-pancreozymin resulting in reduced pancreatic exocrine cell stimulation[81]. In addition, a deficiency of amino acids may result from impaired small intestinal amino acid uptake, leading to a reduction in precursors for pancreatic enzyme synthesis[55,80]. Also, protein malnutrition may lead to structural changes in the pancreas, including atrophy of acinar cells and pancreatic fibrosis[55], resulting in impaired pancreatic exocrine function. In a more recent study[86], pancreatic enzyme measurements were reduced with mucosal atrophy and could be inversely correlated with the degree of intestinal damage.

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EDITORIAL

Exacerbation of inflammatory bowel disease by nonsteroidal anti-inflammatory drugs and cyclooxygenase-2 inhibitors: Fact or fiction?

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Abstract

The existence of a possible link between inflammatory bowel disease (IBD) and nonsteroidal anti-inflammatory drugs (NSAIDs) has been repeatedly suggested. Recently, a few studies have addressed the issue of a possible, similar effect by selective cyclooxygenase-2 inhibitors (COXIBs). The present article reviews the available scientific evidence for this controversial subject.

Key words: COX-2 inhibitor; Inflammatory bowel disease; Non-steroidal anti-inflammatory drugs

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The use of nonsteroidal anti-inflammatory drugs (NSAIDs) has been associated with the onset of inflammatory bowel disease (IBD) or with a clinical flare-up of IBD in a number of case reports[1]. Both rectal and colonic frank ulcerations[2], small bowel ulcers[3] and intestinal, diaphragm-like strictures[1,3,4] have been reported after prolonged NSAID intake. On the other hand, no relationship is reported between NSAID treatment and exacerbation of underlying IBD[5,6].

The absence of controlled, prospective trials makes it difficult to draw definitive conclusions. Uncontrolled clinical experience suggests that anti-inflammatory agents can occasionally elicit relapse of IBD[7] and therefore should be employed with caution in patients with either ulcerative colitis or Crohn's disease. A recent systematic review of the available medical literature concluded that the epidemiological evidence for a positive link between NSAID exposure and relapse of IBD is weak, while admitting that "some patients with IBD do relapse when given NSAIDs[8].

Given the inconsistency of the conflicting data concerning the relationship between NSAIDs and IBD, the possible effect of selective cyclooxygenase-2 inhibitors (COXIBs) in this respect remains even more controversial. In order to better understand the relationship between anti-inflammatory treatment and IBD it is necessary to consider the possible pathogenetic mechanisms involved in the adverse effects on the bowel by non-selective NSAIDs. Several mechanisms have been postulated, such as enhanced intestinal permeability[9], enterohepatic recirculation of NSAIDS and formation of drug enterocyte adducts , the latter phenomena having been observed in animal studies[9] but never demonstrated in humans.

The major mechanism involved, however, is thought to be the inhibition of colonic prostaglandin synthesis[10], in particular of the COX-2 isoform. In the inflamed colon COX-2 expression is upregulated in an effort to repair mucosal damage[11] and its inhibition may result in exacerbation of colonic injury and in impairment of the mucosal repair processes elicited by the COX-2 enzyme[12]. In this respect both NSAIDs and COX-2 inhibitors could hamper the progression of the inflammatory state toward healing. On the other hand, if COX-2 is important in the reparative mechanisms in IBD, then patients with quiescent disease should have a lower risk of flare-up when taking NSAIDs[13].

The studies on the effect of COX-2 inhibitors on animal models of colitis have yielded conflicting results[9,14] even taking in account the differences in experimental conditions, type and dosages of the employed compounds. The only available study on human colonic mucosa, carried out on colonic biopsies taken in IBD patients, found that a highly selective COX-2 inhibitor, L-745337 inhibits local release of PGE2 and PGI2 to the same extent as indomethacin, a nonselective NSAID[15], an effect which would likely promote aggravation of mucosal damage..

In a clinical setting a perspective, open-label study in IBD patients with associated arthropathy rofecoxib, administered at a dose of up to 25 mg daily for 20 d, failed to elicit any flare-up of the intestinal disease[16]. Similarly, a retrospective analysis of IBD patients treated with

either celecoxib or rofecoxib for periods ranging from one week to 22 mo[17]. apparently confirmed the safety of COX-2 inhibitors in this respect. By contrast, a clinical exacerbation of the underlying IBD that subsided after the drug was discontinued, has been reported in 19% of patients taking rofecoxib[18]. In keeping with this finding a recent retrospective study in IBD patients taking either celecoxib or rofecoxib has found clinical relapse of the intestinal disease in 39% of cases, again with resolution of symptoms after COX-2 inhibitor withdrawal[19].

On the other hand, the first multicenter, random, double-blind, placebo-controlled study performed in USA ,taking into consideration of both clinical and endoscopic parameters, has shown that celecoxib 200 mg bid for 2 wk is as safe as placebo in patients with ulcerative colitis in remission[20].

Thus, as with nonselective NSAIDs, the available data remain conflicting and confusing. Summing up, on theoretical ground both NSAIDs and COX-2 inhibitors appear capable of triggering a flare-up of IBD by inhibiting the intestinal production of prostaglandins involved in the tissue reparative processes. In clinical practice, although clear-cut evidence is difficult to obtain due to the variable incidence of IBD reactivation and the paucity of prospective, controlled studies, both types of anti-inflammatory agents may precipitate recurrence of intestinal symptoms and therefore should be avoided, when possible, in patients with ulcerative colitis or Crohn's disease.

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REVIEW

Role of radiation therapy in gastric adenocarcinoma

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Abstract

Outcomes in patients with gastric cancer in the United States remain disappointing, with a five-year overall survival rate of approximately 23%. Given high rates of local-regional control following surgery, a strong rationale exists for the use of adjuvant radiation therapy. Randomized trials have shown superior local control with adjuvant radiotherapy and improved overall survival with adjuvant chemoradiation. The benefit of adjuvant chemoradiation in patients who have undergone D2 lymph node dissection by an experienced surgeon is not known, and the benefit of adjuvant radiation therapy in addition to adjuvant chemotherapy continues to be defined.

In unresectable disease, chemoradiation allows long-term survival in a small number of patients and provides effective palliation. Most trials show a benefit to combined modality therapy compared to chemotherapy or radiation therapy alone.

The use of pre-operative, intra-operative, 3D conformal, and intensity modulated radiation therapy in gastric cancer is promising but requires further study.

The current article reviews the role of radiation therapy in the treatment of resectable and unresectable gastric carcinoma, focusing on current recommendations in the United States.

Key words: Radiation therapy; Gastric cancer; Stomach cancer; Chemoradiation; Adjuvant therapy; Neoadjuvant therapy; Intra-operative radiation therapy; 3D conformal radiation therapy; Intensity modulated radiation therapy

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EPIDEMIOLOGY

Worldwide, gastric cancer is the 5th the most common malignancy and the 2nd leading cause of cancer death. Significant geographic variation exists, with high risk areas including Japan, Central and South America, and Eastern Asia, and low risk areas including Kuwait, Israel, and the United States[1, 2]. Despite its relatively low incidence in the United States, gastric cancer is a significant cause of morbidity and mortality, with 22 000 cases per year, resulting in 13 000 deaths[3].

According to the Surveillance, Epidemiology, and End Results (SEER) database, 5-year survival rate for all gastric cancer patients treated in the United States from 1995 to 2001 was 23.2%. This represents an improvement from 15.3% in the time period from 1974-1976[4]. As expected, patients with localized disease have a higher 5-year survival rate (59%) compared to patients with regional (21.9%) or distant disease (3.1%)[4]. Unfortunately, only 25-40% of patients have localized disease at diagnosis.

Outcomes for patients in high risk countries such as Japan are generally better than outcomes in low incidence countries[2, 5]. This variation is likely due to earlier diagnosis (due to aggressive screening programs) and greater clinical experience in high risk countries, as well as differences in the etiology and biology of the tumors in high versus low risk countries[1,2]. The relative contributions of these factors to outcome and the implication for therapeutic decisions remains ill-defined.

Despite the poor prognosis for those patients with gastric cancer, progress has been made in their management. Improved surgical expertise, better systemic therapy, and the integration of radiation therapy have resulted in modest, but significant improvements in local control and survival. The current review article focuses on the role of radiation therapy in the management of gastric carcinoma in the United States.

PATTERNS OF FAILURE

Following surgical resection, both local and distant recurrences are common, indicating the importance of both local and systemic treatment. Patterns of failure have been reported in clinical series, re-operation series, and autopsy series. As expected, recurrences are greatest in autopsy series and least in clinical series, reflecting limitations in

Table 1 Patterns of relapse based on University of Minnesota re-operation series and autopsy series

	Univ. of Minnesota Re-operation series[10] (*n*=107)	Autopsy series[6-9]
Pattern of relapse	%	%
Gastric bed	55	52-68
Anastomosis	27	54-60
Abdominal wound	5	-
Peritoneal seeding	42	30-43
Lymph nodes	43[1]	52
Local-regional as any site	69	80-93
Local-regional only	23	-
Lymph Nodes only	7	-
Lymph nodes only in nodal dissection sites	3	-
Distant metastases	36	50

[1]Most common nodal sites of relapse were porta-hepatis, celiac, and para-aortic regions. Other sites were suprapancreatic, pancreaticoduodenal, and splenic hilar regions.

the ability to detect recurrences clinically. Patterns of recurrence based on re-operation and autopsy studies are summarized in Table 1.

In the University of Minnesota re-operation series gastrectomy patients underwent a "second look" procedure in the absence of known recurrence or a "symptomatic look" in the setting of known recurrence. Local-regional failure alone was detected in 23% of patients and local-regional failure as any component of failure was detected in 69% of patients. Autopsy series report even higher local-regional failure rates (80-93%)[6-9]. These findings suggest that improvements in local-regional control may be potentially curative and provide a strong rationale for adjuvant radiation therapy.

Distant metastases are reported in approximately 50% of patients by autopsy series[6, 7, 9]. The predominant site of distant organ failure is the liver, involved in 30% of patients at initial exploration[5]. Peritoneal seeding, considered distant metastatic disease, is also common, occurring in 42% of patients from the University of Minnesota re-operation series[10], and in 30-43% of patients based on autopsy series[6-9], Lung metastases are less common, observed in only 10% of cases in one autopsy series[5], These findings provide a strong rationale for adjuvant chemotherapy.

ADJUVANT THERAPY

Radiation alone

At least 2 randomized trials compared surgery alone to surgery plus radiation therapy in gastric carcinoma. These results are summarized in Table 2. The first trial, by the British Cancer Stomach Group, randomized patients to surgery alone, surgery followed by radiation therapy, or surgery followed by chemotherapy (mitomycin, adriamycin, and 5-FU). Of note, 40% of patients on this trial had gross or microscopic residual disease following surgery. No survival difference was detected, but the addition of radiation therapy to surgery resulted in a significant reduction in local recurrence (27% with surgery versus 10% with surgery plus radiation therapy)[11, 12]. Interestingly, 24% of patients on the radiation therapy arm did not receive any radiation, and 32% received a dose < 40.5 Gy (well below that required to control microscopic or gross disease). The benefit of adjuvant radiotherapy may be been greater if given to all patients and in adequate doses.

The second trial, by Zhang *et al* in Beijing, randomized 370 patients with gastric cardia carcinoma to neoadjuvant radiation therapy or surgery alone. Neoadjuvant radiotherapy resulted in improved survival (5-year overall survival was 30% *vs*. 20%, $P=0.009$), improved local control (61% *vs*. 48%, $P<0.025$), and improved regional nodal control (61% *vs*. 45%, $P<0.005$)[13]. Additionally, resection rates were higher in the neoadjuvant radiation arm (89.5%) compared to the surgery alone arm (79%, $P<0.01$). Operative mortality and morbidity did not differ between the two arms. While encouraging, this trial included only cardiac lesions, and it is unknown if these results can be generalized to include distal lesions.

While the British and Beijing trials yielded conflicting results regarding a survival advantage with radiation, both demonstrate significant improvement in local control. Local recurrence in gastric cancer has the potential for substantial morbidity, and thus local control alone is a valid endpoint in evaluating the value of neoadjuvant and adjuvant therapy, including radiation.

Chemoradiation

Three randomized trials have compared surgery alone to surgery plus adjuvant chemotherapy and radiation in gastric carcinoma. These trials are summarized in Table 2. The Mayo Clinic trial randomized 62 patients to surgery alone versus surgery plus adjuvant radiation therapy concurrent with 5-FU chemotherapy[14]. A significant improvement in overall survival and disease free survival was identified with the addition of chemoradiation when analyzed by intention to treat. The five-year survival rate for patients randomized to adjuvant chemotherapy and radiation was 23% compared to 4% for those randomized to no adjuvant therapy. When evaluated by actual treatment received, these benefits were no longer statistically significant. Local control was superior in the chemoradiation arm, although this difference did not reach statistical significance.

The trial by Walsh *et al* included primarily patients with adenocarcinoma of the esophagus, but 35% of patients had lesions involving the gastric cardia. Patients were randomized to surgery alone or neoadjuvant chemotherapy and radiotherapy followed by surgery. The addition of chemoradiation resulted in a statistically significant improvement in overall survival[15]. The median survival of patients assigned to preoperative chemoradiation therapy was 16 mo compared with 11 mo for those assigned to surgery alone ($P=0.01$). Three-year survival was 32% versus 6% favoring combined modality therapy ($P=0.01$).

The landmark trial in the combined modality treatment of gastric cancer in the U.S. is Intergroup 0116 study[16]. In this study, 556 patients with resected adenocarcinoma of the stomach or gastroesophageal junction (stage IB through IVM0 disease) were randomly assigned to surgery

Table 2 Randomized trials of adjuvant radiation therapy in gastric carcinoma

Reference	*n*	Survival			Local-regional relapse		
		Median (mo)	5-year (%)	*P* value	%	*P* value	
Mayo Clinic[14]							
Surgery alone		23	15	4	0.05	54	NS
Post-op EBRT + CT		39	24	23		39	
British stomach group[11, 12]							
Surgery alone		145	15	20	NS	27	<0.01 for
Post-op CT		138	13	19		19	
Post-op EBRT		153	17	12		10	EBRT
China-Beijing[13]							
Surgery alone		199	NR	20	0.009	52[1]	<0.025
Pre-op EBRT		171	NR	30		39	
Intergroup 0116[16]							
Surgery alone		275	27	41 (3 yr)	0.005	29	NR
Postop EBRT + CT		281	36	50 (3 yr)		19	
Walsh *et al*[15]							
Surgery alone		55	11	6 (3 yr)	0.01	NR	-
Preop EBRT + CT		58	16	32 (3 yr)			

Abbreviations: CT = chemotherapy, EBRT = external beam radiation therapy, NS = not significant, NR = not reported.

[1]Refers to local relapse. Regional relapse with 54% with surgery alone versus 39% with chemoradiation, $P < 0.05$.

plus postoperative chemoradiotherapy or surgery alone. The adjuvant treatment consisted of two cycles of 5-FU and leucovorin followed by two more cycles of chemotherapy concurrent with radiation therapy. Patients received 45 Gy of radiation in 25 fractions to the preoperative tumor volume, surgical bed, and regional lymph nodes. The majority of patients on this trial had advanced disease (66% were T3/4 and 85% were node positive) and surgical margins were required to be negative.

Three-year overall survival was improved from 41% to 50% with combined modality therapy ($P = 0.005$). The median survival was improved from 27 mo in the surgery only group to 36 mo in the chemoradiotherapy group ($P = 0.005$). Local regional relapse was decreased from 29% to 19% (*P* value not reported). Interestingly, distant metastases were higher in the chemoradiation arm (18% versus 33%), likely a result of patients living long enough to develop clinically detectable distant metastases.

Toxicity was significantly higher with chemoradiation, with nearly three-quarter of patients experiencing grade 3/4 toxicity, and 17% of patients were unable to complete radiation due to toxicity. However, treatment related mortality was low (1% on the chemoradiation arm versus 0% on the surgery alone arm) and overall chemoradiation appeared tolerable in light of its benefits. Postoperative chemoradiotherapy should be considered for all patients at high risk for recurrence of adenocarcinoma of the stomach who have undergone a potentially curative resection.

An important consideration in the interpretation of the Intergroup 0116 trial is the extent of surgery. At least 54% of patients received suboptimal lymph node dissection based on current National Comprehensive Care Network (NCCN) guidelines, raising the question of whether chemoradiation was simply compensating for inadequate surgery. The extent of lymph node dissection as it relates to outcome is discussed further below.

Extent of lymph node dissection and its impact on adjuvant therapy

A D1 lymph node dissection includes perigastric lymph nodes along the lesser and greater curvature. In addition to those nodes included in a D1 lymph node dissection, a D2 dissection includes lymph nodes along the left gastric artery, the common hepatic artery, the celiac trunk, the splenic hilum, and the splenic artery. A D3 lymph node dissection further includes lymph nodes along the hepatoduodenal ligament and the root of mesentery. A D4 lymph node dissection is a D3 dissection plus dissection of para-aortic and para-colic lymph nodes.

While D2 dissection (at a minimum) is considered standard in Japan, randomized Western trials have not shown a survival difference between D1 and D2 dissection, and have demonstrated a substantial increase in post-operative mortality (Table 3)[17, 18]. These trials have been criticized due to the unexpectedly high morbidity and mortality rates, which may be in part explained by the frequent use of pancreatectomy and splenectomy in patients undergoing D2 dissection (which is no longer considered necessary) and by surgeon inexperience[19-21].

In Japan, Maruyama *et al* reported an improvement in 5 year overall survival from 20.3% with D0 dissection to 41.2% with D1 dissection to 63.8% with D2 dissection[22]. Perhaps the greatest criticism of the Japanese data supporting extended lymph node dissection has been its retrospective and/or non-randomized nature. To address these criticisms, the Japanese recently completed a prospective, randomized trial comparing D2 versus D4 dissection. Although disease control data has not yet been reported, acute toxicity data show no difference.[21] Of note, D2 patients had in-hospital mortality rate of 0.8%, which is substantially lower than that reported in the MRC and Dutch trials.

The results of this trial will represent a tremendous contribution to the treatment of gastric cancer. Unfortuna-

Table 3 Randomized trials of D1 versus D2 lymph node dissection in gastric cancer

	MRC[18] (*n*=711)			Dutch[17] (*n*=400)		
Endpoint	D1	D2	*P* value	D1	D2	P value
5 year overall survival	45%	47%	NS	35%	33%	NS
Post-operative morbidity	25%	43%	<0.001	28%	46%	<0.001
Post-operative mortality	4%	10%	0.004	6.5%	13%	0.04

Abbreviations: NS = non-significant.

tely, it is unclear how and if the results of this trial can be applied to Western countries given significant differences in physician experience and patient populations compared to Japan. While surgeon training at tertiary centers in the United States may aid in addressing issues of surgeon experience, hospitals in the United States simply do not have the volume of gastric cancer patients seen in Japan, limiting the ability for surgeons to gain experience. Furthermore, patient related issues such as body mass index and age at diagnosis may differ by geographic location and may influence the risks and benefits of extensive nodal dissection[23].

Evaluation of the benefit of more extended nodal dissection is confounded by stage migration. Bunt *et al* reported that up to 75% of patients with stage IIB disease on D1 dissection are upstaged to IV on D2 dissection[24]. Therefore, when compared stage for stage, results with more extensive nodal dissection may appear superior.

Given these considerations, the optimal extent of lymph node dissection remains elusive. Per NCCN guidelines in the United States, at a minimum a D1 dissection is recommended. Furthermore, it is recommended that at least 15 lymph nodes be evaluated.

On the Intergroup 0116 trial, 10% of patients underwent D2 dissection, 36% underwent D1, and 54% underwent less than a D1 dissection[16]. Subset analysis did not detect differing effects of adjuvant treatment based on the type of lymph node dissection performed (adjuvant treatment appeared to be of benefit regardless of type of lymph node dissection)[25]. This observation is difficult to interpret based on the small number of patients actually treated with D2 dissection. The role of post-operative chemoradiation in patients who undergo D2 dissection by an experienced surgeon is, therefore, unknown.

Lim *et al* reported the outcome of 291 patients treated in Korea with D2 dissection and post-operative chemoradiation identical to that delivered on the Intergroup 0116 trial[26]. 5-year overall survival and local-regional failure were superior to that observed on the chemoradiation arm of the Intergroup 0116 trial, but it is unknown if these outcomes were related primarily to the use of D2 dissection, chemoradiation, or both. It is also unknown if the outcomes were related to surgeon experience and other confounding variables in Korea compared to the United States. The value of chemoradiation in patients who have undergone D2 dissection by an experienced surgeon can only be fully answered by a prospective, randomized trial.

Chemotherapy alone

While the randomized Intergroup 0116 trial showed a benefit in both local control and overall survival with adjuvant chemoradiation, the relative contributions of radiation versus chemotherapy to this benefits are unknown.

The aforementioned British Stomach Group trial, which randomized patients to surgery alone, surgery followed by radiation therapy, or surgery followed by chemotherapy, found no improvement in overall survival with chemotherapy compared to surgery alone or surgery and radiation[11, 12]. A randomized Southwest Oncology Group (SWOG) trial showed no benefit to adjuvant chemotherapy (5-FU, adriamycin, and mitomycin-c) following surgical resection[29]. To date, meta-analyses evaluating the role of adjuvant chemotherapy have demonstrated no or minimal benefit[27, 28].

More recently, however, a British MRC randomized trial (the MAGIC trial) showed an improvement in both overall survival and disease free survival with the addition of pre- and post-operative chemotherapy (epirubicin, cisplatin, and 5-FU) to surgery[30, 31]. Five-year overall survival was improved from 23% to 36% ($P=0.009$). This trial suggests a survival benefit with adjuvant chemotherapy in the absence of radiation therapy. Newer chemotherapeutic regimens may be superior and therefore provide a greater benefit compared to epirubicin, cisplatin, and 5-FU[30, 32-36].

While the results of the MAGIC trial demonstrate a survival advantage to adjuvant chemotherapy in the absence of radiation therapy, only an adequately powered, prospective randomized trial of chemotherapy with or without radiation therapy can fully evaluate the benefit of radiation treatment, and to date no such trial exists. A recent Korean trial treated 81 patients with resection (including D2 dissection) followed by chemotherapy alone (FU, cisplatin) versus chemotherapy (FU, cisplatin) before and after radiation concurrent with capecitabine. With short follow-up (15 mo) there is no difference in disease free or overall survival. Further follow-up is necessary to confirm these results, and radiation therapy should not be omitted on the basis of this trial alone[37].

Currently in the United States, NCCN guidelines recommend adjuvant chemoradiation following complete surgical resection in T3, T4, or node positive gastric cancer patients or in patients with microscopically positive margins. Adjuvant chemotherapy alone or adjuvant radiation therapy alone are considered investigational.

Pre-operative versus post-operative adjuvant radiation therapy

Based on the Intergroup 0116 trial, chemoradiation in the United States is most commonly delivered post-operatively. However, pre-operative radiation therapy offers a number

Table 4 Prospective trials of pre-operative chemoradiation in gastric cancer

Reference	Chemo	RT dose (Gy)	Overall survival % (2 yr)	Median survival time (mo)	Complete response (%)	Partial response (%)	R0 resection (%)	% Distant metastases during therapy	rate (%)
RTOG 99-04[46] (n=49)	5FU/LV/cis×2 prior to RT; 5FU with RT	45	NR	NR	27	NR	77	NR	
Roth[45] (n=19)	Cis/5FU	31.2, 38.4, 45.6[1]	71	NR	5	50	NR	0	
Lowy[41] (n=23)	5FU	45	NR	NR	11	63	NR	17	
Ajani 2004[43] (n=33)	5FU/LV/cis×2 prior to RT; 5FU with RT	45	54	33.7	30	24	NR	12	
Ajani 2005[44] (n=41)	5FU/cis/tax×2 prior to RT; 5FU/tax with RT	45	68[2]	Not reached	20	15[3]	78	0	
Balandraud[47] (n=42)	5FU/cis	45	45.6	23	14	-	81	-	
Klautke[67] (n=21)	Cis/5FU or tax	50.4	42	18	14	62	53	-	

Abbreviations: Cis = cisplatin, 5FU = 5-fluorouracil, LV = leucovorin, tax = paclitaxol, RT = radiation therapy, R0 = resection with negative margins.
[1]Given 1.2 Gy b.i.d. in a phase I dose escalation trial; [2]At a median follow-up of 36 mo; [3]PR defined as <10% residual cells in resected specimen.

of theoretical advantages. First, target tissues are better oxygenated prior to surgery, and radiation therapy is more effective against oxygenated tissues[38]. Second, pre-operative therapy may enhance the ability to perform R0 resection (defined as removal of all tumor such that surgical margins are histologically negative). The MAGIC trial demonstrated an improvement in potentially curative resection rates from 60% with surgery alone to 70% with pre-operative chemotherapy[30], and the Beijing trial demonstrated an improvement in resection rates from 79% with surgery alone to 89.5% with pre-operative radiation therapy[13]. A phase II, multi-institutional trial by the Radiation Therapy Oncology Group (RTOG 99-04) of pre-operative chemoradiation demonstrated a pathologic complete response rate of 27% and an R0 resection rate of 77%, which compares well to historical controls[39, 40]. Table 4 summarizes trials evaluating pre-operative chemoradiation in the setting of gastric carcinoma.

Third, preoperative treatment is generally better tolerated than postoperative treatment and patients are more likely to receive the prescribed doses of chemotherapy and radiation. For example, 88% of patients treated on a phase II trial of pre-operative 5-FU and radiation therapy were able to complete full dose chemoradiation[41], compared to 65% on the Intergroup 0116 trial, which delivered radiation and chemotherapy post-operatively[42]. Furthermore, during pre-operative therapy, distant metastases may manifest clinically and therefore spare patients unwarranted surgery. Prospective trials have reported the development of distant metastatic disease in 12-17% of patients during pre-operative chemoradiation, despite the use of staging laparoscopy at diagnosis[41, 43].

Pre-operative radiation also has potential risks. Perhaps most notably, a significant proportion of patients are under-staged clinically, and based on clinical stage may undergo unnecessary irradiation. Bonenkemp *et al* report that 29% of eligible surgical patients were discovered at laparotomy to have peritoneal, hepatic, or distant lymph node metastases[17]. Careful evaluation including endoscopic ultrasound, CT scans, and laparoscopy is therefore indicated prior to initiation of radiation therapy.

While the potential exists for increased surgical morbidity and mortality with pre-operative treatment, trials of pre-operative chemoradiation have yielded conflicting results in this regard[41, 43-47]. The RTOG 99-04 reported a 21% grade 4 toxicity rate but no treatment-related deaths, demonstrating that pre-operative therapy can be delivered in a multi-institutional setting with acceptable toxicity[46].

The timing of chemoradiation can only be definitively answered through a prospective, randomized trial. An ongoing European trial (SWS-SAKK-43/99) is randomizing gastric cancer patients to pre- versus post-operative chemotherapy; however, this trial does not use radiation and will not address the optimal timing of radiation therapy.

THE ROLE OF RADIATION THERAPY IN UNRESECTABLE GASRTIC CARCINOMA

Only 25-40% of patients with gastric adenocarcinoma will be able to under go potentially curative surgical resection[48]. While the prognosis of these patients is poor, radiation therapy with or without chemotherapy occasionally results in long-term survival (5 year survivals of 5-15%). However, this is not a viable alternative to resection (and adjuvant therapy as indicated) in patients who have resectable disease (Table 5).

Gastric adenocarcinoma is a radioresponsive tumor.

Table 5 Treatment results: Unresectable or residual gastric cancer

Reference	*n*	Treatment	Radiation therapy	Chemotherapy	Results
Randomized					
Mayo Clinic[51]	48	EBRT±CT	35-37.5 Gy in 4-5 wk	5FU 1st wk of EBRT	Median survival 13 *vs* 6 mo and 5-year overall survival 12% *vs* 0% favoring EBRT+5FU
GITSG[53]	90	CT±EBRT	50 Gy split course in 8 wk	5FU during EBRT, maintenance 5FU/MeCCNU	4-year survival 18% *vs* 7%, favoring CT+EBRT
EORTC[52]	90	EBRT±CT	55.5 Gy in 6 wk	5FU	14% long-term survival (3 patients) with EBRT and 5FU
Retrospective					
MGH[55]	32	EBRT±CT	45-55 Gy in 5-6 wk	5FU during EBRT, maintenance 5FU/MeCCNU	EBRT+CT: Unresectable disease 14 mo median survival; unresectable and residual disease 10% 4-year survival

Abbreviations: EBRT=external beam radiation therapy; CT=chemotherapy; 5FU=5-fluorouracil; MeCCNU=semustine; GITSG=gastrointestinal study group; EORTC=European organization for research and treatment of cancer; MGH=Massachusetts General Hospital; Gy=gray.

Wieland and Hymmen used radiotherapy alone in patients with unresectable gastric cancer. The radiation dose was 60 Gy when feasible, in 1.5 to 2.0 Gy fractions. They noted an 11% (9 of 82) 3-year and 7% (5 of 72) 5-year survival[49]. Abe and Takahashi reported a 14.7% 5-year survival rate with intraoperative radiation therapy (28 to 35 Gy) in a group of 27 patients with stage IV disease. In the same study, there were no 5-year survivors in the 18 stage IV patients randomized to a surgery-alone control arm[50].

Most phase III trials for unresectable or residual gastric cancer show an advantage for combined-modality treatment over single-modality treatment. Two randomized trials compared radiation therapy alone to chemoradiation. The seminal trial in the treatment of unresectable gastric cancer is from the Mayo clinic[51]. All patients were randomized to radiotherapy (35 to 37.5 Gy in 4 to 5 wk) with or without 5-FU chemotherapy during the first 3 d of radiation. Mean and overall survival was improved in the combined-modality treatment group compared to radiotherapy alone (13 *vs* 6 mo median survival and 12% *vs* 0% 5-year survival, respectively). The EORTC performed a randomized trial of radiation therapy with or without 5-FU chemotherapy following attempted surgical resection[52]. Residual disease after resection was identified in 22 patients and the three long-term survivors (14%) received both irradiation and 5-FU.

Two randomized trials compared chemotherapy alone to chemoradiation. In a study by the GITSG, 90 patients were randomized to radiation therapy and 5-FU followed by maintenance 5-FU/MeCCNU or 5-FU/MeCCNU alone[53]. The radiotherapy dose was 50 Gy given in a split course. This trial included patients with unresectable, gross, microscopic, or no residual disease after resection, though only 7 patients had no residual disease. Radiation and chemotherapy resulted in a statistically significant improvement in 4-year survival compared with chemotherapy alone (18% *vs* 6%, respectively). A second trial performed by the GITSG comparing radiation and chemotherapy to chemotherapy alone did not demonstrate a survival advantage to combined modality therapy[54]. However, the results from this second trial are difficult to interpret as nearly 50% of patients on the combined modality arm either did not receive the prescribed dose of radiation or had a major deviation in radiation delivery.

Data from retrospective series also suggest an improvement in disease control and survival with the combination of radiation therapy and chemotherapy. In published series from the Mayo Clinic and Massachusetts General Hospital (MGH), long-term survival of 10% or more has been demonstrated in patients receiving external beam radiation and chemotherapy following subtotal surgical resection with residual disease (MGH) or unresectable disease[55,56]. In the MGH experience, patients with unresectable or gross residual disease treated with combined radiation and chemotherapy had median survivals of 14 mo and 15 mo, respectively, and the overall four-year survival rate was 10%.

In the University of Pennsylvania experience with unresected adenocarcinoma of the GE junction or esophagus, both local control and overall survival was superior with combined modality versus single modality therapy[57]. Local control was 52% with radiation and chemotherapy, compared to 4% with radiation alone and 0% with chemotherapy alone. Median survival with radiation and chemotherapy was 10 mo compared with 5 mo for radiation alone. Overall, considering the randomized and retrospective data, there appears to be improved survival with combined radiation and chemotherapy for unresectable gastric cancer. However, the use combined-modality therapy in unresectable gastric cancer is tempered by the fact that relatively few of these patients will be long-term survivors.

To the best of our knowledge, there are no randomized trials of neoadjuvant radiation and chemotherapy in unresectable gastric adenocarcinoma. Neoadjuvant chemotherapy alone has been used in unresectable and locally advanced disease in numerous phase II and in at least one phase III trial. Wilke *et al* treated 34 patients with unresectable gastric cancer confirmed at exploratory laparotomy with etoposide, adriamycin and cisplatin chemotherapy[58]. Following chemotherapy and second look operation, fifteen patients (44%) underwent potentially curative resection and five patients (15%) were pathologic complete respon-

ders. Median survival in this phase II trial was 18 mo for all patients. In an update, 79% of these patients had local-regional failure[58].

Taken as a whole, neoadjuvant chemotherapy for unresectable gastric cancer results in clinical response rates of 30% to 68%, potentially curative resections in as few as 8% or as many as 73% of patients, high rates of local relapse, and except for the Wilke study, pathologic complete response rates of <15%. In view of the high incidence of local recurrence and relatively low pathologic complete response rates, radiation therapy has been integrated into neoadjuvant studies of unresectable gastric cancer. A recent US Intergroup trial, to confirm the Walsh trial[15], of neoadjuvant combined-modality therapy in esophageal and GE junction carcinoma was closed due to inadequate accrual.

In the purely palliative setting for gastric cancer, total or subtotal gastric resection can successfully relieve symptoms of obstruction, hemorrhage, and ulceration. In advanced gastric cancer, total gastrectomy for bulky or proximal tumors has resulted in good quality of life, but was less helpful for relief of symptoms due to linitis plastica[59]. In the absence of surgery, radiation therapy is effective in 50% to 75% of patients in the palliation of gastric outlet or biliary obstruction, pain, and bleeding[48]. In select patients with good performance status, the administration of concurrent 5-FU chemotherapy may improve response.

RADIATION THERAPY TECHNIQUES

Intra-operative radiation therapy

Intraoperative radiation therapy (IORT) delivers a single high dose of radiation to areas at high risk relapse. A substantial advantage of intraoperative radiation therapy is that normal tissues can be largely excluded from the radiation field, such that larger doses of radiation can be delivered to the target tissues and toxicity minimized. Although appealing, the role of intraoperative radiation therapy in the setting of gastric cancer remains ill-defined. Several studies suggest an advantage to IORT in locally advanced gastric carcinoma, but these trials do not define the role of IORT in patients receiving EBRT.

A randomized trial by Sindelar *et al* compared gastrectomy with IORT to gastrectomy with or without EBRT (EBRT was not used in early stage lesions confined to the stomach) in forty-one patients[60]. Survival was equivalent between the 2 arms, but local-regional control was superior on the IORT arm versus the control arm (64% versus 8% respectively; $P < 0.001$). Skoropad *et al* randomized patients with gastric cancer to pre-operative EBRT (20 Gy in 5 d) followed by IORT (20 Gy) plus gastrectomy versus gastrectomy alone[61]. The addition of radiation therapy had a statistically significant survival advantage in more advanced stages (T3-4 and/or node positive). In a randomized trial of 211 patients from Kyoto, Japan, patients were randomized to 28-35 Gy of IORT or surgery alone[62]. This study also demonstrated improved survival in patients with disease penetrating the wall of the stomach or in patients with involved lymph nodes, but not in patients with disease limited to the wall of the stomach. While these trials suggest an advantage to radiation therapy in locally advanced disease, they do not answer the question of whether EBRT alone, IORT alone, or EBRT plus IORT should be used. To date, no randomized trials exist evaluating EBRT with or without IORT.

Notably, prospective and retrospective trials have not demonstrated increases in morbidity and mortality with IORT[63]. Given its potential benefits and limited morbidity, IORT in the setting of gastric cancer continues to be used in select centers in the United States.

3D conformal and intensity modulated radiation therapy (IMRT)

Radiation therapy technique is important in evaluating the benefits of radiation. In the Intergroup 0116 trial, 2D radiation therapy with an anterior-posterior (AP) and a posterior-anterior (PA) radiation beam was utilized. Radiation therapy portals were centrally reviewed prior to radiation, and 34% had major protocol violations prior to revision. These findings demonstrate that trials without central review may suffer from inadequate radiation therapy field design, and suggest that the skill and experience of the treating radiation oncologist is important.

Technology has evolved over time to allow increasingly conformal radiation therapy, potentially sparing normal tissues and decreasing toxicity and/or allowing dose escalation to the target volume. However, as technology allows progressively more conformal treatment, the radiation oncologist must be increasingly mindful of known patterns of recurrence.

Two such targeted techniques are 3D conformal radiation therapy and intensity modulated radiation therapy (IMRT). 3D conformal radiation therapy uses 3D reconstruction of the target volume and normal tissues from CT data to plan multiple radiotherapy beams conforming to the target and sparing, as much as possible, normal tissues. Leong *et al* compared a 6-field 3D conformal radiation therapy plan a 2D AP:PA radiation therapy plan in 15 patients and noted lower radiation doses to the kidneys and spinal cord, both important radiation dose-limiting normal tissues[64]. Dose was higher to the liver with the 3D plans, but still well below tolerance. Princess Margaret Hospital treated 50 patients per the chemoradiation arm of the INT 0116 protocol, but used a 5-field 3D conformal radiation technique. Grade 3 or 4 toxicity occurred in 52% of patients, similar to the INT 0116 trial, suggesting minimal impact on toxicity compared to 2D radiation therapy.

Intensity modulated therapy, like 3D conformal radiation therapy, uses multiple beams with the shape of each beam conforming to the target. Unlike 3D radiation therapy, the intensity of radiation within any given beam varies such that normal organs are spared compared to target tissues. Planning studies of IMRT in the use of gastric cancer demonstrate decreased dose to the kidneys compared to conventional plans[65-67]. In treating abdominal organs, however, IMRT must be used with caution as intra-abdominal motion can be significant and highly conformal treatments can miss targeted areas. The role of IMRT in gastric cancer is not yet defined and remains investigational.

To date, 3D conformal and IMRT techniques have not

demonstrated a convincing reduction in acute or late radiation toxicity. However, both techniques offer great potential to decrease radiation toxicity by avoiding normal tissues and to allow dose escalation to target tissues.

CONCLUSION

From the available clinical data in the treatment of gastric carcinoma, the following conclusions can be reached: (1) Patterns of failure following surgical resection demonstrate high local and distant failure rates, suggesting a need for both local and systemic adjuvant treatment. (2) Adjuvant radiation therapy improves local control based on randomized trials. (3) Adjuvant chemoradiation improves survival in the randomized Intergroup 0116 trial, and adjuvant chemoradiation following complete resection with negative margins is currently considered standard of care in the United States. (4) Chemoradiation allows long-term survival in a small number of patients with locally advanced, unresectable gastric carcinoma.

However, each advancement in the treatment of gastric cancer leads to further questions, which should serve as the basis for future clinical trials: (1) In the adjuvant or neoadjuvant setting, what is the benefit of adding radiation therapy to chemotherapy? (2) Does adjuvant chemotherapy and/or radiation therapy provide benefit in patients who have undergone a D2 lymph node dissection? (3) Is pre-operative radiation therapy superior to post-operative radiation therapy? (4) Will conformal radiation technologies including IORT, 3D conformal, and intensity modulated radiation therapy improve outcomes?

Despite its disappointing survival rates, the treatment of gastric carcinoma has improved modestly over time. Radiation therapy has a role in the adjuvant setting for improving local control and, in combination with chemotherapy, survival. Radiation therapy in unresectable disease provides effective palliation and, in combination with chemotherapy, long term survival in a small number of patients.

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REVIEW

Pathogenesis of columnar-lined esophagus

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Abstract

Since its initial description, the pathogenesis of the columnar-lined esophagus (CLE) has been surrounded by many controversies. The first controversy is related to the existence of the condition itself. The second controversy centers on whether the CLE is a congenital or an acquired condition. In this article, we review the congenital and acquired theories of development of CLE and discuss the various factors in acquisition of CLE. The bulk of evidence in the literature suggests that CLE is an acquired condition.

Key words: Barrett's esophagus; Risk factors; Esophageal adenocarcinoma; Pathogenesis; Reflux esophagitis; Gastroesophageal reflux

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INTRODUCTION

The lower esophagus lined by columnar epithelium is a condition poor in fact but rich in theory. It invites speculation but resists laboratory reproduction[1]. Columnar-lined esophagus (CLE) is commonly called "Barrett's esophagus". The same eponym is also used to describe the metaplastic epithelium and complications of this condition. Barrett's epithelium, Barrett's mucosa, Barrett's ulcer, Barrett's stricture and Barrett's carcinoma are used frequently in the literature. It is a pathological premalignant condition in which the lower segment of the esophagus becomes lined by a metaplastic columnar epithelium in the form of either circumferential extension of the gastric columnar epithelium or islands of columnar mucosa or both. The presence of specialized columnar epithelium anywhere in the esophagus represents a true metaplasia of the normal squamous epithelium and is highly suggestive for the diagnosis of CLE.

Since its initial description, the pathogenesis of CLE has been surrounded by many controversies. The first controversy is related to the existence of the condition itself. For more than six years, Norman Barrett[2,3] argued that the columnar-lined structure is an intrathoracic stomach due to a congenitally short esophagus. Earlier in 1943, Allison *et al*[4] described peptic ulceration in the 'short' esophagus. Allison[5] introduced the term reflux esophagitis and showed that peptic ulcer is in the esophagus. Our current concept of CLE is based on the demonstration by both Allison and Johnstone[6]. They have proven that the columnar-lined structure is esophagus on the basis of the following criteria: (1) The tubular structure grossly resembles the esophagus; (2) absence of peritoneal covering; (3) presence of squamous islands within the columnar mucosa; (4) presence of muscularis propria and submucosal glands of esophageal type; and (5) absence of the acid-producing oxyntic cells in the gastric mucous membrane, which line the lower esophagus.

The second controversy center on whether the CLE is a congenital or an acquired condition.

CONGENITAL THEORY OF COLUMNAR-LINED ESOPHAGUS

This theory is based upon the fact that the embryonic esophagus has a columnar epithelial lining, which may later be incompletely replaced with squamous epithelium leaving the lower esophagus lined by the fetal columnar epithelium. Many investigators showed that the embryonic columnar and glandular epithelium might persist in the esophagus after birth. Islets of ciliated columnar epithelium have been reported in post-mortem examination of esophagus from premature and newborn infants. There are four types of congenital columnar epithelium in the esophagus.

Embryonic ciliated columnar epithelium

Johns[7] showed that the fetal esophagus is lined by ciliated columnar epithelium until the 17th wk of development, and is later replaced by squamous epithelium. Initially, this replacement starts in the middle of the esophagus and then extends in both directions. Although, at birth, the esophagus is entirely lined by squamous epithelium, remnants of this embryonic ciliated columnar epithelium sometimes remain in the esophagus after birth. Rector and Connerley[8] found such columnar epithelium in the

esophagus of 7.8% of infants and children. It was more than 8 times commoner in the upper end than the lower end. A sheet of ciliated columnar epithelium has also been found lining the lower esophagus of one asymptomatic adult[9].

Heterotopic or ectopic gastric epithelium

Occasional foci of gastric epithelium were also reported to be present in the esophagus. Carrie[10] reported islands of ectopic gastric mucosa in 50% of his patients. Bosher and Taylor[11] reported the presence of heterotopic gastric mucosa associated with esophageal ulcer and stricture formation. Moreover, Abrams and Heath[12] also showed such epithelium.

Superficial cardiac glands

Normally superficial cardiac glands are present in the lamina propria, particularly in the lower end of the esophagus.

Congenital Barrett's epithelium

Although there is no similarity between the ciliated columnar epithelium and Barrett's epithelium, some investigators considered Barrett's epithelium to be a congenital remnant of the ciliated columnar epithelium.

Initially, Norman Barrett argued that since the early embryonic esophagus is lined by columnar epithelium, and that the metamorphosis to squamous epithelium only occurs after proliferation and recanalization of the original layer, the presence of columnar epithelium in the esophagus of an adult might be explained on the basis of a failure of this process to achieve completion[13]. Later on in 1962, Barrett changed his views and argued that the difficulty in accepting this explanation is that if it were true, one might expect the whole esophagus, and not simply the lower end to be abnormally lined more often than it is[14].

EVIDENCE SUPPORTING CONGENITAL ORIGIN OF COLUMNAR MUCOSA

Epidemiological evidence

Several studies have reported the cases of CLE in patients who had no GER[21-23]. Many authors reported the cases of CLE in children[24-26]. Two reports showed that a 9-year-old boy[24] and a 10-year old girl[24] had experienced dysphagia since infancy. The authors who favored the congenital theory assumed that the columnar mucosa is present since birth, but it becomes symptomatic later in life as a result of the development of a hiatal hernia and GER. Only a very small proportion of patients with CLE develop esophageal adenocarcinoma.

Morphological evidence

Postlethwait and Musser[27] in an autopsy study described a neonate with CLE. Haque and Merkel[28] reported a case of total CLE, where an adult patient had typical Barrett' s mucosa involving the esophagus. The authors argued that such a case support a congenital origin. However, a review of this case shows that this patient had long-standing, severe GER requiring antireflux surgery. Wolfe *et al*[29] and Mangla[22] described the presence of islands of heterotopic/ectopic gastric mucosa in the esophagus. Mangla[22] also described the cases with squamous epithelium interposed between Barrett's and gastric mucosa. Stadelmann *et al*[23] described patients with a linear, smooth squamo-columnar junction rather than the irregular junction commonly found in patients with CLE, and they argued that this observation support the congenital origin. Other several studies[22,30,31] argued that the presence of parietal and chief cells in Barrett's mucosa, which are highly differentiated cells, is unlikely to be an acquired condition.

Experimental evidence

Failure of experimental models has been reported by Van de Kerckhof and Gahagan by injuring the esophageal mucosa[32]. The absence of reversion of Barrett's mucosa to squamous epithelium after antireflux surgery has been cited as evidence supporting a congenital origin[22].

Details of evidence for production of CLE experimentally will be presented when discussing the acquired origin of columnar mucosa.

ACQUIRED THEORY OF COLUMNAR-LINED ESOPHAGUS

The arguments against the congenital theory include the fact that the embryonic studies have shown that the squamous replacement of the fetal columnar epithelium begins in the mid esophagus and progresses toward each end[7]. In addition, despite the fact that the islets of congenital ectopic gastric mucosa are more common in the cervical region of the esophagus which appears to be the last area to lose the embryonic columnar lining, the columnar epithelium is always found in the lower esophagus.

The association between CLE and gastroesophageal reflux (GER) was recognized early and led to the concept that CLE is an acquired condition. Tileston[15] suggested that the first requisite for the formation of peptic ulcer of the esophagus is an insufficiency of the cardia. He concluded that hyperacidity of the gastric juice plays a part in preventing healing of the esophagus ulcer seems probable. In the same article, he noticed with interest the close resemblance of the mucous membrane around the esophageal ulcer to that normally found in the stomach. In their historical paper, Allison and Johnstone[6] concluded that peptic esophagitis and peptic ulceration of the squamous epithelium of the esophagus are secondary to regurgitation of digestive juices, are most commonly found in those patients in whom the competence of the cardia has been lost through herniation of the stomach into the mediastinum. In the past, there has been some discussion about gastric heterotopia as a cause of peptic ulcer of the esophagus, but this point was very largely settled when the term reflux esophagitis was coined. It describes accurately in two words the pathology and etiology of a condition which are a common cause of digestive disorder. They reported seven patients in whom the lower esophagus

Table 1 Various evidences to support the congenital and acquired theories

Evidences	Congenital theory	Acquired theory
Epidemiological	1 Cases in children	1 Association with gastroesophageal reflux
	2 Cases without demonstrated reflux	2 Usual occurrence after middle age
Morphological	1 Neonate with columnar-lined esophagus	1 Absence of typical cases in fetuses
	2 Adult with involvement of entire esophagus	2 Presence of intestinal-type goblet cells and sulphomucins.
	3 Islands of heterotopic/ectopic gastric mucosa	3 Absence of gastrin-containing cells.
	4 Cases with squamous epithelium interposed between Barrett's and gastric mucosa	4 Endoscopic demonstration of upwards migration of Barrett's mucosa with ongoing gastroesophageal reflux
	5 Cases with linear, smooth squamo-columnar junction	
	6 Presence of parietal and chief cells in Barrett's mucosa	
Experimental	1 Failure of experimental models	1 Animal models.
	2 Absence of reversion to squamous epithelium after antireflux operation	2 Regression after successful antireflux surgery.
		3 Acquisition of Barrett's mucosa after onset of reflux following esophagogastrostomy, Heller myotomy and esophagojejunostomy.

Modified from Hamilton SR. Pathogenesis of columnar cell-lined (Barrett's) esophagus. In: Spechler S J, Goyal R K, (Eds.) Barrett's esophagus: Pathophysiology, diagnosis, and management. New York: Elsevier Science, 1985: 29-37.

was lined by gastric mucous membrane, and all these patients had reflux esophagitis. Four of these patients had progressed to stricture formations and one patient had ulceration in the esophageal part, which was lined by gastric epithelium. They termed such ulcers as Barrett's ulcer although they stressed that the use of this eponym does not imply agreement with Barrett's description of an esophagus lined with gastric mucous membrane as "stomach".

Although Allison and later on Barrett recognized the association between CLE and hiatal hernia and reflux esophagitis, nevertheless, they assumed that the condition is congenital in origin. Moersch *et al*[16] were the first to suggest that the columnar lining might be an acquired condition as a sequel of reflux esophagitis. Hayward[17] suggested that the CLE is an acquired condition due to reflux esophagitis that destroys the squamous epithelium. Moreover, he suggested that the denuded area is re-epithelialized from the columnar cells below, and that the lower 1 to 2 cm of the esophagus is lined by columnar epithelium which acts as a buffer zone of junctional epithelium, does not secrete acid or pepsin but is resistant to them.

The principal of the acquired theory is that the columnar epithelium is an adaptive change in response to the continued harmful environment present in the esophagus due to ongoing GER. Following repeated cycles of destruction and regeneration, in some individuals, a simple columnar epithelium more resistant to digestive action finally replaces the destroyed squamous epithelium[1].

Many earlier observations documented the ascent up the esophagus of a stricture at the squamo-columnar junction as squamous epithelium is replaced by columnar epithelium, supporting the concept of an acquired dynamic epithelium[18,19]. Adler[1] reported a series of 11 patients with CLE associated with strictures, in three of them, progression and ascent of the strictures were documented over 3 to 5 years. Moreover, he also suggested that the occurrence of islets of squamous epithelium within the sheet of columnar epithelium could be interpreted as the more resistant remnants of squamous epithelium that survived the erosive action of the digestive secretions. He stated that the findings of these squamous islets are less easily explained by the congenital theory.

Hamilton[20] summarized the epidemiological, morphological and experimental evidence cited in the literature to support the congenital and acquired theories (Table 1).

EVIDENCE SUPPORTING ACQUIRED ORIGIN OF COLUMNAR MUCOSA

Epidemiological evidence

(1) Many investigators reported the frequent association between CLE and GER[33]. Allison and Johnstone[6] suggested that if gastric reflux occurs, and causes ulceration of squamous epithelium without producing stenosis, is healing of the ulcer in an acid medium more likely to be by overgrowth of gastric rather than of esophageal epithelium? If this were so it might be that some examples of the gastric mucosa in the esophagus were acquired rather than congenital. (2) The usual occurrence of CLE in patients of middle and older ages. If the congenital columnar epithelium lining the esophagus produces acid and pepsin itself as some believe it is difficult to understand why complications do not occur early in life. (3) Dahms and Rothstein[34] reported that CLE in children is a consequence of chronic GER.

Morphological evidence

There is no typical cases of CLE in fetuses[33]. The histopathologic and histochemical characteristics of Barrett' s epithelium have also been quoted to support an acquired origin. The presence of intestinal-type goblet cells, and sulphomucins has suggested an acquired origin[35,36]. Dalton *et al*[37] and Dayal and Wolf[38] suggested that the absence of gastrin-containing cells from Barrett's mucosa as contrasted with congenital gastric heterotopia such as in a Meckel's diverticulum, support an acquired origin for CLE. However, Mangla *et al*[30] have demonstrated the presence of gastrin in Barrett's mucosa. The strongest morphological evidence for acquired origin has been

provided by cases with endoscopic demonstration of cephalic migration of the peptic esophagitis and stricture above an ascending boundary of CLE with continuing GER[19,26,39]. These cases provided strong evidence that the condition could be progressive and result from persistent severe GER and complicating esophagitis. The concept of erosive reflux esophagitis healing by upward migration of adjacent columnar epithelium was advanced by Hayward[17]. Adler[1] reported that out of five patients with CLE associated with strictures, three patients had progression and ascent of the strictures, which were documented over 3 to 5 years. He proposed the possibility of repair by extension from esophageal glands following reflux esophagitis.

Experimental evidence

Bremner *et al*[40] and Wong and Finckh[41] successfully produced columnar-lined mucosa in the esophagi of dogs and rats in the presence of GER, respectively. Li *et al*[42] showed that seven of the 10 dogs developed Barrett's mucosa after a reflux procedure was created, suggesting that a squamous injury in the esophagus is repaired by columnar epithelium in the presence of reflux. The columnar epithelium is acquired when the squamous epithelium is injured and during repair it undergoes columnar metaplasia. Although damage to squamous mucosa is a requisite factor for the development of columnar metaplasia, acute injury alone is not a sufficient stimulus for columnar metaplasia. The acquisition of the columnar epithelium requires a chronically abnormal esophageal environment during the period of mucosal repair. In an experimental study on dog esophagus, Gillen *et al*[43] found that a surgical injury in the esophageal squamous mucosa healed by the regeneration of squamous epithelium in normal dogs. However, if the surgical injury is preceded by alteration of the gastroesophageal junction to cause chronic GER, then the injured squamous mucosa is replaced by a columnar lining. Radigan *et al*[44] and Ransom *et al*[45] reported restoration of squamous mucosa after a successful antireflux operation. The acquisition of Barrett's mucosa after onset of GER following esophagogastrostomy, Heller myotomy, and esophagojejunostomy provide strong evidence for an acquired origin[46-49].

The arguments regarding the etiology of CLE have centered on an acquired *versus* congenital origin; the possibility remains that examples of both do occur[50]. However, the available evidence suggests that the vast majority of cases, if not all, are acquired due to chronic GER. The question remaining to be answered is why Barrett's mucosa develops in only 8-20% of patients with reflux, but not in other patients with reflux of similar severity[20].

FACTORS IN ACQUISITION OF CLE

Patients with CLE frequently have anatomical and physiological abnormalities which either predispose to or exaggerate the severe GER, which in turn leads to the acquisition of the columnar epithelium. Another group of these patients acquire the columnar epithelium secondary to esophagitis or mucositis due to irritant or cytotoxic agents. Given the combination of these anatomical and physiological abnormalities, it is not surprising that severe esophagitis commonly accompanies Barrett's epithelium.

The major risk factors that can lead to acquisition of the columnar epithelium are: (1) GER with acid and pepsin; (2) extreme lower esophageal sphincter incompetence; (3) alkaline reflux (duodeno-gastroesophageal reflux of bile and pancreatic juices); (4) gastric acid hypersecretion; (5) abnormal esophageal motility and delayed acid clearance; (6) diminished esophageal pain sensitivity; and (7) alcohol consumption.

The minor risk factors that can lead to acquisition of the columnar epithelium are: (1) Caustic injury by lye; (2) cytotoxic chemotherapy agent; and (3) acid and pepsin secretion by Barrett's epithelium.

GER with acid and pepsin

Much attention has been directed to reflux of the gastric juices containing acid and pepsin. Patients with CLE frequently have an extremely incompetent lower esophageal sphincter, and therefore, are exceptionally predisposed to GER. In most patients with CLE, GER is judged to be the principal factor that causes injury to the squamous epithelium and provides the abnormal esophageal environment necessary for columnar metaplasia. It is now widely accepted that a substantial proportion of patients with Barrett's esophagus do have severe GER. Schnell *et al*[51] conducted a study to test the hypothesis that since acid contact time is related to formation of Barrett' s epithelium, then a greater esophageal acid contact time might result in longer segments of Barrett's epithelium. They studied 116 patients with CLE and demonstrated a definite positive correlation between the length of Barrett' s esophagus and the duration of esophageal acid exposure. Recently some of the published Swedish studies linked adenocarcinoma of esophagus with duration, frequency and severity of GER symptoms[52,53].

Extreme lower esophageal sphincter incompetence

An important factor that contributes to mucosal injury in patients with CLE is lower esophageal sphincter (LES) hypotension. Stein *et al*[54] found that 11 of 12 patients with CLE had an incompetent LES. The abnormalities were defined as LES pressure of less than 6 mm Hg, overall LES length of less than 2 cm, or abdominal LES length of less than 1 cm. They concluded that this predisposes to GER. This study also confirmed earlier reports documenting extreme LES hypotension in patients with CLE. Attwood *et al*[55] reported that 90% of patients with CLE had a mechanically defective LES and 93% had increased esophageal exposure to gastric juice on esophageal pH monitoring.

Alkaline reflux (duodeno-gastro-esophageal reflux of bile and pancreatic juices)

Most of the earlier studies on CLE traditionally focused on the role of gastric acid and pepsin, but other reports suggested that other factors contributed to the esophageal damage in patients with CLE. Bile reflux into the esophagus was reported as an important causative

factor in acquisition of CLE by many investigators[39,47]. DeMeester *et al*[56] using 24-h esophageal pH monitoring found abnormal alkaline reflux (esophageal pH >7 for more than 10.5% of a 24-h period) in 34% (14/41) cases. Gastric secretions normally are not alkaline, suggesting that bile and other alkaline materials in the duodenum frequently reflux into the stomach and esophagus in patients with CLE and this alkaline reflux has a role in the esophageal injury. Waring *et al*[57] also documented the frequent occurrence of duodeno-gastric reflux in patients with CLE. In their study, radionuclide appeared within 95 min of an intravenous injection of ^{99m}Tc-diisopropyl iminodiacetic acid in the stomach of 46% (6/13) patients with CLE. In contrast, 11% (2/19) persons in the control group had radionuclide evidence of duodeno-gastric reflux. The role of alkaline reflux is particularly evident in those cases of CLE acquired after total gastrectomy in which gastric juices were eliminated[49]. Tada *et al*[58] also provided evidence that refluxed substances other than acid and pepsin play a pathogenic role in CLE. They reported a patient with severe GER disease who developed CLE at age 47, 22 years after a total gastrectomy. At age 64, an endoscopic examination revealed adenocarcinoma in Barrett's epithelium. This report confirms previous reports that acid and pepsin are not necessarily the only pathogenic factors for either Barrett's esophagus or esophageal adenocarcinoma, and further supports the important role of alkaline reflux in the development of CLE and adenocarcinoma. Stoker and Williams[59] suggested that a mixture of gastric and duodenal secretions causes esophageal mucosal disruption and intracellular damage through a toxic synergism between the two secretions. The clinical importance of the alkaline reflux is that the treatment of GER in patients with CLE should involve shifting or neutralizing the alkaline substances as well as the acid. As with duodeno-gastric reflux, the stomach contents available for reflux into the esophagus will include bile and pancreatic enzymes in addition to acid. Therefore, suppressing the acids alone may be ineffective in healing esophagitis for patients with CLE. Hetzel *et al*[60] showed that omeprazole, a drug that is dramatically effective in reducing gastric acid secretion and in healing mild esophagitis, was far less reliable for healing ulcerations in Barrett's epithelium. van der Veen *et al*[61] reported that previous gastric surgery represents an increased risk factor for the development of adenocarcinoma in patients with CLE. Attwood *et al*[62] conducted a study to determine the role of intra-esophageal alkalinization in the genesis of complications in Barrett's esophagus. They found that the esophageal acid exposure (% time pH<4) was similar in patients with or without complications (19.2% *vs* 19.3%). In contrast, the esophageal alkaline exposure (% time pH>7) was greater in patients with complications (24.2% *vs* 8.4%). In 81% of patients, there was a clear relationship between gastric and esophageal alkalinization. They concluded that complications in Barrett's esophagus develop in association with increased exposure of the esophagus to an alkaline environment, which appears to be secondary to duodeno-gastric reflux.

Gastric acid hypersecretion

Patients with CLE may have elevated gastric acid secretion. Collen *et al*[63] suggested that hypersecretion of gastric acid may contribute to the frequency of complications in Barrett's esophagus. In a prospective study, 75% (9/12) patients who had heartburn intractable to treatment with conventional doses of ranitidine (150 mg twice per day) were found to have gastric acid hypersecretion (basal acid output > 10 mEq/h). Among those 12 refractory patients, 10 had Barrett's esophagus, and increasing the conventional dose of ranitidine 2 to 6 times controlled their heartburn, which reduced the gastric acid output to less than 1 mEq/h. With gastric acid hypersecretion and duodeno-gastric reflux of bile and pancreatic enzymes, the gastric material available for reflux can be unusually caustic and harmful.

Abnormal esophageal motility and delayed acid clearance

Many patients with CLE have abnormal motility in the distal esophagus that can delay esophageal clearance, and so the refluxed material may have prolonged contact with the esophageal mucosa. Winter *et al*[64] reported on the importance of delayed acid clearance from the esophagus as a contributory factor in the causation of CLE. Stein *et al*[54] using 24-h esophageal motility monitoring reported that there was an increase in the frequency of abnormally weak contractions (<30 mm Hg) of the distal esophagus in patients with CLE compared with patients with uncomplicated reflux disease. These weak contractions may not empty the esophagus effectively, and could be responsible for the delayed esophageal acid clearance that has been observed in patients with Barrett's esophagus.

Diminished esophageal pain sensitivity

There are large proportions of patients with CLE who are asymptomatic. Cameron *et al*[65] estimated that for every case identified clinically, there are approximately 20 cases in the general population that go unrecognized. Patients with CLE usually seek medical advice when they develop complications, such as esophagitis, stricture, ulcer, bleeding or adenocarcinoma. Many patients with CLE have diminished esophageal pain sensitivity. Skinner *et al*[66] suggested that persistence of severe reflux after antireflux surgery in some patients with Barrett's esophagus who are asymptomatic is due to the Barrett's epithelium not being sensitive to acid after ulceration or strictures heal. Iascone *et al*[67] found that as compared to patients with reflux esophagitis without Barrett's epithelium, patients with Barrett's esophagus have less severe symptoms of heartburn and regurgitation in spite of significantly greater duration of acid reflux in the distal esophagus. Johnson *et al*[68] suggested that the CLE has diminished pain sensitivity and that acid reflux may not cause heartburn for many patients with CLE. Perhaps most patients with Barrett's esophagus escape medical attention because their reflux symptoms are minimal. Diminished pain sensitivity may play a role in the development of the complications that frequently accompany CLE. Patients whose reflux is not heralded by heartburn are unlikely to take antacid or seek medical advice. With no symptomatic warning that caustic material is damaging the esophagus, patients are unlikely to take medications that may prevent further injury[69]. The decrease in pain sensitivity may explain the

lack of reflux symptoms before the onset of dysphagia in some of the patients who develop adenocarcinoma.

Alcohol consumption

Martini and Wienbeck[70] noted the frequent history of chronic alcohol abuse in patients with CLE. Whether this association is related to a direct effect of alcohol on the mucosa or to promotion of GER by the effects of alcohol on the LES is uncertain. It is interesting to notice that alcohol abuse was quoted as early as 1906 as an important cause for peptic ulcer of the esophagus. Tileston[15] in 1906 suggested that the abuse of alcohol deserves a mention among the predisposing causes, occurring with considerable frequency. Alcohol probably acts in two ways: first, by irritation of the esophagus, and second, by the production of gastritis, with vomiting and insufficiency of the cardia.

Caustic injury by lye

Spechler *et al*[71] reported a case with fundic-type Barrett's epithelium in the mid esophagus with sparing of the distal esophagus complicating caustic injury by lye ingestion.

Cytotoxic chemotherapy agent

Cases of CLE have been reported in 63-75% of patients who received a prolonged course of chemotherapy for breast carcinoma. Sartori *et al*[72] reported that CLE could be acquired through an iatrogenic injury by prolonged chemotherapy with anti-neoplastic agents (cyclophosphamide, methotrexate and 5-fluorouracil). These agents injured the esophageal mucosa and caused columnar epithelium secondary to the drug-induced mucositis. In this regard, they published two reports suggesting that chronic chemotherapy with these agents frequently causes columnar metaplasia of the esophagus. In the first report, they described a woman who, because of duodenal ulcer disease, had an endoscopic examination before the beginning of prolonged chemotherapy for breast cancer. The esophagus was normal, but after 4 mo of chemotherapy, a second endoscopy revealed an esophageal ulcer with columnar epithelium. Endoscopic examinations in another eight women who had been treated with the same chemotherapy agents showed columnar epithelium in six of them. The length of the columnar epithelium was more than 3 cm in all six patients[72]. In the second paper, they reported a prospective endoscopic study carried out to document the development of CLE in women receiving the same chemotherapy drugs**.** Ten of sixteen women (63%) who had no endoscopic or histologic evidence of CLE before chemotherapy were found to develop a CLE after 6 mo of chemotherapy[73].

Acid and pepsin secretion by Barrett's epithelium

Secretion of acid and pepsin by the parietal and chief cells in the Barrett's mucosa has been considered as possible factors in development of CLE by some investigators like Mangla *et al*[30] and Ustach *et al*[74]. However, there are no recent data to support this.

SUMMARY

The incidence of CLE and adenocarcinoma is rising at an epidemic rate, and the cause for this rapid rise is unclear and the answer to this principle question remains to be determined. Due to the causal relationship between CLE and GER, risk factors which enhance reflux such as tobacco smoking, alcohol consumption, dietary changes including high fat diet, obesity, and the wide spread usage of the medications that affect the upper gastrointestinal tract could be partially responsible for this rise. Although other several factors can be offered as possible explanations for this rise in incidence such as increased awareness of the condition, widespread use of endoscopy, and improved diagnostic techniques, this rapid increase in the incidence of CLE and adenocarcinoma raises serious concern that the current management for GER is inadequate and deserves reassessment.

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Reduced expression of β-catenin inhibitor Chibby in colon carcinoma cell lines

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Abstract

AIM: To analyse the Chibby expression and its function in colon carcinoma cell lines and colorectal carcinoma (CRC).

METHODS: Chibby expression levels were investigated by quantitative RT-PCR in a panel of seven different colon carcinoma cell lines. By sequencing, we analysed mutational status of Chibby. To test whether Chibby exhibited effects on β-catenin signalling in colon carcinoma cells, we transfected SW480 cells with Chibby expression plasmid and, subsequently, analysed activity of β-catenin and tested for alterations in cellular phenotype. In addition, we examined Chibby mRNA levels in samples of colorectal carcinomas and adjacent normal tissues by using quantitative RT-PCR and hybridised gene chips with samples from CRC and normal tissues.

RESULTS: Chibby mRNA expression was strongly downregulated in colon carcinoma cell lines in comparison to normal colon epithelial cells and no mutation in any of the examined colon carcinoma cell lines was found. Further, we could show that Chibby inhibited β-catenin activity in TOPflash assays when over-expressed in SW480 cells. Proliferation and invasion assays with Chibby transfected SW480 cells did not reveal profound differences compared to control cells. In contrast to these *in vitro* data, quantitative RT-PCR analyses of Chibby mRNA levels in CRC tumor samples did not show significant differences to specimens in adjacent non-cancerous tissue. Consistent with these findings, gene chips analysing tissue samples of tumors and corresponding normal tissue did not show altered Chibby expression

CONCLUSION: Altered Chibby expression might be observed *in vitro* in different colon carcinoma cell lines. However, this finding could not be confirmed *in vitro* in CRC tumors, indicating that Chibby is not likely to promote CRC tumor development or progression. As Chibby is an important inhibitor of ß-catenin signalling, our data implicate that the usability of colon carcinoma cell lines for *in vitro* studies analysing the Wnt/β-catenin pathway in colorectal carcinoma needs extensive verification.

Key words: Chibby; Wnt; β-catenin; Colorectal carcinoma; Colon carcinoma cell lines

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INTRODUCTION

The Wnt/Wingless pathway has been shown to exhibit important functions in embryonic and cancer development. In the absence of Wnt signalling, β-catenin is phosphorylated by glycogen synthase kinase-3 (GSK-3) and targeted for ubiquitin-mediated proteasomal degradation[1]. Upon Wnt binding to its cellular receptor Frizzled, the degradation complex is destabilised, and, as a result, unphosphorylated β-catenin accumulates and translocates into the nucleus. There, it functions as a co-factor of lymphoid enhancer factor/T-cell factor (LEF/TCF) transcription factors leading to upregulation of a variety of genes. It has been shown that mutations in different genes of the Wnt pathway are involved in development of various cancers. One of those tumor entities is colorectal carcinoma where approximately 90% of the tumors have been shown to harbour activating mutations within the canonical Wnt signalling pathway[2-4].

The most potent transcriptional activation domain of β-catenin is located within the C-terminal region of β-catenin and has been used as bait in a yeast screening to identify interacting proteins. In this screening, Chibby, a novel protein that is conserved from Drosophila to human, was isolated [5]. It is a nuclear protein of 126 amino

acids, and as it inhibited Wnt signalling by preventing the interaction between β-catenin and LEF/TCF factors, it was suggested as a potential tumor suppressor gene. Chibby is encoded by the human *C22orf2* gene located at chromosome 22q12-q13. This chromosomal region is known to be mutated frequently in colorectal cancer[6], and therefore, Chibby might be an interesting candidate as a tumor suppressor in CRC. Gad *et al*[7] performed loss of heterozygosity (LOH) analyses of tumors of 36 patients involving the *C22orf2* region in CRC and were not able to detect differences between normal and CRC tumor tissues, but did not analysed effects of Chibby on cellular functions.

In this study, we aimed to analyse whether Chibby is mutated in CRC, to evaluate Chibby mRNA expression levels in colon carcinoma cell lines and tumor samples, and to investigate whether Chibby over-expression in colon carcinoma cell lines has an effect on cell behaviour in functional assays.

MATERIALS AND METHODS

Isolation and cultivation of colon epithelial cells

Isolation and cultivation of colon epithelial cells (CEC) were performed as previously described[8,9].

Cell lines and culture conditions

The colon carcinoma cell lines SW48 (ATCC CCL-231), SW480 (ATCC CCL-228), CaCo-2 (ATCC HTB-37), LoVo (ATCC CCL-229), HCT116 (ATCC CCL-247), and HT29 (ATCC HTB-38) were used for *in vitro* experiments. The cell line HT29M3 was chosen as a highly differentiated colon carcinoma cell line[10-13].

For tissue culture, the cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with penicillin (400 U/mL), streptomycin (50 μg/mL), L-glutamine (300 μg/mL), and 100 mL/L fetal calf serum (FCS) (Sigma, Deisenhofen, Germany) and split 1 : 5 every three days.

For demethylation assays, the cells were treated with 5-azacytidine at a final concentration of 10 μmol/L for 48 h (Sigma)[8]. HT29 M3 cells were used as control cells in the demethylation experiments.

RNA isolation and reverse transcription

For RT-PCR, total cellular RNA was isolated from cultured cells or laser microdissected tissue samples using the RNeasy kit (Qiagen, Hilden, Germany). The integrity of the RNA was controlled on 10 g/L agarose/formaldehyde gel, and subsequently cDNAs were generated by reverse transcriptase reactions. The reverse transcription (RT) reaction was performed in 20 μL reaction volume containing 2 μg of total cellular RNA, 4 μL of 5x first strand buffer (Gibco), 2 μL of 0.1 mol/L DTT, 1 μL of dN_6 primer (10 mmol/L), 1 μL of dNTPs (10 mmol/L) and DEPC water. The reaction mix was incubated for 10 min at 70 ℃. Then 1 μL of Superscript II reverse transcriptase (Gibco) was added and RNAs were reverse transcribed for 1 h at 37 ℃. Subsequently, reverse transcriptase was inactivated at 70 ℃ for 10 min and RNA was degraded by digestion with 1 μL of RNase A (10 mg/mL) at 37 ℃ for 30 min. cDNAs were controlled by PCR amplification of ß-actin.

Chibby mRNA mutational analysis

The entire coding region of Chibby was amplified by RT-PCR from cDNA using16, ATGCCTTTCTTTGGGAATTACGTTC (forward) and chibby496, TCATTTTCTCTTCCGGCTGATC (reverse), which resulted in a 380-bp fragment. The PCR reaction was performed in 50 μL reaction volume containing 5 μL of 10x Taq buffer, 1 μL of cDNA, 0.5 μL of each primer (0.2 μmol/L), 0.5 μL of dNTPs (10 mmol/L), 0.5 μL of Taq polymerase (5 U/μL), and 41 μL of water. The amplification reactions were performed by 35 repetitive cycles of denaturation for 30 s at 94 ℃, annealing for 30 s at 64 ℃, extension for 30 s at 72 ℃, and a final extension step at 72 ℃ for 5 min. The PCR products were resolved on 15 g/L agarose gels. For sequencing, the products were purified by PEG precipitation to remove unincorporated primers and dNTPs. Both strands were sequenced for each PCR product from at least two independent PCR reactions. Sequences were compared with the gene data bank by means of BLAST search (National Center of Biotechnology Information).

Analysis of Chibby expression by quantitative PCR

Quantitative real-time PCR was performed on a LightCycler (Roche, Mannheim, Germany). Two microliters of cDNA template, 1.6 μL of 25 mmol/L $MgCl_2$, 0.2 μmol/L forward and reverse primers {chibby125, TTTGGGAATACGTTCAGTCCG (forward) and chibby395, TCAGCCGCAAGAGATTGTTC (reverse)} and 2 μL of SybrGreen LightCycler mix in 20 μL volume were PCR-amplified through 40 cycles, each cycle consisting of initial denaturation at 95 ℃ for 30 s, 20 ℃/s temperature transition rate up to 95 ℃ for 15 s, 64 ℃ for 3 s, 72 ℃ for 5s, 85 ℃ acquisition mode single. The PCR product was evaluated by melting curve analysis following the manufacturer's instructions and by checking the PCR products on 18 g/L agarose gels. All quantitative PCR experiments were repeated three times.

Transient transfection of SW480 cells

A total of 2×10^5 cells were seeded onto six-well plates one day before transfection. Transfections with pCMX PL2 or pCMX PL2 flag Chibby plasmid (0.5 μg per well) were performed using the LipofectAMINE plus method (Life Technologies) according to the manufacturer's instructions. Cells were harvested 48 h after transfection and the amount of Chibby expression was determined by RT-PCR and Western blot analysis.

Protein analysis in vitro (Western-blotting)

For protein isolation, 2×10^6 cells were washed in 1x PBS and lysed in 200 μL RIPA buffer (Roche). The protein concentration was determined using the BCA protein assay reagent (Pierce, USA). Equal amounts of cellular protein (40 μg total protein) were denatured at 94 ℃ for 10 min after addition of Rotiload buffer (Roth, Karlsruhe, Germany) and subsequently, separated on NuPage SDS gels (Invitrogen, Groningen, The Netherlands). After transferring the proteins onto PVDF membranes (BioRad, Richmond,

USA), the membranes were blocked in 50 g/L dry milk in TBS-T and incubated overnight with primary monoclonal anti flag antibody (1 : 4500 dilution, Sigma) at 4 ℃. A 1 : 4 000 dilution of anti IgG AP (Sigma) was used as secondary antibody. Staining was performed using 5-bromo-4-chloro-3-indolyl phosphate/ nitroblue tetrazolium tablets (Sigma). All Western blot experiments were repeated at least three times.

Immunofluorescence staining of flag-tagged Chibby

A total of 2×10^4 SW480 cells were seeded per chamber of four-well-Falcon chamber slides (Becton Dickinson, Le Pont de Plaix, France) and cultivated overnight before transfection with either control vector or Chibby flag pCMX PL2. After 48 h of transfection, cells were washed with PBS, fixed with 40 g/L paraformaldehyde, permeabelised with 1 ml/L Triton X-100, washed again, and blocked for 30 min in 10 g/L BSA in PBS. Anti flag antibody (dilution 1 : 1 000) was used as primary antibody, and anti mouse FITC-conjugated antibody (dilution 1 : 40) was used as secondary antibody. Incubations took place at room temperature for 1 h each. Staining of nuclei occurred with DAPI.

Reporter gene assays

For transient transfections, 2×10^5 cells per well were seeded into six-well plates and transfected with 0.5 μg of TOPflash or FOPflash reporter gene plasmids using the LipofectAMINE plus method (Gibco) according to the manufacturer's instructions. For cotransfection, 0.125 μg, 0.25 μg, 0.5 μg, and 1 μg of Chibby expression plasmid per well were used, respectively. At 48 h post-transfection, the cells were lysed and the luciferase activity in the lysate was measured. To normalise transfection efficiency, 0.2 μg of a pRL-TK plasmid (Promega, Mannheim, Germany) was cotransfected and renilla luciferase activity was measured by a luminometric assay (Promega). All transfection experiments were repeated at least three times.

Proliferation assay

Proliferation was measured as previously described[14]. Proliferation of Chibby transfected SW480 cells was compared to non or mock transfected SW480 cells in an XTT assay. Results were expressed as mean ± SD (range). All experiments were repeated three times.

Invasion assay

Invasion assays were performed as previouly described[14]. Briefly, the assays were performed in Boyden chambers containing polycarbonate filters with an 8-μm pore size. In brief, filters were coated with a commercially available reconstituted basement membrane (Matrigel; Becton Dickinson, Heidelberg, Germany), and the lower compartment was filled with fibroblast-conditioned medium as chemoattractant. SW480 cells and Chibby transfected SW480 cells were harvested by trypsinization, resuspended in DMEM without FCS at a density of 2×10^5 cells/mL, and placed in the upper compartment of the chambers. After incubation for 4 h at 37 ℃, the filters were removed. The cells adhering to the lower surface were fixed, stained, and counted.

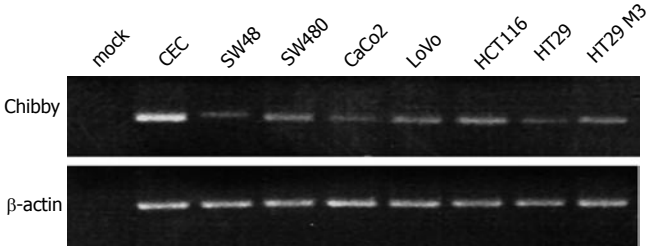

Figure 1 PCR amplification of the complete coding region of Chibby mRNA. The Chibby coding region was amplified using RT-PCR. All colon carcinoma cell lines were shown to express full-length Chibby mRNA. Expression levels cannot be estimated from this experiment as the PCR was carried out to saturation in order to illustrate full-length mRNA expression and to obtain enough PCR products for sequencing. β-actin amplification served as control to ascertain the integrity of the cDNA in all samples.

Fifteen random fields were counted at 200-fold magnification. Each sample was assayed in triplicate, and all experiments were repeated three times.

RESULTS

It is well known that the Wnt/β-catenin signalling pathway is constitutively active in a large number of colorectal cancers. The most commonly mutated proteins within the pathway are β-catenin and APC. Intriguingly, the activation of the cascade cannot in all cases of CRC be explained by mutations in these proteins. It remains unclear what other proteins of the pathway have to be altered in addition to the aforementioned mutations to lead to activation of the Wnt/β-catenin signalling pathway. Hence, we were interested in analysing other potential endogenous inhibitory factors that might accomplish regulatory functions within the Wnt/β-catenin signalling pathway and thus influence signal transduction. Due to these reasons, we analysed the role of the β-catenin-associated antagonist Chibby in colorectal carcinoma cell lines and compared our *in vitro* findings of Chibby expression with the *in vivo* situation by analysing colorectal tumor samples and the corresponding normal tissue.

Analysis of Chibby mutational status and expression level in colon carcinoma cells

We analysed a panel of seven colon carcinoma cell lines, such as SW48, SW480, CaCo2, LoVo, HCT116, HT29, and HT29 M3, that are frequently used as model systems to study effects in colorectal carcinoma. We evaluated the panel of colon carcinoma cell lines for alterations in the Chibby mRNA expression. The complete Chibby coding region was amplified by RT-PCR. All cell lines examined expressed Chibby mRNA at the expected length, indicating that no deletions or mutational insertions had occurred (Figure 1). As a control the Chibby coding sequence was amplified using CEC cDNA as template. The same cDNAs were used to amplify β-actin as a control for the integrity of the cDNA.

The cell lines were evaluated for levels of Chibby mRNA expression using quantitative RT-PCR in comparison to normal colon epithelial cells (CEC). Chibby

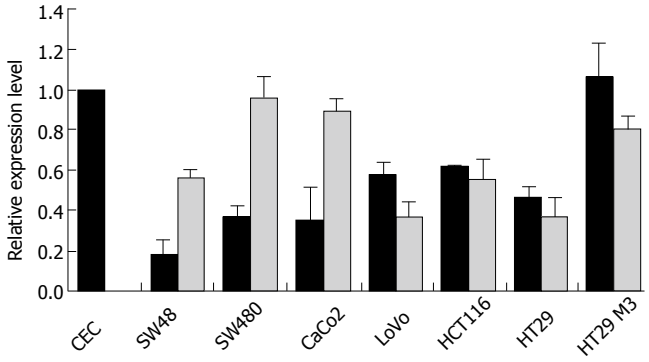

Figure 2 Quantification of Chibby expression in colon carcinoma cell lines. The amount of Chibby mRNA expression was carefully quantified by real-time PCR. All colon carcinoma cell lines showed down-regulation of Chibby mRNA expression in comparison to colon epithelial cells (CEC) and HT29 M3 cells, a cell line derived from HT29 cells that is considered to be a highly differentiated colon carcinoma cell line (black bars). Treatment of cells with the demethylating agent 5-azacytidine caused a modest increase in Chibby mRNA levels in SW48, SW480, and CaCo2 cells, but not in the other cell lines (grey bars). HT29 M3 cells were used as controls for the demethylation experiments as the experiment could not have been performed with primary CEC due to culture conditions.

mRNA was found to be down-regulated in all cell lines in comparison to CEC and HT29 M3 cells which are considered to be a highly differentiated colon carcinoma cells and express Chibby in amounts similar to those of CEC (Figure 2, black bars). Three of the analysed cell lines, SW48, SW480, and CaCo2 cells, showed residual expressions of Chibby mRNA of 18%, 37%, and 35%, respectively, in comparison to CEC or HT29 M3 (100% and 106%, respectively). LoVo, HCT116, and HT29 cells were also shown to have reduced Chibby mRNA levels, but down-regulation was not as explicit as in the other examined cell lines (57%, 62%, and 46%).

To investigate the reason for down-regulation of Chibby expression in colon carcinoma cell lines, we amplified and analysed the exons of the *chibby* gene. All PCR products were purified and subsequently sequenced using two different primers. The analysis of sequenced PCR products did not reveal any sequence variation within the coding region of the *chibby* gene locus (data not shown).

From these results, we concluded that Chibby does not harbour mutations within the coding sequence in all seven analysed colon carcinoma cell lines.

As this reduction in mRNA expression in colon carcinoma cell lines could not be explained by loss or mutation of the *chibby* gene, we hypothesised that promoter hypermethylation could have silenced gene expression. To test this hypothesis, all cell lines were exposed to the demethylating agent 5-azacytidine. Treated and untreated cells were tested for Chibby mRNA expression which was quantified by quantitative RT-PCR. HT29 M3 cells were used as controls in these experiments. HT29 M3 cells are highly differentiated cells that were used as an additional control as CEC could not be used in demethylation experiments as controls due to culturing issues.

Our results showed that 5-azacytidine treatment did not lead to induction of Chibby expression in LoVo, HCT116, HT29, or HT29 M3 cells, but to a moderately increased expression in SW48, SW480, and CaCo2 cells (Figure 2, grey bars). Therefore, we conclude that promoter hypermethylation might play a minor role for altered Chibby expression in some of the analysed cell lines, but we do not consider this silencing mechanism to be a general event in down-regulation of Chibby mRNA expression in the colon carcinoma cell lines.

Effect of Chibby expression on β-catenin transcriptional activity

As colon carcinoma cell lines display reduced levels of Chibby expression in comparison to normal CEC, we were interested in whether over-expression of Chibby in these cells influences functional processes. To test for the potential effects, we over-expressed Chibby in the colon carcinoma cell line SW480 by transfecting a flag-tagged Chibby expression construct. Successful over-expression was observed by quantitative RT-PCR (data not shown) and Western blotting (Figure 3A). Exogenous Chibby protein was not detected following mock or control transfection with pCMX PL2 flag plasmid which did not alter Chibby levels. Strong over-expression of Chibby was observed after transfection of SW480 cells with Chibby flag expression plasmid. Immunofluorescence staining revealed correct expression patterns of Chibby flag-tagged protein in the nucleus of SW480 cells (Figure 3B), also displaying high transfection efficiency.

As Chibby as endogenous antagonist of β-catenin is expected to exert negative functions on the Wnt/β-catenin signalling pathway, we performed LEF/TCF luciferase reporter gene assays using the TOPflash/FOPflash system. Analysing the activity of β-catenin in control and Chibby over-expressing cells, we observed reduced activation levels in the TOPflash system in accordance to the amount of transfected Chibby expression plasmid (Figure 3C). Transfection with 0.125 μg or 0.25 μg of Chibby plasmid per well did not affect the activity of TOPflash reporter, whereas activity dropped to 44% and 48%, respectively, when 0.5 μg and 1 μg expression plasmids were applied. FOPflash reporter construct activity was not affected by Chibby expression (Figure 3C). These results showed that transfection of SW480 cells with Chibby expression plasmid reveals functional Chibby protein.

Functional assays with Chibby over-expressing SW480 cells

To assay whether altered Chibby levels had an impact on proliferation and invasion potential of SW480 cells, we performed functional experiments utilizing mock and Chibby transfected cells. Boyden chamber assays were performed to examine the effect of Chibby on the invasiveness of SW480 cells. In this assay, the tumor cells have to invade through matrigel mimicking the basal lamina. Comparing mock treated cells, control transfected cells, and Chibby over-expressing cells, we did not observe any differences with regard to their invasiveness.

As the Wnt/β-catenin signalling pathway is known to influence cell proliferation, we next assayed for alterations of proliferation and performed *in vitro* proliferation assays with Chibby transfected SW480 cells and mock or control transfected cells. Modifying the Chibby protein level had

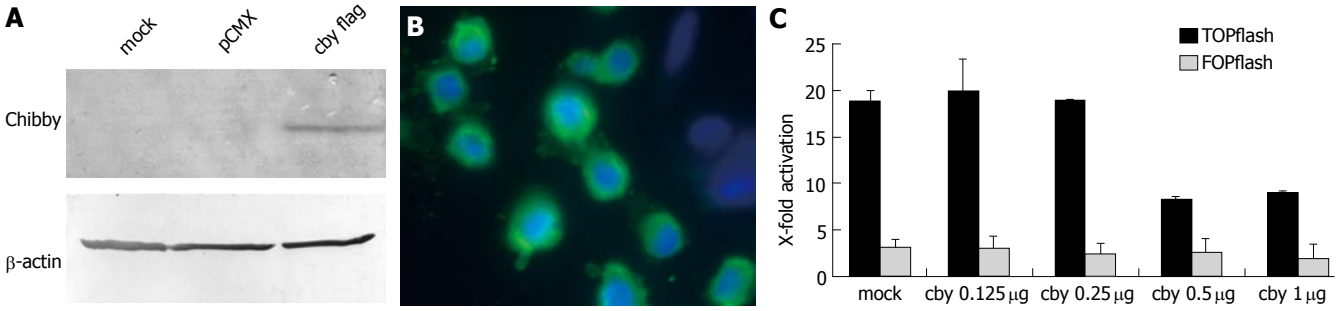

Figure 3 Expression of Chibby in transfected SW480 cells. A: Western blot analysis showing Chibby protein expression levels in untransfected (mock), control vector, or Chibby transfected SW480 cells. B: immunofluorescence staining of flag-tagged Chibby transfected SW480 cells. Nuclei were stained with DAPI in blue, flag-tagged Chibby was detected with FITC-labelled anti-flag antibody (green). C: TOPflash reporter gene assays showing inhibition of β-catenin signalling after transfection of SW480 cells with Chibby expression plasmid.

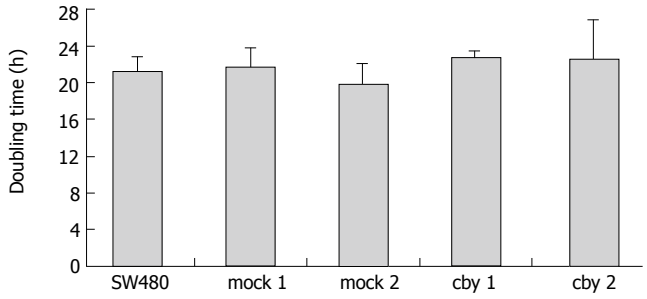

Figure 4 Proliferation Assay. Analysis of the cells over-expressing Chibby could not altered doubling time in comparison to mock or control transfected SW480 cells. Assays were performed in triplicates.

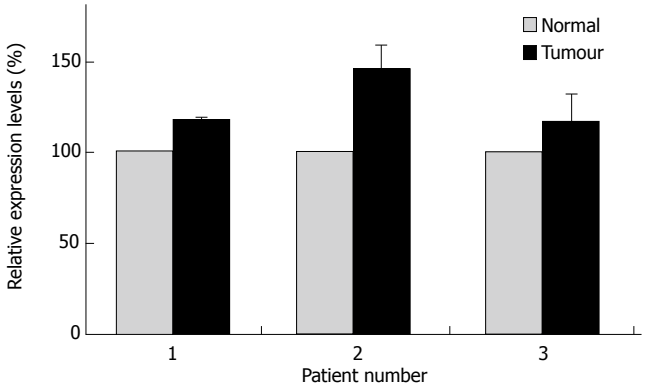

Figure 5 Chibby mRNA expression levels in colorectal tumor and corresponding normal tissue samples. Quantitative RT-PCR showing Chibby mRNA expression levels in matched pairs of tumor and normal colorectal tissue of 3 different patients.

no significant effect on cell proliferation (Figure 4). Doubling times were as follows: SW480: 21.2 h, mock 1: 21.7 h, mock 2: 19.7 h, cby 1: 22.6 h, and cby 2: 22.5 h, respectively. Thus, we did not consider proliferation to be affected by Chibby expression levels in SW480 cells.

Chibby mRNA expression in vivo

As our experiments in colon carcinoma cell lines had revealed sound down-regulation of Chibby expression, the absence of effects on cell functions of over-expressed functional Chibby protein in one of these cell lines (SW480) was rather unexpected. We next performed experiments with samples from patients with diagnosed colorectal carcinoma to assess the question whether Chibby expression was altered not only in colon carcinoma cell lines, but also in coloreactal tumors and might therefore contribute to or support development and progression of colorectal tumors.

We analysed Chibby transcript expression levels in tumor samples and corresponding normal colonical tissue from the same patients by hybridisation of an oligonucleotide-based array (Affymetrix HG-U133A) with 43 samples of colorectal cancer and 34 matched normal mucosa (unpublished data). The ratio of expression levels between tumors and normal tissues was 1.11, displaying no altered Chibby expression in CRC tumors in comparison to adjacent normal colon tissue. In addition, when tumor samples with microsatellite stability (MSS; $n = 24$) and microsatellite instability (MSI; $n = 19$) were separately assessed, again no difference in Chibby expression was observed.

To further validate this result, using quantitative RT-PCR, we tested whether expression levels of Chibby mRNA differed between normal colorectal tissue and adjacent cancerous tissue. We manually microdissected frozen material and analysed the extracted mRNAs. Figure 5 shows the results of 3 examined matched samples, displaying no profound alterations between tumor and adjacent normal tissue. Relative Chibby levels ranged from 100% in normal tissue to 117.4% (patient 1), 146.1% (patient 2), and 116.4% (patient 3), respectively.

DISCUSSION

The cellular antagonist and β-catenin interacting factor Chibby, a small nuclear protein, has been shown to be an endogenous inhibitor of the canonical Wnt signalling pathway. It binds to the LEF/TCF interaction domain at the C-terminus of β-catenin and thereby eliminates interaction of β-catenin with LEF/TCF transcription factors[5]. This leads to a block of transcriptional activation within the signalling cascade. It has been shown for colorectal carcinoma that more than 90% of all tumors harbour activating mutations within proteins of the canonical Wnt/β-catenin signalling cascade[2] and most mutations affect either the β-catenin or the APC

protein[14,15]. Interestingly, tumor development cannot in all cases be solely explained by these mutations, but there need to be further and additional disrupting cellular events. Hence, we were analysing other factors within the Wnt/β-catenin pathway and this study showed our results on the cellular antagonist of β-catenin, Chibby. Due to its function, Chibby has been suggested as a potential tumor suppressor[5]. The aim of our study was to analyse whether Chibby expression is altered in colon carcinoma cell lines and tumors, and if so, whether restoration of Chibby amounts in colon carcinoma cell lines affects cell functions and behaviour.

Examining six colon carcinoma cell lines and one highly differentiated cell line (HT29 M3) that was used as additional control with regard to the mutational status, we could not detect any alterations within Chibby coding sequence. Using quantitative RT-PCR methods, we detected down-regulation of Chibby mRNA expression levels in all six cell lines in comparison to normal colon cells (CEC) or HT29 M3. We demonstrated in another study that gene silencing via promoter hypermethylation might be responsible for down-regulated transcript expression if the coding region does not harbour any mutations[8]. We, therefore, speculated that promoter hypermethylation might be a mechanism for Chibby down-regulation in the examined cell lines, but treating cells with the demethylating agent 5-azacytidine did not lead to sound induction of Chibby mRNA expression in all cell lines. SW48, SW480, and CaCo2 cells showed the lowest default expression levels among all the cell lines tested and displayed a moderate increase in Chibby mRNA expression upon 5-azacytidine treatment. But we do not reckon epigenetic alterations as a common mechanism for Chibby down-regulation.

To assess the functional implication of reduction of chibby expression, we used the cell line SW480 to re-express Chibby via transfection of a Chibby expression plasmid. Tests for correct expression of Chibby were followed by functional assays for proliferation and invasion. We were not able to detect altered invasion potential of Chibby over-expressing cells in comparison to control cells or mock transfected cells. Also, proliferation rate of control and Chibby transfected cells was not different (Figure 4) by means of doubling times. These findings were unexpected as Chibby seemed to be a promising candidate as tumor suppressor in colorectal carcinoma. We speculate that the experimental over-expression of Chibby in SW480 cells does not lead to changes in cell behaviour as mutations in other molecules further downstream within the cascade could overcome Chibby effects. This hypothesis is based on the knowledge that the SW480 cell line is known to harbour several mutations, not only in the Wnt/β-catenin signalling pathway but also in a number of other proto-oncogenes[16,17].

In the very contrast to our findings concerning Chibby expression in a panel of colon carcinoma cell lines, the *in situ* and *in vivo* analyses of colorectal tumors did not support the *in vitro* conclusions. Neither quantitative RT-PCRs, nor hybridisation of an oligonucleotide-based array gave an indication of dysregulation of Chibby in colorectal tumors in comparison to normal colorectal tissue. Our results are supported by a recent study by Gad *et al*[17] who have reported neither somatic mutations in samples of colorectal tumors from 36 patients, nor have they been able to correlate Chibby expression levels to tumorigenicity. Gad *et al*[17] have reported very similar findings to our data that also display no differences in expression levels between normal colon tissue and tumor material.

Taking these data together, we conclude that in contrast to colon carcinoma cell lines where Chibby expression was down-regulated in comparison to normal colonical epithelial cells, *in vivo* Chibby expression is not altered in colorectal tumors and therefore neither a cause nor an indicator for tumor development in colorectal carcinoma.

Our data point out that the use of colon carcinoma cell lines to analyse events within the Wnt/β-catenin signalling pathway has to be considered very carefully. The status of protein expression in these cell lines, at least with regard to Chibby, varies compared to that of tumors and may end up in different phenotypical behaviour of the cells. Thus, direct conclusions on *in vivo* situations could be misleading. Therefore, these differences have to be taken into account when performing *in vitro* studies on cellular signalling pathways.

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COLORECTAL CANCER

Identification of serum proteins discriminating colorectal cancer patients and healthy controls using surface-enhanced laser desorption ionisation-time of flight mass spectrometry

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Abstract

AIM: To detect the new serum biomarkers for colorectal cancer (CRC) by serum protein profiling with surface-enhanced laser desorption ionisation - time of flight mass spectrometry (SELDI-TOF MS).

METHODS: Two independent serum sample sets were analysed separately with the ProteinChip technology (set A: 40 CRC + 49 healthy controls; set B: 37 CRC + 31 healthy controls), using chips with a weak cation exchange moiety and buffer pH 5. Discriminative power of differentially expressed proteins was assessed with a classification tree algorithm. Sensitivities and specificities of the generated classification trees were obtained by blindly applying data from set A to the generated trees from set B and vice versa. CRC serum protein profiles were also compared with those from breast, ovarian, prostate, and non-small cell lung cancer.

RESULTS: Mass-to-charge ratios (m/z) 3.1×10^3, 3.3×10^3, 4.5×10^3, 6.6×10^3 and 28×10^3 were used as classifiers in the best-performing classification trees. Tree sensitivities and specificities were between 65% and 90%. Most of these discriminative m/z values were also different in the other tumour types investigated. M/z 3.3×10^3, main classifier in most trees, was a doubly charged form of the 6.6×10^3-Da protein. The latter was identified as apolipoprotein C-I. M/z 3.1×10^3 was identified as an N-terminal fragment of albumin, and m/z 28×10^3 as apolipoprotein A-I.

CONCLUSION: SELDI-TOF MS followed by classification tree pattern analysis is a suitable technique for finding new serum markers for CRC. Biomarkers can be identified and reproducibly detected in independent sample sets with high sensitivities and specificities. Although not specific for CRC, these biomarkers have a potential role in disease and treatment monitoring.

Key words: Proteomics; Colorectal cancer; Biomarker; Sensitivity; Specificity

Engwegen JYMN, Helgason HH, Cats A, Harris N, Bonfrer JMG, Schellens JHM, Beijnen JH. Identification of serum proteins discriminating colorectal cancer patients and healthy controls using surface-enhanced laser desorption ionisation-time of flight mass spectrometry. *World J Gastroenterol* 2006; 12(10): 1536-1544

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INTRODUCTION

Colorectal cancer is the third most common cause of cancer-related death in both men and women, accounting for about 10% of all cancer deaths annually. When diagnosed and treated early, the overall 5-year survival rate is around 90%. However, most patients present with locally advanced or metastasised disease at the time of diagnosis, or develop metastasis during follow-up. Suitable tumour markers will facilitate colorectal cancer detection, determination of prognosis, and disease and therapy evaluation.

However, currently used non-invasive methods, such as measurement of serum carcinoembryonic antigen (CEA) levels, faecal occult blood testing and faecal DNA analysis,

have low sensitivities and/or specificities for colorectal cancer[1-4]. Although CEA is currently the best available marker for follow-up of resected colorectal cancer and monitoring of chemotherapy, its use to determine eligibility for adjuvant therapy or its routine use as a single parameter for treatment monitoring has significant clinical limitations[5,6].

Detection of so-called biomarker proteins in serum may lead to new and better tumour markers for colorectal cancer. The proteome, contrary to the genome, is not a static parameter: it reflects not only the presence of active or inactive (mutated) genes, but also their extent of expression at a specific time point. In addition, the proteome reflects all proteins and peptides that may rise from only one gene, i.e. different cleavage products and proteins with different post-translational modifications. Both characteristics allow a more detailed evaluation of a disease status using the human proteome.

Protein profiling in complex biological matrices has become more easily achievable with the Surface-Enhanced Laser Desorption Ionisation (SELDI) ProteinChip technology in combination with a time of flight (TOF) mass spectrometer. This is a relatively new technique, which lacks the disadvantages of 2D-gel-electrophoresis for proteomic research in that it has high sensitivity in the low molecular weight range and high throughput capability, and proteins with extreme characteristics (highly hydrophobic, acidic or basic) can be analysed more easily[7,8]. With this technique, whole serum is applied to protein chips with different chromatographic affinities in a suitable binding buffer. Selectively bound proteins are retained on the surface and non-selectively bound proteins are washed off. In the mass spectrometer, a laser desorbs the bound proteins from the chip surface, which are subsequently detected in the TOF analyser by their respective mass-to-charge ratios (m/z). Since a whole pattern of proteins is analysed, more than one biomarker can be detected. Combination of several of these biomarkers for the evaluation of a patient's status may result in enhanced sensitivities and specificities.

SELDI-TOF MS has already been applied to several forms of cancer, including breast, ovarian, prostate, and lung cancer[9-12]. In the obtained protein profiles, proteins with high sensitivity and specificity for disease have been detected. For colorectal cancer, a discriminative protein of $12*10^3$ Da has been found in tumour cell lines, the identity of which was prothymosin-alpha[13]. Comparing epithelial colorectal carcinoma cells with normal tissue, 3.48×10^3-, 3.55×10^3- and 3.6×10^3-Da proteins were found to be increased in cancer tissue[14]. In Asian patients with colorectal cancer and healthy controls, discriminating serum protein profiles have been recently reported, m/z 5911, 8922, 8944 and 8817 being the most important biomarkers[15-18]. These results were obtained in a single sample set and the reported sensitivities and specificities resulted from cross-validation within this single set. In addition, the identities of the reported biomarker proteins remain unknown.

The objective of this study was to detect biomarker proteins for colorectal cancer in serum using SELDI-TOF MS, and to validate these with an independent sample set. In addition, we aimed at identifying any found biomarkers so that further insight into the pathological processes involved in colorectal cancer can be obtained.

MATERIALS AND METHODS

Patient samples

Two independent serum sample sets were analysed for their protein profiles on different occasions. The first set consisted of samples from 40 patients with colorectal cancer (all Dukes' D) and 49 healthy controls. The second set consisted of samples from 37 patients with colorectal cancer (1 Dukes' A, 2 Dukes' B, 12 Dukes' C, 17 Dukes' D, 5 unknown) and 31 healthy controls. For comparison of colorectal cancer protein profiles with those from other tumour types, a third sample set consisting of serum samples from 8 non-small cell lung cancer (NSCLC) patients (stage III and IV), 10 breast cancer patients (stage II and III), 10 prostate cancer patients (stage I-IV), and 10 ovarian cancer patients (stage I-IV) was analysed. All serum samples were obtained from the serum bank at the Netherlands Cancer Institute, where they were stored at -30 ℃ until analysis. Sample collection was performed after taking individuals' informed consent under approval of the Institutional Review Board. Samples were drawn before surgery or chemotherapy was started, except for 9 patients with metastatic disease in sample set B who had already had surgery.

Protein profiling

Protein profiling was performed using SELDI-TOF MS (Ciphergen Biosystems Inc., Freemont, CA, USA). Several chromatographic chip surfaces and binding conditions were screened for discriminative m/z values between colorectal cancer patients and healthy controls. The most discriminating peaks were seen on CM10 chips, a weak cation exchange chip, which contains anionic carboxylate groups that bind positively charged proteins in serum. Best results were obtained using a sodium phosphate binding buffer (pH 5) and a 500 mL/L solution of sinapinic acid (SPA; Ciphergen Biosystems) in 500 mL/L acetonitrile (ACN) + 5 mL/L trifluoracetic acid (TFA) as energy absorbing molecule.

All serum samples were denatured by adding 180 μL of 9 mol/L urea, 20 g/L CHAPS, 10 g/L DTT (all from Sigma, St. Louis, MO, USA) to 20 μL of serum. CM10 chips were assembled in a 96-well format bioprocessor (Ciphergen Biosystems) which can hold twelve 8-spot protein chips. During all steps of the protocol, the bioprocessor was placed on a platform shaker at 350 r/m. Chips were equilibrated twice with 200 μL of binding buffer consisting of 20 mmol/L sodium phosphate (Sigma) buffer (pH 5) with 1 g/L TritonX-100 (Sigma) for 5 min. Subsequently, 180 μL of binding buffer and 20 μL of denatured sample were applied to the chip. Sample allocation was at random. Incubation was set to 30 min. After binding, the chips were washed twice for 5 min with binding buffer, followed by two 5-min washes with binding buffer without TritonX-100. Lastly, chips were rinsed with deionised water. After air-drying, two times 1 μL of the SPA was applied to the spots.

Protein chips were analysed using the PBS-IIC

ProteinChip Reader (Ciphergen Biosystems). Data were collected between 0 and 200 000 Da. Data collection was optimised for detection of discriminating peaks, resulting in an average of 65 laser shots per spectrum at laser intensity 150 and detector sensitivity 8, and laser focusing at 3000 Da. M/z values for the detected proteins were calibrated externally with a standard peptide mixture (Ciphergen Biosystems) containing [Arg8] vasopressin (1 084.3 Da), somatostatin (1 637.9 Da), dynorphine (2 147.5 Da), ACTH (2 933.5 Da), insulin β-chain (bovine) (3 495.9 Da), insulin (human recombinant) (5 807.7 Da), and hirudin (7 033.6 Da).

Statistics and bioinformatics

Data were analysed with ProteinChip Software package, version 3.1 (Ciphergen Biosystems). For each sample set, all acquired spectra were compiled and analysed as a whole. Spectra were baseline subtracted and normalised to the total ion current between 1 500 and 200 000 Da. For validation of either sample set, the normalisation factor from the training set was applied to the spectra of the validation set. The Biomarker Wizard (BMW) software application (Ciphergen Biosystems) was used to autodetect m/z peaks with a signal-to-noise ratio of at least 5. Peak clusters were completed with peaks with a signal-to-noise ratio of at least 2 in a 0.5% cluster mass window. For validation purposes, peak clusters of the training set were applied in the validation set. Group differences were calculated with the same application, comparing mean intensities of all detected peaks between groups with non-parametric statistical tests. *P* values less than 0.01 were considered statistically significant.

Next, Biomarker Patterns Software (BPS; Ciphergen Biosystems) was used to generate classification trees from the BMW files. A classification tree is built of nodes with an m/z value and a cut-off value for the peak intensity. An example of such a tree is shown in Figure 1. When an analysed spectrum has a peak intensity at the specified m/z below the cut-off value, the sample is placed in the left tree branch. Otherwise, it is placed to the right and its peak intensity at the next m/z value is evaluated. Peaks that result in a maximum separation of the two groups, with a minimum of misclassification are chosen for the nodes. The branch consists of new nodes with an m/z value until a final classification can be made for the spectrum: originating from a colorectal cancer patient or from a healthy control. For every tree, the BPS performs a ten-fold cross-validation in the tree building process, in which ten times another tenth of the data set is used for testing of the tree and these results are combined to yield a cross-validation sensitivity and specificity as a measure for the tree's discriminative power. However, to obtain a more realistic sensitivity and specificity, classification trees built with one sample set as the training set were validated with the blinded data from the remaining set. In addition, both sample sets were combined to form a training set with two thirds of all samples keeping a random third behind in the tree building process for independent validation afterwards.

Biomarker purification and identification

Biomarkers detected in the profiling experiments were purified by fractionation of denatured serum on QhyperD beads (Biosepra; Ciphergen Biosystems), a strong anion exchange surface, with decreasing buffer pH. Subsequently, fractions containing the markers were concentrated on microcon YM-50 filters (Millipore, Billerica, MA, USA) and eluted with increasing concentrations of ACN + TFA (1: 0.001, v/v). The purification process was monitored by profiling each fraction on NP20 chips, containing a non-selective, silica chromatographic surface. Relevant eluates were evaporated and redissolved in loading buffer for SDS-PAGE. Gel electrophoresis was performed on Novex NuPage gels (Invitrogen, San Diego, CA, USA). Gels were stained using colloidal Coomassie staining (Simply Blue; Invitrogen) and protein bands of interest were excised and collected for either passive elution, followed by in-solution digestion or in-gel digestion with trypsin (Promega, Madison, WI, USA).

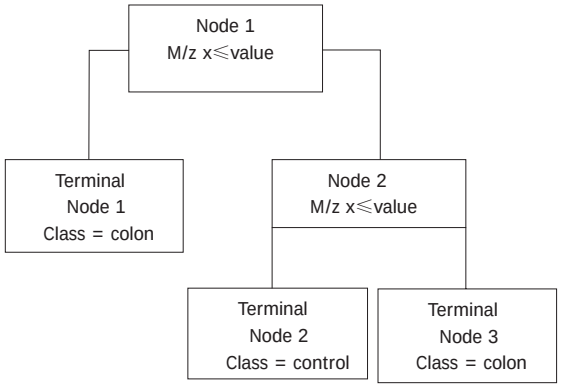


Figure 1 Example of a BPS-generated classification tree distinguishing colorectal cancer patients and healthy controls. If the peak intensity of an analyzed sample is below the cut-off value at the m/z in the node, the sample proceeds to the left. If not, it proceeds to the right, where its peak intensity at the next m/z is evaluated.

For passive elution, bands were washed twice with 300 mL/L ACN + 100 mmol/L NH_4HCO_3 (Sigma), followed by dehydration in ACN. Samples were heated at 50 °C and then eluted with 15 μL of formic acid/ACN/isopropanol/deionised water (4.5:3:1:1.5, v/v) under sonification for 30 min. The eluate was left for 3 h at room temperature before profiling on NP20 chips. Then, the eluate was left overnight. In-solution digests were obtained by evaporation of the supernatant in a SpeedVac, resuspending it in 20 mg/L trypsin in 100 mL/L ACN + 25 mmol/L NH_4HCO_3 and incubation for 4 h at room temperature.

In-gel digestion was performed after washing the excised band with methanol/acetic acid/deionised water (4:1:5, v/v) twice, followed by a wash with 300 mL/L ACN + 100 mmol/L NH_4HCO_3. Samples were dried on a SpeedVac and immersed in a 20 mg/L-solution of trypsin in 100 mmol/L NH_4HCO_3. Digestion was allowed for 12 h at room temperature. All tryptic digests were profiled on NP20 chips, using 1 μL 200 g/L CHCA (Ciphergen Biosystems) solution in 500 mL/L ACN + 5 mL/L TFA as matrix.

Peptides in the resulting digest were investigated with the MASCOT and ProFound search engines (http://www.matrixscience.com; http://prowl.rockefeller.edu/profound_bin/WebProFound.exe), using the Swiss-Prot

Table 1 Classification trees generated with the Biomarker Patterns Software

Tree	Included m/z's ($\times 10^3$ Da): cut-off intensity values and class assignment				Sensitivity (%)	Specificity (%)
	Node 1	Node 2	Node 3	Node 4		
I	3.3 ⩽ 15.035	-	-	-	77.8	73.3
	Colorectal cancer					
II	28 ⩽ 1.558	4.5 ⩽ 29.791	3.1 ⩽ 9.866	6.6 ⩽ 33.233	77.8	73.3
	Colorectal cancer	to Node 3	to Node 4	Colorectal cancer		
III	3.3 ⩽ 12.757	-	-	-	66.7	83.3
	Colorectal cancer					
IV	3.3 ⩽ 12.757	28 ⩽ 1.285	-	-	75.0	83.3
	Colorectal cancer	Colorectal cancer				
V	3.3 ⩽ 12.981	4.5 ⩽ 28.599	-	-	84.2	83.3
	Colorectal cancer	Healthy control				
VI	28 ⩽ 1.529	4.5 ⩽ 28.577	6.6 ⩽ 44.685	-	89.5	88.9
	Colorectal cancer	to Node 3	Colorectal cancer			

Sensitivities and specificities for trees I to IV were obtained by blinded testing with data from the remaining sample set.

and NCBI databases, respectively. Data were searched against the Homo sapiens subset of the database, defining fixed modification of the cysteine residues with propionamide and variable modification of methionine residues (oxidation). Peptide mass tolerance of the average MH^+ masses was 0.5 - 3 Da; the number of tryptic miscleavages allowed was 1 or 2.

Identification of proteins was confirmed either by immunoassay on protein A beads (Biosepra; Ciphergen Biosystems) with an appropriate antibody (Abcam Ltd, Cambridge, UK), or by sequencing of the most important peptides in the tryptic digest with tandem MS on both a Q-TOF™ II, (Micromass Ltd, UK) and a QSTAR ™ (AB/Sciex, Foster City, CA, USA), both equipped with a PCI 1000 interface (Ciphergen Biosystems). For the immunoassay, beads were loaded with antibody in phosphate-buffered saline (PBS, Sigma), and washed twice with PBS, followed by a 30-min incubation with whole serum, 5 subsequent washes with PBS and one with deionised water. Finally, bound proteins were eluted using 1 mol/L acetic acid and the eluate was profiled on NP20 chips.

Serum CRP, TRF and CEA quantification

Serum CEA was quantified using an electrochemiluminiscence immunoassay on a Modular analytics E170 analyser (Roche Diagnostics, Mannheim, Germany). A cut-off value of 5 μg/L was employed. Levels of the acute phase reactants C-reactive protein (CRP) and transferrin (TRF) were assessed by a near infrared particle immunoassay and a turbidimetric immunoassay, respectively, using the Beckman Synchron LX20 analyser (Beckman-Coulter Inc., Fullerton, CA, USA). CRP levels below 8 mg/L were considered clinically normal, as are TRF levels between 2.1 and 3.8 g/L. All statistical analyses for these data were performed with SPSS, version 11.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Biomarker detection

In the first and second sample set respectively, 15 and 6 proteins of which the expression differed in colorectal cancer patients compared to healthy controls ($P < 0.01$) were detected with the BMW application. Peaks below 2 000 Da were discarded, as they result mainly from the SPA matrix. In either sample set, m/z values of $3.2*10^3$, $3.3*10^3$, 6.4×10^3, 6.6×10^3, 6.8×10^3, and 28×10^3 were differentially expressed. Expressions of m/z 2.7×10^3, 3.1×10^3, 4.2×10^3, 4.3×10^3, 4.5×10^3, 8.0×10^3, 8.9×10^3, 14×10^3, and 16×10^3 significantly differed only in sample set A.

With the BPS several classification trees were built. Tree characteristics of the best-performing trees with accompanying sensitivities and specificities are described in Table 1. Tree I and II were generated from sample set A, tree III and IV from sample set B, and tree V and VI from the combination of sample sets A and B. Tree sensitivities and specificities for trees I to IV were obtained using the second sample set as validation set. For trees V and VI, the sensitivity and specificity were calculated by randomly choosing one third of all data to be excluded from the tree building process for use as validation data afterwards. As shown in Table 1, m/z 3.3×10^3 was the most frequently observed classifier among these best trees. When removing this classifier from the tree-building model, equally- or better-performing trees were seen with m/z 28×10^3 as main classifier (trees II and VI). Other biomarkers used in the trees include m/z 3.1×10^3, 4.5×10^3, and 6.6×10^3. Of these, m/z 3.1×10^3 and 4.5×10^3 were more abundant in colorectal cancer serum samples compared to healthy controls, whereas the others were less abundant. Parts of representative MS-spectra for patients and controls are shown in Figure 2.

Biomarker selectivity

To determine the selectivity of the observed protein profiles for colorectal cancer when compared to other cancer forms, additional samples from patients with other tumours were analyzed. This third sample set was analyzed concomitantly with 17 of the previously analyzed samples from colorectal cancer patients (10 from sample set A, 7 from B) and 20 previously analyzed control samples (10 from either sample set) using the same assay procedures.

When examining peak intensity differences between cancer patients and healthy controls by means of the Biomarker Wizard application, most of the biomarkers for

Table 2 BMW expression differences of colorectal cancer biomarkers in other tumors

Group	3.1×10³ Da		3.3×10³ Da		4.5×10³ Da		6.6×10³ Da		28×10³ Da		5.9×10³ Da	
	Intensity	P (×10⁻³)	Intensity	P (×10⁻³)	Intensity	P (×10⁻³)	Intensity	P (×10⁻³)	Intensity	P (×10⁻³)	Intensity	P (×10⁻³)
HC (n=20)	5.51 (2.4)		14.9 (4.73)		20.6 (9.16)		52.1 (7.4)		2.73 (1.25)		7.26 (4.23)	
CRC (n=17)	7.95 (4.64)	211	9.05 (3.81)	1.11	25.5 (5.99)	12.4	37.6 (9.17)	0.0388	1.44 (0.65)	0.801	10.84 (7.66)	161
BC (n=10)	8.87 (2.59)	1.13	11.51 (3.4)	103	19.44 (8.16)	538	42.7 (7.58)	5.59	1.44 (0.59)	3.69	4.51 (2.55)	43.0
NSCLC (n=8)	5.00 (2.93)	508	7.93 (3.38)	2.27	25.1 (7.93)	93.3	30.9 (11.2)	0.205	0.942 (0.45)	0.450	1.91 (2.13)	0.305
OC (n=10)	7.29 (3.77)	218	9.89 (2.52)	3.69	27.7 (8.67)	27.8	40.6 (8.65)	2.07	1.31 (0.45)	0. 968	2.98 (1.47)	1.32
PC (n=10)	7.65 (3.25)	131	9.59 (4.35)	7.21	29.6 (10.1)	18.4	36.5 (11.5)	0.967	1.26 (0.56)	1.35	2.73 (2.01)	1.86

For each m/z value, mean peak intensities (SD) are given with their P-values for the intensity difference with healthy controls (HC). BC: breast cancer; NSCLC: non-small cell lung cancer; OC: ovarian cancer; PC: prostate cancer.

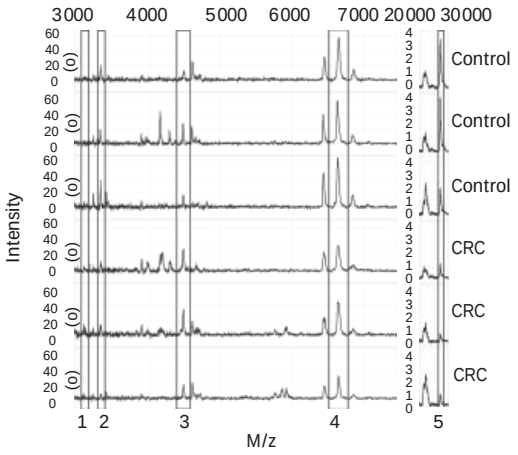


Figure 2 Spectra from colorectal cancer patients and controls. Biomarker proteins are boxed: 1=3.1×10³ Da, 2=3.3×10³ Da, 3=4.5×10³ Da, 4=6.6×10³ Da, 5=28×10³ Da.

colorectal cancer were found to be discriminative for other cancer forms as well (Table 2). Except for breast cancer samples, m/z 3.3×10³, 6.6×10³, and 28×10³ were markedly less abundant in all types of cancer compared to the control samples (P<0.01). For these tumour types, mean peak intensities were not significantly different from those for colorectal cancer, independent of patient characteristics (data not shown). There was a tendency for a significant increase of m/z 4.5×10³ in ovarian and prostate cancer (P<0.05). In breast cancer patients, no significance was reached for peak intensity differences of m/z 3.3×10³, and 4.5×10³ (P=0.10 and P=0.54, respectively). However, m/z 3.1×10³ was found to be significantly increased only in breast cancer samples (P=0.0011), but not in other cancer types.

A decision tree combining data from all tumour types was built with the BPS. Although most of the earlier observed biomarkers were discriminative for all other cancer forms as well, 76% of samples from colorectal cancer patients could be correctly distinguished from those of other cancers based on a classifier peak at m/z 5.9×10³. In samples from colorectal cancer, peak intensities for this m/z were slightly higher compared to the controls, whereas they were significantly lower than the controls in the other cancers (Table 2). In addition, data from the other tumour types were applied to the trees in Table 1. For all trees, more than 89% of patients with other cancers than colorectal cancer were classified as having colorectal cancer, except for tree VI, in which this was 78.4%.

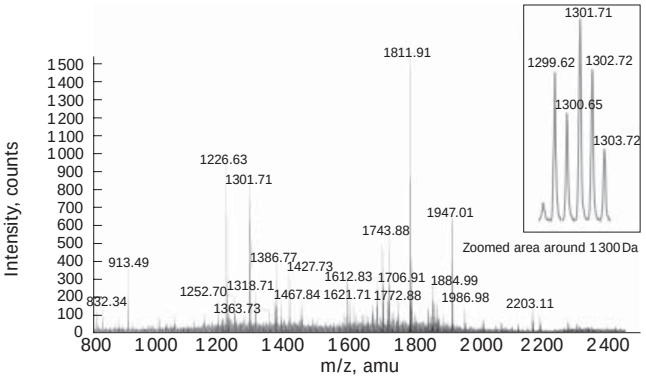
Biomarker purification and identification

Fractionation of whole serum from colorectal cancer patients and controls resulted in elution of the 6.6×10³-Da marker mainly in the flowthrough (pH 9), and the 28*10³-Da marker in the pH 4 fraction. Following concentration on YM-50 filters, the 6.6×10³-Da marker was seen in the 200 mL/L- and 300 mL/L-ACN eluates mostly, more purified from surrounding masses in the latter. The 28×10³-Da marker was present in the filter wash (1 mL/L TFA).

SDS-PAGE of selected eluates was performed on a 120 g/L Bis-Tris gel for the 28×10³-Da marker and a 180 g/L Tris-glycine gel for the 6.6×10³-Da marker. For this marker, both the 200 mL/L- and 300 mL/L-ACN eluates were placed on gel. A clear band was seen for the 28×10³-Da protein, which was divided in half for both passive elution and in-gel digestion. For the 6.6×10³-Da marker, a number of faint bands was seen in the 6000-Da region, in both the 200 mL/L- and 300 mL/L-ACN eluates. All were excised. Bands from the 200 mL/L-ACN eluate were subjected to passive elution, and from the 300 mL/L-eluate to in-gel digestion. Profiling of gel eluates on NP20 confirmed the masses to be indeed 6.6×10³ and 28×10³ Da.

Peptide mapping results revealed the identity of the 28×10³-Da marker to be apolipoprotein A-I. The theoretical mass of this protein is 28 078.62 Da in the SwissProt database and its pI=5.27. The apolipoprotein A-I identity was confirmed by tandem MS of the 1 299.62-, 1301.71-, 1612.83- and 1386.77-Da peptides in the tryptic digest (Figure 3).

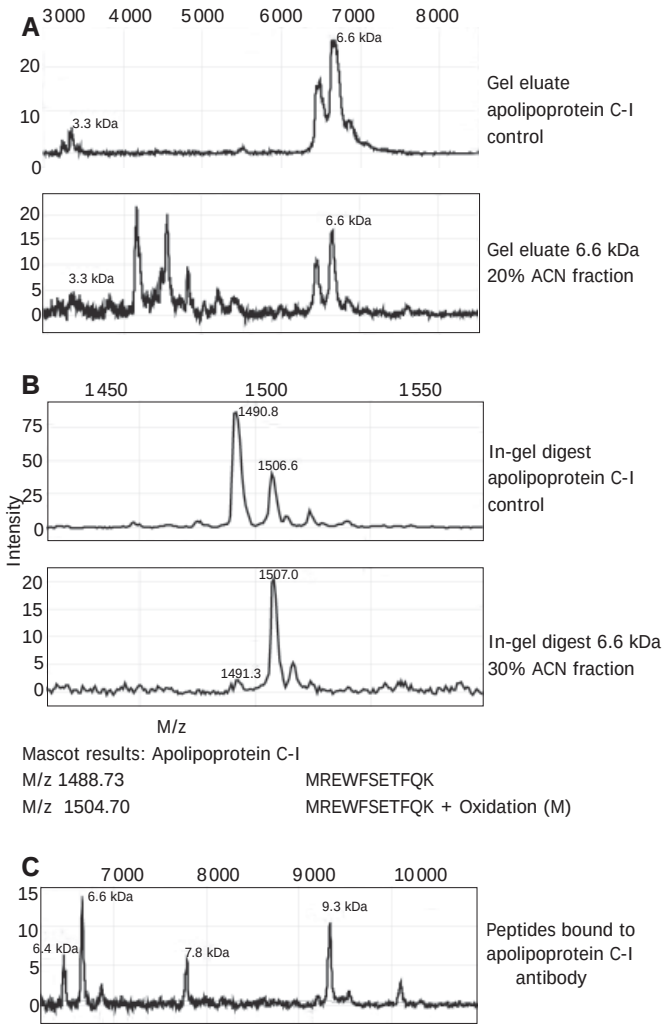
The 6.6×10³-Da marker was identified as apolipoprotein C-I, with a theoretical mass of 6 630.58 Da and pI=7.93. Spectra of this identification are shown in Figure 4. Confirmation of the apolipoprotein C-I identity was performed on protein A beads using a goat apolipoprotein C-I polyclonal antibody. The eluate's MS-spectrum clearly showed a large peak at 6.6×10³ and another prominent peak at 9.3×10³. The mass of the latter peak corresponded to that of apolipoprotein C-I precursor. In addition, the passive elution of the apolipoprotein C-I control (Figure 4A) showed a peak at 3.3×10³. The 3.3×10³-Da biomarker found in our sample sets A and B, as well as the set combined with other tumour types, consistently showed a high correlation with the 6.6×10³-Da one: the ratio of their



Mascot peptide mapping results: apolipoprotein A-I

Start-End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
70-83	1612.83	1611.82	1611.78	0.04	0	**LLDNWDSVTSTFSK**
84-101	2203.11	2201.10	2201.11	-0.01	1	LREQLGPVTQEFWDNLEK
131-140	1427.73	1426.72	1426.66	0.07	1	KWQEEMELYR Oxidation (M)
132-140	1299.62	1298.61	1298.56	0.05	0	**WQEEMELYR Oxidation (M)**
143-155	1467.84	1466.83	1466.78	0.05	1	VEPLRAELQEGAR
251-262	1386.77	1385.76	1385.71	0.05	0	**VSFLSALEEYTK**
185-195	1301.71	1300.70	1300.64	0.06	0	**THLAPYSDELR**

Figure 3 Peptide mapping of 28×10^3-Da apolipoprotein A-I. MS spectrum of the 28×10^3-Da in-gel tryptic digest. Results from the MASCOT search for protein identification include start and end positions of the found peptide sequence starting from the amino acid terminal of the whole protein, the observed m/z, transformed to its experimental mass [Mr(expt)], the calculated mass [Mr(calc)] from the matched peptide sequence, as well as their mass difference (Delta), the number of missed cleavage sites for trypsin (Miss) and the peptide sequence. Peptides in bold were sequenced with tandem MS using Q-TOF for confirmation.



Mascot results: Apolipoprotein C-I

M/z 1488.73	MREWFSETFQK
M/z 1504.70	MREWFSETFQK + Oxidation (M)

Figure 4 Identification of apolipoprotein C-I. **A:** Parts of the MS spectra of the gel eluates of an apolipoprotein C-I control and the 6.6×10^3-Da protein isolated from HC serum run on the same gel. **B:** Parts of the MS spectra of the in-gel digests of an apolipoprotein C-I control and the 6.6×10^3-Da protein isolated from HC serum and the results of sequencing of these two peptides with tandem MS. **C:** Part of the MS spectrum of the eluate from the apolipoprotein C-I antibody. Apart from the expected peaks at 9.3×10^3, apolipoprotein C-I precursor, and 6.6×10^3, a 6.4×10^3-Da peak is seen, which is a known fragment of apolipoprotein C-I missing two N-terminal amino acids. The mass at 7.8×10^3 is unknown and does not correspond to any of the apolipoproteins with which antibody cross-reactivity can occur. It is possibly an intermediate splice form of the precursor protein.

peak intensities was quite constantly ranging between 3.5 and 4.0 (Table 2). This supports the fact that the observed 3.3×10^3-Da marker is actually a doubly charged artefact of the 6.6×10^3-Da protein. The 3.1×10^3-Da protein was lost during the purification process and was therefore directly sequenced on-chip. It was identified as a 27-amino acid N-terminal fragment of albumin with sequence: DAHKS EVAHRFKDLGEENFKALVLIAF. The identity of the 4.5×10^3-Da protein is still under investigation.

Serum CRP, TRF and CEA levels

Evaluation of the extent of a possible acute phase reaction was done by measurement of CRP and TRF levels. Mean TRF levels in the patient and control group were 2.37 g/L (range, 1.20-3.60 g/L) and 2.59 g/L (range, 1.90-4.00 g/L), respectively (P=0.037, non-parametric Mann-Whitney U test). Mean CRP levels in either group were 29.0 mg/L (range, 0 - 213 mg/L) and 3.70 mg/L (range, 0 - 29.2 mg/L) (P<0.000, non-parametric Mann-Whitney U test). Although there was a significant difference in the levels of these acute phase reactants in the patients and controls, the mere presence of an acute phase response was not a good predictor for colorectal cancer: the sensitivities of CRP and TRF were 51.9% (40/77) and 22.1% (17/77), respectively, using the clinical cut-off values [specificities 88.8% (71/80) and 96.3% (77/80), respectively]. In addition, CRP and TRF levels were included in the BMW data files for the tree-building process with the BPS, in order to evaluate their capability to distinguish colorectal cancer patients and healthy controls. Neither CRP, nor TRF concentrations were as good a classifier as the m/z values in the generated trees.

Mean serum CEA in the colon group was significantly higher (mean 326.2 μg/L, range <1-9 452) compared to the control group (mean 2.23 μg/L, range <1-18.98) (P<0.001, non-parametric Mann-Whitney U test). Using a cut-off value of 5 μg/L, its sensitivity and specificity were found to be 75.3% and 95.0%, respectively, for all samples combined. This sensitivity was lower than that for the proteins in the classification trees generated with all samples (V and VI), but the specificity of CEA in this population was higher. Assessing CEA sensitivity in the total sample set according to colorectal cancer stage, using the 5 μg/L cut-off, resulted in correct classification of 0 of 1 Dukes' A, 0 of 2 Dukes' B, 3 of 12 (25.0%) Dukes' C, and 51 of 57 (89.5%) Dukes' D. In comparison, using the total sample set and the trees generated with the sets (V and VI), 1 of 1 Dukes' A, 1 of 2 Dukes' B, 11 of 12 (91.7%)

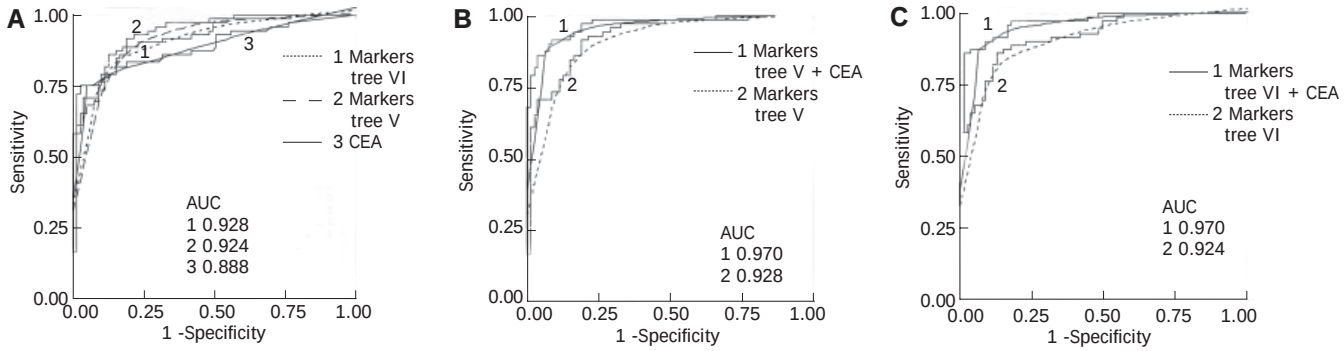


Figure 5 ROC curves for biomarker proteins from trees V and VI with and without log(CEA). Areas under the curve (AUC) are given for each model.

Dukes' C, and 47 of 57 (82.5%) Dukes' D were correctly classified. In addition, logistic regression was performed for CEA and the markers from tree V and VI, and receiver operating characteristic (ROC) curves were generated. As shown in Figure 5, combining the markers from each tree with log-transformed CEA values yielded a higher area under the ROC curve than for either alone.

DISCUSSION

In this study, five biomarker proteins were detected that were able to reliably distinguish colorectal cancer patients and healthy controls using the SELDI-TOF MS technique for protein profiling. Two of these were identified as apolipoprotein C-I (6.6×10^3 Da) and apolipoprotein A-I (28×10^3 Da). Using the ProteinChip Software, the 3.3×10^3-Da protein could be identified as a doubly charged form of the 6.6×10^3-Da apolipoprotein C-I, which was confirmed by its appearance in the MS spectrum of pure apolipoprotein C-I isolated from a gel. The m/z of 3.1×10^3 was found to be an N-terminal fragment of albumin. In addition, the detection of these biomarkers' expression difference was shown to be reproducible on two separate occasions, considering the obtained classification tree sensitivity and specificity between 65% and 90% when using the second sample set as a blinded validation set. Such reproducible detection is imperative for any future use as a clinical tool. Sensitivities and specificities obtained with data from the blinded sample set were comparable to those obtained by cross-validation within one sample set (data not shown), which supports the fact that there was no additional misclassification of samples due to experimental variability between the two sample sets.

Several reports have been made of differential expression of the same m/z values in colorectal cancer, even though different chip surfaces were used. Yu *et al*[17] reported a 3 329- and 6 669-Da protein to be differentially expressed on a hydrophobic chip surface, that were not selected in the final diagnostic pattern. In the same study, a 4 477-Da protein, which was a classifier in the final pattern, was also up-regulated in colorectal cancer patients[17,18]. The 6.6×10^3- and 3.3×10^3-Da proteins were detected by Yu *et al*[17] but not identified. It is likely that these are apolipoprotein C-I and its doubly charged form, since this is a very hydrophobic protein and retention on a hydrophobic chip surface is very plausible. In fact, in our study population, these m/z values were also seen on hydrophobic chips (data not shown). This appearance of (likely) the same colorectal cancer biomarkers in different laboratories underlines their validity. Also, a 5.9×10^3-Da protein was reported, which was an up-regulated biomarker in serum of colorectal cancer patients[17,18]. Despite lack of significance in our study, there was a tendency for our 5.9×10^3-Da protein to be higher in patients than controls, which is consistent with the result from Yu *et al*[17] and indicates this may be the same protein. Data from a proteomic study by our group[19] on breast cancer patients have shown a 5.9×10^3-Da down-regulated protein in this cancer type, which is in concordance with the 5.9×10^3-Da protein in the current study. The protein in the former study was identified as a fragment of fibrinogen alphaE chain.

Apolipoprotein C-I is primarily synthesised in the liver and, to a minor degree, in the small intestine. Its function resides mainly in lipoprotein metabolism[20]. It is originally formed as a pro-peptide of 9.3×10^3 Da, which generates the mature protein upon cleavage during translation. To our knowledge, no previous reports about apolipoprotein C-I down-regulation in cancer have been made as yet. However, a 6.6×10^3-Da protein was detected and identified as apolipoprotein C-I in another SELDI-TOF MS study, being decreased in hemorrhagic *versus* ischemic stroke and hemorrhagic stroke *versus* controls on a strong anion exchange surface at pH 9[21]. Apolipoprotein A-I is synthesised both in the liver and small intestine and is a major constituent of HDL apolipoprotein. It is a known negative acute phase reactant, of which decreased expression has been described in several cancers, including a SELDI-TOF MS study on ovarian cancer[22-25]. In the latter study an immunoassay was peformed. In contrast to our data, the authors found no decreased apolipoprotein A-I levels in colorectal cancer. However, apolipoprotein A-I levels assessed by immunoassay may reflect concentrations of both bound pro-apolipoprotein A-I and apolipoprotein A-I. Increased expression of apolipoprotein A-I has been described in tissue of both liver metastases and, to a lesser extent, primary tumours of colorectal origin[26]. The observed decrease in serum levels in our study thus may be due to decreased liver synthesis. Other human proteomics studies in which differential expression

of apolipoprotein A-I has been described include a SELDI-TOF MS analysis of plasma from patients with diabetes, and several studies using 2D-gel electrophoresis in old *versus* young brain tissue, cerebrospinal fluid of patients with Alzheimer's disease, serum during infection with hepatitis B virus, and plasma during acute coronary syndrome[27-31]. In all these diseases, decreased levels of apolipoprotein A-I were observed. To our knowledge, the albumin fragment that was found in this study has not been described in the literature before. Albumin is synthesised with an 18-amino acid signal peptide and a 6-amino acid pro-peptide. Over-expression of this specific fragment may be caused by enhanced proteolytic activity, as increased proteolysis is common in cancer invasion and metastasis[32]. However, for this fragment, a correlation with sample age was seen in the colorectal cancer group (data not shown). Thus, it cannot be ruled out that it is a product of protein degradation upon storage.

Although the identification of apolipoprotein C-I and apolipoprotein A-I as biomarkers suggests an acute phase response, comparison with routine markers for establishing such a response, CRP and TRF, shows that our biomarkers are much more sensitive for colorectal cancer than these. The value of our biomarkers for detection of colorectal cancer was also evaluated by comparison with the predictive value of CEA. Sensitivity of our biomarkers was higher than that of CEA considering all samples. Stratification by Dukes' stages showed a significant better sensitivity of our classification trees (91.7%, 11/12) compared to CEA (25.0%, 3/12) in Dukes' C colorectal cancer, although at stage D CEA performed better. Combining log-transformed CEA in a logistic regression model with the markers in the trees resulted in a higher AUC in the ROC curve than for either log(CEA) or the combined tree classifiers alone. This indicates that our markers provide additional information to CEA values. CEA sensitivity has been reported to be lower in earlier stages of colorectal cancer. Sensitivity has been reported to vary between 3% and 66.7% for Dukes' A to D staged disease[2,18,33]. No conclusions can be drawn on the performance of our classification trees at earlier stages of colorectal cancer due to limited samples, but 2 of 3 patient samples from stage A and B were correctly classified by the trees and none when using the clinical cut-off for CEA.

Our results showed that it is very important to compare any biomarkers found for a certain type of cancer with those for other tumour types. This is lacking in most of the SELDI-TOF MS studies published so far. We found that most of our biomarker proteins were differentially expressed in other cancers as well. Lack of a significant difference at m/z 3.3×10^3 and 4.5×10^3 in breast cancer patients could be explained by the large proportion of early disease (9 of 10 stage 2) in this group compared to the others (mainly stage 3 and 4). However, since m/z 3.3×10^3 is the doubly charged 6.6×10^3-Da protein, which was significantly less expressed in breast cancer, this lack of significance is more likely due to slight differences in ionisation of this protein in this group. The fact that the 3.1×10^3-Da protein is not significantly different in ovarian and prostate cancer may result from the limited sample size, as for the smaller colorectal cancer group in this analysis with comparable mean peak intensity no significant difference at m/z 3.1×10^3 was observed either, although in sample sets A and B it was.

Even though most of our biomarkers are not specific for colorectal cancer, a potential role for them lies in therapy evaluation, disease surveillance or prognosis, possibly combined with CEA or other available markers. At present, CEA is recommended for monitoring chemotherapy, but no studies showing any benefit on survival, quality of life, or reduction of costs are available, although serial CEA testing may lead to earlier detection of progressive disease[5]. In addition, specificity of CEA for treatment monitoring can be compromised by transient increases during treatment with various chemotherapeutic drugs, such as 5-flourouracil and levamisole[5]. Since the expression profiles of our reported markers reliably reflect presence of cancer, be it colorectal cancer or not, changes in expression levels may correspond to response to therapy or disease progression and provide additional information to CEA levels.

In conclusion, our results show that SELDI-TOF MS is a suitable technique to find new serum biomarkers for colorectal cancer. The markers we have found in this study reliably distinguish colorectal cancer patients from healthy persons. Although not specific for colorectal cancer, they have a potential role as markers in treatment monitoring, disease surveillance, or prognosis, possibly in combination with other available markers. To extend the study population and evaluate the ability of our biomarkers to detect early-stage tumours and polyps, a prospective study is currently ongoing.

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Psychological impact of chronic hepatitis C: Comparison with other stressful life events and chronic diseases

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Abstract

AIM: To examine the psychological impact of chronic hepatitis C (CHC) diagnosis in a large cohort of CHC patients as compared with other stressful life events and chronic diseases carrying a risk of life-threatening complications.

METHODS: One hundred and eighty-five outpatients with compensated CHC were asked to self-grade, using a 100-mm visual analogue scale (VAS), the degree of stress caused by the learning of CHC diagnosis and the perceived severity of their disease. Diagnosis-related stress was compared to four other stressful life events and perceived CHC severity was compared to four other common chronic diseases.

RESULTS: Learning of CHC diagnosis was considered a major stressful event (mean ± SD scores: 72±25), significantly less than death of a loved-one (89±13, $P < 0.0001$) and divorce (78 ± 23, $P < 0.007$), but more than job dismissal (68 ± 30, $P < 0.04$) and home removal (26±24, $P < 0.0001$). CHC was considered a severe disease (74±19), after AIDS (94±08, $P < 0.001$) and cancer (91±11, $P < 0.001$), but before diabetes (66±23, $P < 0.001$) and hypertension (62±20, $P < 0.001$). Perceived CHC severity was not related to the actual severity of liver disease, assessed according to Metavir fibrosis score. In multivariate analysis, diagnosis-related stress was related to perceived disease severity ($P < 0.001$), trait anxiety ($P < 0.001$) and infection through blood transfusion ($P < 0.001$).

CONCLUSION: Our results show the considerable psychological and emotional burden that a diagnosis of CHC represents, even in the absence of significant liver disease. They should be taken into account when announcing a diagnosis of CHC in order to reduce its negative effects.

Key words: Hepatitis C; Stressful life event; Perceived severity; Visual analogue scale

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INTRODUCTION

Despite the fact that chronic hepatitis C (CHC) may lead to life-threatening complications such as cirrhosis, liver failure and hepatocellular carcinoma, most patients with CHC are asymptomatic and unaware of their liver disease prior to diagnosis. Nonetheless, these patients consistently report a significant reduction in health-related quality of life (HRQOL) as compared with population controls[1-6]. This impairment of HRQOL has been documented even in the absence of significant liver disease[3] and is usually improved after sustained viral clearance[4,5]. In addition, clinically significant emotional distress and depressive disorders have been reported in untreated CHC patients with a prevalence ranging from 11 to 53%[2,7-15], questioning the role of HCV itself in their occurrence[16]. Whether these disorders are due to the uncertainty of living with a chronic disease with potential life-threatening complications or to other psychosocial factors remains unclear. However, it has been recently shown that CHC patients aware of their diagnosis had worse HRQOL scores as compared with unaware seropositive patients[6,17], suggesting that the psychological impact of diagnosis knowledge may play an important role.

The aim of this prospective study was to examine the psychological impact of CHC diagnosis in a large cohort of CHC patients as compared with other stressful life events and chronic diseases carrying a risk of life-threatening complications.

MATERIALS AND METHODS

Study population

A total of 185 patients with a confirmed diagnosis of HCV infection (defined as detectable hepatitis C antibodies and HCV RNA in serum) attending the Hepatology Clinics at the University of Bordeaux were studied. Exclusion criteria were: age below 18 years, decompensated cirrhosis, co-infection by hepatitis B virus or human deficiency virus (HIV), current psychotic or manic disorders, obvious intellectual impairment, and inability to communicate in French.

Measures

Immediately prior to or following an outpatient clinic appointment, and after their written informed consent had been obtained, participants were asked to self-grade, using a 100-mm visual analogue scale (VAS), the degree of stress caused by the learning of CHC diagnosis. On that scale, the left endpoint 0 was defined as not stressful at all, the right endpoint 100 as extremely stressful. There were no further marks on the line. Instructions were as follows: "Put a mark on the line at the point that best describes how stressful you consider the learning of hepatitis C diagnosis". The degree of stress experienced was indicated by the distance in millimeters from the left endpoint. Perceived disease severity was assessed using the same method. VAS has been shown to be a reliable tool to assess subjective variables, such as stress[18], pain[19] and fatigue[20].

In addition to validate the reproducibility of measurements over time, a second assessment was performed 3 mo later in a subgroup of randomly selected patients.

Trait anxiety was assessed using the Spielberger State Trait Anxiety Inventory[21], a 20-item standardized self-report scale ranging from 20 (low tendency to be anxious) to 80 (high tendency to be anxious).

The influence on diagnosis-related stress of age, gender, marital status, educational level, route of HCV transmission, time since diagnosis, antiviral therapy, perceived severity of illness, actual severity of liver disease (assessed according to the fibrosis score of the METAVIR scoring system[22] in patients in whom a liver biopsy specimen was available), and trait anxiety was also studied.

Comparison with other stressful life events and chronic diseases

Using VAS mean scores, diagnosis-related stress was compared to four other stressful life events (i.e., home removal, job dismissal, divorce, or death of a loved-one) selected from the Paykel life-event scale[23]. Perceived CHC severity was compared to four other common chronic diseases carrying a risk of life-threatening complications (i.e., hypertension, diabetes mellitus, cancer and AIDS).

Statistical analysis

VAS scores for diagnosis-related stress and perceived disease severity were expressed as mean ± SD. Chi-square test was used for categorical variables and one-way analysis of variance (ANOVA) for continuous variables. A P value < 0.05 was considered statistically significant. Using paired Student's t-test, test-retest reliability was assessed for VAS mean scores in a randomly selected sample of patients. For the study of factors associated with diagnosis-related stress, ANOVA was used for both categorical (gender, educational level, marital status, route of transmission, antiviral therapy, and histological severity of liver disease) and continuous variables (age, perceived disease severity and trait anxiety). For the later, patients were divided into two groups according to the median value. Multivariate analysis was performed including variables significantly associated with diagnosis-related stress ($P < 0.10$).

Table 1 Characteristics of the 185 patients studied

	n = 185 (%)
Age (yr)	45±11
Gender (male/female)	111/74
Educational level	
No diploma	24 (13)
High school	83 (45)
College undergraduate	35 (19)
College graduate	43 (23)
Marital status	
Single/divorced	57 (31)
Married	92 (50)
Non-married couple	36 (19)
Routes of transmission for HCV	
Blood transfusion	74 (40)
Intravenous drug use	48 (26)
Others*	63 (34)
Histological severity (Metavir fibrosis score)	n=169
F0-1	39 (23)
F2	83 (49)
F3	19 (11)
F4	28 (17)
Trait anxiety (STAI)	45±11 (20-80)

RESULTS

Study population

The characteristics of the 185 patients are shown in Table 1. There were 111 men and 74 women with a mean age of 45±11 years. The study group had a broad distribution of educational background. Seventy-four (40%) patients were infected through blood transfusion, whereas 48 (26%) were infected through intravenous drug use. The mean time since CHC diagnosis was 2.9±2.6 years. Fifty-nine (32%) patients were receiving antiviral therapy at the time of the study. Of the 169 patients with available liver biopsy data, 28 (17%) had evidence of cirrhosis. The mean STAI-Y score for trait anxiety was 45±11 (range 22-80). This score did not differ from measures obtained in a community sample [24].

VAS scores for diagnosis-related stress and perceived disease severity

Mean±SD scores for diagnosis-related stress were 72±25 (range: 5-100). Mean±SD scores for perceived disease severity were 74±19 (range: 15-100). Three months later, the second assessment in a subgroup of 30 randomly selected patients showed that all VAS scores were reproducible over time (no difference was observed for

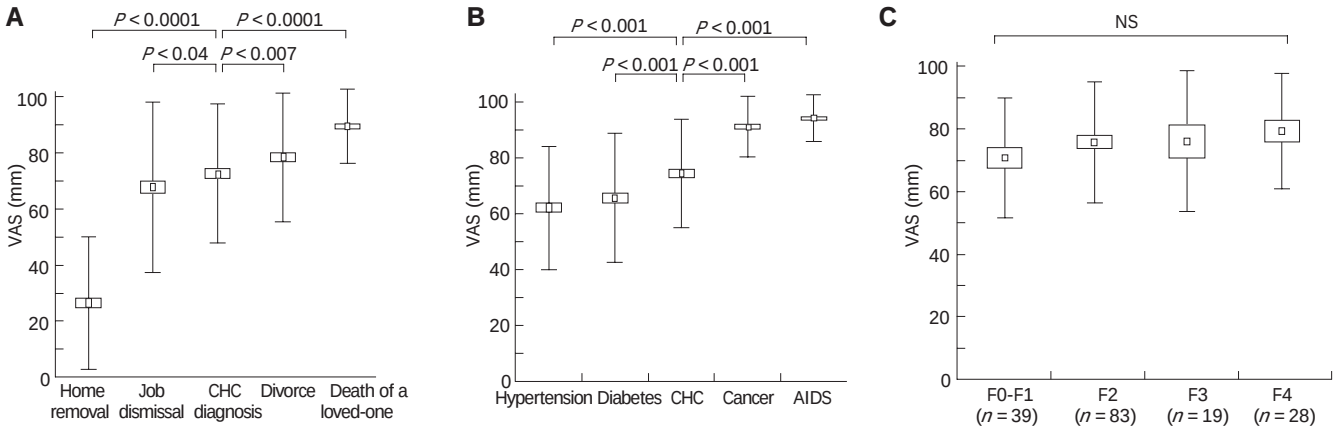

Figure 1 (**A**) Comparison between mean visual analogue scale (VAS) scores for chronic hepatitis C (CHC) diagnosis-related stress and four other stressful life events (i.e., home removal, job dismissal, divorce, and death of a loved-one) selected from the Paykel life-event scale[23] in the 185 patients. *P* values are for post-hoc comparisons made after significant one-way ANOVA ($F = 249.32$, $P < 0.0001$). (**B**) Comparison between mean visual analogue scale (VAS) scores for perceived chronic hepatitis (CHC) severity and four other common chronic diseases carrying a risk of life-threatening complications (i.e., hypertension, diabetes, cancer and AIDS) in the subgroup of 185 patients. *P* values are for post-hoc comparisons made after significant one-way ANOVA ($F = 173.81$, $P < 0.001$). (**C**) Mean visual analogue scale (VAS) scores for perceived chronic hepatitis (CHC) severity according to actual severity of liver disease assessed using METAVIR fibrosis score (F0: no fibrosis; F1: portal fibrosis without septa; F2: portal fibrosis and few septa; F3: numerous septa without cirrhosis; F4: cirrhosis) in the 169 patients with available liver specimen. In Figures A, B and C, the upper and lower limits of the boxes and the middle square indicate the standard error and the mean, respectively. The upper and lower horizontal bars indicate the standard deviation.

any item, using a paired *t* test).

Comparison with other stressful life events and chronic diseases

Mean±SD scores for stress were as follows: death of a loved-one (89±13), divorce (78±23), CHC diagnosis (72±25), job dismissal (68±30) and home removal (26±24) (Figure 1A). CHC diagnosis was significantly more stressful than job dismissal ($P < 0.04$) and home removal ($P < 0.0001$) but less than divorce ($P < 0.007$) and death of a loved-one ($P < 0.0001$).

Mean VAS scores for perceived disease severity were as follows: AIDS (94±08), cancer (91±11), CHC (74±19), diabetes mellitus (66±23) and hypertension (62±20) (Figure 1B). CHC was considered significantly more severe than diabetes mellitus ($P < 0.0001$) and hypertension ($P < 0.0001$) but less than cancer ($P < 0.001$) and AIDS ($P < 0.001$).

Relationship between perceived disease severity and actual severity

Mean VAS scores for perceived disease severity did not differ according to the Metavir fibrosis stages in the 169 patients in whom a liver specimen was available (Figure 1C).

Factors associated with diagnosis-related stress

In univariate analysis, diagnosis-related stress was associated with female sex ($P < 0.003$), infection through blood transfusion ($P < 0.002$), high perceived disease severity ($P < 0.001$) and high trait anxiety ($P < 0.001$) (Table 2). Stress tended to be higher in patients with low education level, although the difference did not reach statistical significance ($P = 0.07$). Scheffé post hoc tests revealed that stress scores were significantly higher in

Table 2 Factors associated with diagnosis-related stress in univariate analysis in the 185 patients

	Diagnosis-related stress (mean VAS scores)	*P*
Age (yrs)		
<46	73±25	
⩾46	72±24	NS
Gender		
Male	66±26	
Female	79±22	<0.003
Educational level		
< High school	75±24	
⩾ High school	69±25	0.07
Marital status		
Single/divorced	73±25	
In couple	72±24	NS
Routes of transmission for HCV		
Blood transfusion	80±20	
Intravenous drug use	65±27	
Others	68±26	<0.002
Antiviral therapy		
Ongoing	69±25	
Untreated	74±25	NS
Histological severity of liver disease ($n = 169$)		
F0-F1-F2 ($n = 122$)	74±23	
F3-F4 ($n = 47$)	70±29	NS
Time since diagnosis (yrs)		
<2	71±26	
⩾2	73±24	NS
Perceived disease severity (VAS)		
<75	59±23	
⩾75	83±21	<0.001
Trait anxiety (STAI)		
<45	64±27	
⩾45	81±19	<0.001

For continuous variables (age, time since diagnosis, perceived disease severity and trait anxiety) patients were divided into two groups according to the median value.

Table 3 Factors associated with diagnosis-related stress in multivariate analysis in the 185 patients

	Diagnosis-related stress (100mm VAS score)[2]	
	β^1	P
Gender : (male: 1, female: 2)	0.02	0.76
Routes of transmission: (drug user = 1; other = 2, blood transfusion = 3)	0.17	< 0.01
Educational level: (< high school:1, ⩾ high school:2)	-0.03	0.58
Anxiety (STAI-Y score)	0.30	< 0.001
Perceived disease severity (100mm VAS score)	0.43	< 0.001

[1]regression coefficient.
[2]Regression model: R = 0.60, R^2 = 0.36, adjusted R^2 = 0.35, F(5, 171) = 19.65, P < 0.001.

patients infected through blood transfusion than in those infected through intravenous drug use (P < 0.001). No difference was observed for patients infected through other routes of transmission. Stress did not differ between untreated and treated patients. In the latter patients, stress did not differ according to virological response.

In multivariate analysis, stress was related only to perceived disease severity (P < 0.001), trait anxiety (P < 0.001) and infection through blood transfusion (P < 0.01). These parameters accounted for 36% of the variance of diagnosis-related stress (R^2 = 0.36; P < 0.001; Table 3).

DISCUSSION

Our results show that learning one has contracted HCV infection, with its implications for future health and behaviour, is a major stressful event (with mean scores of 72 ± 25 on a 100-mm visual analogue scale). By comparison with other stressful life events selected from the Paykel life-event scale[23], ranging from minor (i.e., home removal) to major (i.e., death of a loved one), CHC diagnosis was considered less stressful than death of a loved one (89 ± 13, P < 0.0001) and divorce (78 ± 23, P < 0.007), but more than job dismissal (68 ± 30, P < 0.04) and home removal (26 ± 24, P < 0.0001). These results emphasize the considerable emotional and psychological burden that CHC diagnosis represents in patients' life. So far, the psychological issues that CHC patients face remain poorly understood. In the studies having reported clinically significant emotional distress and depressive disorders in patients with CHC[2,13-15], the psychological impact of diagnosis knowledge was not taken into account. However, it is important to distinguish between psychological reactions to the knowledge that one has been infected with HCV and the direct effects of the virus itself as evidenced by the results of the present study. Our findings are consistent with those of Rodger *et al*[6] who compared 15 patients who were aware of having HCV infection with 19 subjects who were unaware of having HCV infection, and found that the former had significantly poorer HRQOL scores. More recently, Dalgard *et al*[17] have confirmed these results in a cohort of 199 Swedish active drug user patients, showing that those who believed they were infected had a lower HRQOL scores than those who believed they were not infected. Taken together, these results provide evidence of the considerable negative impact of the knowledge of the diagnosis of CHC on patients' perception of their psychological well-being, known for other chronic diseases as the "labeling" effect[25]. Such an effect has recently been illustrated in a study by Zickmund *et al*[26], showing that stigmatization was common in patients with CHC and was associated with a lower quality of life and with a deterioration of social support.

There are increasing evidences that CHC infection may be associated with significant cerebral dysfunction. Several authors have reported cognitive impairment and central nervous system functional abnormalities in CHC patients compared with uninfected controls, using P300 event-related potentials, a sensitive electrophysiologist test of cognitive processing[27] or magnetic resonance spectroscopy[28-31]. Such findings suggest a direct biological effect of HCV infection on cerebral function. Whether cognitive dysfunction is an important determinant of impaired HRQOL in CHC remains to be determined.

As natural history of chronic HCV infection remains poorly defined[32], it is not surprising that CHC diagnosis, a disease with uncertain outcome, raises significant concerns about future health status. Indeed, CHC was perceived as a severe disease (74 ± 19), after AIDS (94 ± 08, P < 0.001) and cancer (91 ± 11, P < 0.001), but before diabetes (66 ± 23, P < 0.001) and hypertension (62 ± 20, P < 0.001). Interestingly, perceived CHC severity was not related to the actual severity of the disease, as assessed by the severity of liver histological lesions. This finding is consistent with the results of Foster *et al*[3] who showed that the significant impairment of HRQOL experienced by CHC patients could not be attributed to the histological severity of liver disease. It is also in keeping with studies[33-35] conducted in patients with hypertension or chronic respiratory diseases, showing that perception of a disease usually correlates poorly with its medically defined characteristics. In addition, the patients' perception of CHC severity seemed to have a more significant impact on their psychological status than did the histological severity of their liver disease as evidenced by multivariate analysis, showing that diagnosis-related stress was mainly related to perceived CHC severity, trait anxiety and infection through blood transfusion. An explanation for that latter finding may be the fact that patients infected through blood transfusion had no reason to expect such a health problem. By contrast, former drug users likely knew, given their past lifestyle, they were at risk of contracting severe diseases, such as HIV/AIDS, hepatitis B or C, with related-consequences for their future health.

Although our patient population does not represent a random sampling of CHC patients, we believe that it is representative of the general population of CHC patients. The demographic and clinical characteristics of our study population, including routes of transmission and proportion of patients with cirrhosis at the time of diagnosis, are similar to that reported in large surveys of patients with CHC in France[36,37]. In addition, all

VAS scores were reproducible over time in a sample of randomly selected patients. Although some of our patients received antiviral therapy (32%), diagnosis-related stress and perceived disease severity mean VAS scores did not differ between treated and untreated patients.

Finally, our findings may have important implications for clinicians who take care of patients with CHC. They highlight the importance of communication between patients and their physician and the crucial role of the specialist in providing adequate and accessible information for patients to cope with their condition[38].

In conclusion, our results show the considerable psychological and emotional burden that a diagnosis of CHC represents, even in the absence of significant liver disease. They should be taken into account when announcing a diagnosis of CHC in order to reduce its negative effects.

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Efficient expression of histidine-tagged large hepatitis delta antigen in baculovirus-transduced baby hamster kidney cells

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Abstract

AIM: To study the baculovirus/mammalian cell system for efficient expression of functional large hepatitis delta antigen (L-HDAg).

METHODS: A recombinant baculovirus expressing histidine-tagged L-HDAg (L-HDAgH) was constructed to transduce baby hamster kidney (BHK) cells by a simplified transduction protocol.

RESULTS: The recombinant baculovirus transduced BHK cells with efficiencies higher than 90% as determined by flow cytometry. The expression level was significantly higher than that obtained by plasmid transfection and was further enhanced 3-fold to around 19 pg/cell by the addition of 10 mmol/L sodium butyrate. Importantly, the expressed L-HDAgH was localized to the cell nucleus and correctly isoprenylated as determined by immunofluorescence labeling and confocal microscopy. Moreover, L-HDAgH interacted with hepatitis B surface antigen to form virus-like particles.

CONCLUSION: The fusion with histidine tags as well as overexpression of L-HDAgH in the baculovirus-transduced BHK cells does not impair the biological functions. Taken together, the baculovirus/mammalian cell system offers an attractive alternative for high level expression of L-HDAgH or other proteins that require extensive post-translational modifications.

Keywords: Baculovirus; Hepatitis delta virus; L-HDAg; Mammalian cell; Protein expression; Transduction

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INTRODUCTION

Hepatitis delta virus (HDV) is a subviral satellite and requires hepatitis B virus to supply envelope proteins (HBsAg) for its packaging, secretion and infection[1, 2]. HDV superinfection of HBV carriers can lead to fulminant hepatic failure and very likely the progression to liver cirrhosis[3], consequently HDV imposes a huge threat to the health of 350 million HBV carriers worldwide. However, effective vaccines or diagnostic assays remain unavailable. The RNA genome of HDV encodes the large and small forms of hepatitis delta antigens (L-HDAg and S-HDAg). The 24 kd S-HDAg and 27 kd L-HDAg are identical in sequence except that L-HDAg contains additional 19 amino acids at the C-terminus[1, 4]. The C-terminus extension of L-HDAg allows for the isoprenylation which is crucial for the interaction with S-HDAg and HBsAg for the formation of virions. It has been established that cell-mediated immunities are important in controlling HDV infection[5, 6], thus an effective T-cell stimulating HDV vaccine would be highly desired. In this regard, L-HDAg may represent an excellent vaccine candidate because L-HDAg (expressed by a DNA vaccine) induces significant titers of anti-HDV antibodies as well as strong cell-mediated immune responses in mice[7]. Moreover, L-HDAg is a dominant repressor for HDV replication[8], thus implicating its application as an anti-HDV drug in addition to being a vaccine. However, efforts to characterize and evaluate the immunological properties of L-HDAg have been hampered due to the lack of a proper method for efficient expression and purification of L-HDAg.

Baculovirus (*Autographa californica* multiple nuclear polyhedrosis virus, AcMNPV) has been widely utilized for protein expression in insect cells. Since the finding that baculovirus is capable of transducing mammalian cells in 1995, baculovirus has been developed as a vector for *in vitro* and *in vivo* gene therapy studies and many other applications (for review, see[9, 10]). Recently, the applications

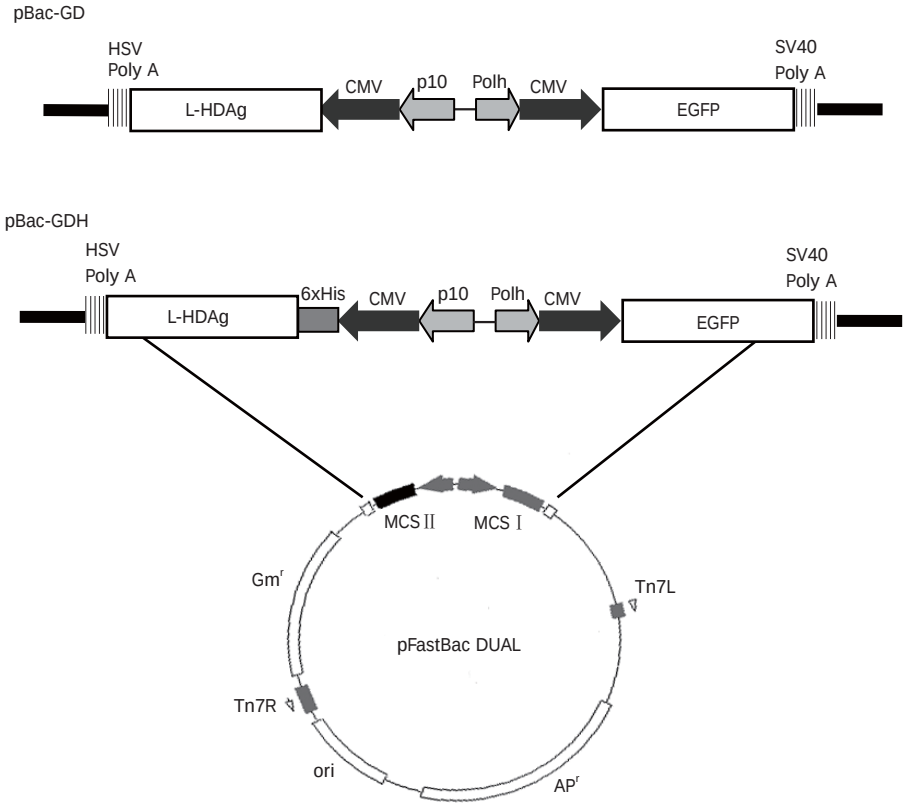


Figure 1 Schematic illustration of recombinant donor plasmids pBac-GDH and pBac-GD

of baculovirus have been further expanded to the identification of MHC class I mimotopes[11] and genetic modification of primary chondrocytes[12]. Furthermore, our group has also demonstrated the feasibility of producing HDV virus-like particles in Huh-7 cells[13]. All these studies suggest that baculovirus may be an ideal tool for the expression of functional, complex eukaryotic proteins owing to the following advantages: (1) Baculovirus transduction is highly efficient for many cell types and generally causes no appreciable cytopathic effect[10,14]; (2) The large AcMNPV genome (about 130 kb) confers the virus huge cloning capacity to harbor multiple genes or large inserts[15]; (3) The construction, manipulation and production of recombinant baculovirus are easy.

Considering the need for efficient expression of L-HDAg and the advantages of baculovirus/mammalian cell system, we constructed a recombinant baculovirus encoding histidine-tagged L-HDAg (L-HDAgH) for rapid and high level expression in transduced BHK cells. The high level expression and subsequent purification may allow us to obtain sufficient quantities of functional L-HDAgH for further evaluation of its immunological properties and its potential as a vaccine candidate. The success of this study supports the notion that the baculovirus/mammalian cell system can be a platform for efficient expression of eukaryotic proteins requiring post-translational modifications.

MATERIALS AND METHODS

Generation of recombinant baculoviruses

The transfer plasmid was generated using pFastBac™ DUAL (Invitrogen) as the backbone, which contained two multiple cloning sites (MCS). The gene encoding enhanced green fluorescent protein (EGFP), along with the upstream cytomegalovirus immediate-early (CMV-IE) promoter, was amplified by polymerase chain reaction (PCR) from pEGFP-C1 plasmid (Clontech) and inserted into MCS I under the polyhedrin promoter. The cDNA encoding L-HDAg was PCR-amplified from p2577-1L[16] and subcloned into pcDNA4/HisMax-TOPO (Invitrogen), thus a His_6 tag was fused at the N-terminus of L-HDAg under the control of CMV-IE promoter. The whole expression cassette was PCR-amplified again and subcloned into *Kpn* I site of MCS II. The resultant plasmid was designated pBac-GDH (Figure 1). Another plasmid, pBac-GD, was previously constructed to harbor the genes encoding L-HDAg and EGFP[13] (Figure 1). The recombinant baculoviruses were subsequently generated using the Bac-to-Bac® system (Invitrogen) and designated as Bac-GDH and Bac-GD. Another recombinant baculovirus co-expressing HBsAg and EGFP, Bac-GB, was constructed previously[13].

Cells and medium

BHK cells were cultured in Dulbecco's minimal essential medium (DMEM, Sigma) supplemented with 100 mL/L heat inactivated fetal bovine serum (FBS; Gibco), 2 mmol/L L-glutamine (Gibco) and non-essential amino acids (Gibco). The cells were maintained at 37 ℃ in a humidified incubator containing 5 % CO_2. Insect cell Sf-9 was maintained in TNM-FH medium (Sigma) supplemented with 10 mL/L FBS for baculovirus generation, amplification and titration according to standard protocols[17]. The viruses were not concentrated by ultracentrifugation. The titer of Bac-GDH was 8.3×10^8

plaque forming units per ml.

Baculovirus transduction

A simple process eliminating the need for virus ultracentrifugation[18] was adopted for transduction. Briefly, BHK cells cultured in 6-well plates (5×10^5 cells per well) were washed with PBS, and then transduced simply by adding 100 μL unconcentrated virus solution and 400 μL PBS. The plates (or dishes) were shaken on a rocking platform for 6 h at 25 ℃. After the virus incubation, the virus solution was aspirated. The wells were replenished with 2 mL complete medium with or without sodium butyrate and incubated at 37 ℃. The cells grown on 10-cm dishes were transduced similarly, except that larger volumes of virus (800 μL) and PBS (3.2 mL) were used.

Western blot analysis

BHK cells were singly transduced by Bac-GDH (or Bac-GD), or co-transduced by Bac-GDH and Bac-GB. To detect intracellular L-HDAgH (or L-HDAg), the cells were harvested at 2 dpt (days post-transduction) and resuspended in TE buffer containing phenylmethylsulfony lfluoride (PMSF). The proteins were released by sonication on ice and separated from debris by centrifugation. To detect secreted HBsAg and L-HDAgH, virus-like particles released into conditioned medium from co-transduced cells were concentrated by 200 g/L sucrose-cushioned ultracentrifugation as described previously[13]. The pellet was resuspended in TE buffer for Western blot. The intracellular or extracellular samples were resolved on 120 g/L gels and transferred onto nitrocellulose membranes. The L-HDAgH (or L-HDAg) was probed by human anti-HDV sera[19] or mouse anti-His_6 mAb (Amersham Biosciences). The HBsAg was probed by anti-HBsAg mAb (A10F1, a kind gift from Prof. S.C. Lee, National Taiwan University). The secondary antibodies were AP-conjugated goat anti-human or anti-mouse IgG (Kirkegaard and Perry Laboratories). The bands were developed using BCIP/NBT reagent (Sigma). The densitometry was performed by scanning the bands on the membranes and analyzing by Scion Image Shareware (Scion Corporation). For estimation of the product yield, serially diluted purified protein was used as the standard and analyzed by scanning densitometry.

Flow cytometry

The transduced cells were harvested by trypsin/EDTA, washed and resuspended in PBS for flow cytometry (FACSCalibur, Becton Dickinson) analysis according to the manufacturer's instruction. The percentage of cells emitting fluorescence (% GFP + cells) and mean fluorescence intensity (MFI) of each sample were measured 3 times by counting 10 000 cells in each measurement. The mean± standard deviation (SD) of 3 independent experiments are presented as arbitrary units (au).

Immunofluorescence labeling

BHK cells were seeded onto 22 mm×22 mm glass slides and transduced in duplicate by Bac-GDH at a multiplicity of infection of 280. The cells were fixed at 24 h post-transduction (hpt) with methanol/acetone (1:1) for 5 min at -20 ℃, rinsed with PBS and then blocked with PBS containing 20 g/L bovine serum albumin (BSA) for 30 min at 37 ℃. For the detection of subcellular localization, L-HDAgH was probed with 150 μL anti-HDAg mAb (HP6A1, 1:300 dilution)[16] for 1 h at 37 ℃ and washed with PBS 3×5 min. L-HDAgH was subsequently labeled using Cy3-conjugated goat anti-mouse IgG (1:25 dilution, Sigma). For simultaneous nuclear staining, SYBR green I (1: 40 000 dilution, BioWhittaker) was added together with the secondary antibody. For isoprenylation analysis, L-HDAgH was first probed with primary antibody solution containing HP6A1 and anti-farnesyl mAb (Sigma) for 1 h at 37 ℃, followed by the labeling with secondary antibody solution containing FITC-conjugated anti-mouse and TRITC-conjugated anti-rabbit mAb (Jackson ImmunoResearch) for 1 h at 37 ℃. The slides were washed with PBS 3×5 min, mounted using glycerol/PBS (3:1) mounting solution and observed by a confocal microscope (TCS SP2, Leica, Germany).

Protein purification

The His_6-tagged L-HDAg was purified by immobilized metal affinity chromatography (IMAC) under denaturing conditions. The cells were resuspended in 10 ml binding buffer A (20 mmol/L Tris-HCl, 500 mmol/L NaCl, 8 mol/L urea and 10 mL/L PMSF, pH 7.8), sonicated for 5 min and centrifuged (12000 r/min for 30 min). After centrifugation, the supernatant was loaded cyclically for 1 h at room temperature to the Fast-flow Protein Liquid Chromatography (ÄKTA FPLC, Amersham Biosciences) system in which the column contained 5 mL Ni^{2+}-coupled Chelating Sepharose Fast Flow resin. Bound proteins were eluted by a mixture of elution buffers A and B (20 mmol/L Tris-HCl, 500 mmol/L NaCl, 500 mmol/L imidazole, pH 7.8) at 1 mL/min at 4 ℃. The FPLC system generated step gradients by varying the volume ratios of buffers A and B so that the urea concentration decreased while the imidazole concentration increased stepwise. The eluate was collected in 1.5 mL fractions and then analyzed by Western blots and SDS-PAGE.

RESULTS

Confirmation of protein expression

The recombinant baculoviruses designed to express L-HDAg with or without His_6 tags were constructed and designated Bac-GDH and Bac-GD (Figure 1). To confirm the correct expression of L-HDAgH in BHK cells, the cells grown on 10-cm dishes were transduced by Bac-GD or Bac-GDH, harvested at 2 dpt and the cell lysates were analyzed by Western blot. Figure 2A depicts that the mock-transduced cells expressed no protein recognized by the anti-HDV serum, while the Bac-GD-transduced cells expressed a protein whose molecular mass (27 kd) corresponded to that of authentic L-HDAg, indicating the specificity of the serum. Similarly, the Bac-GDH-transduced cells expressed a protein recognized by the antibody, but with a slightly higher molecular mass (about 31 kd), thus suggesting the fusion of the His_6 tag to L-HDAg. The identities of the proteins were further

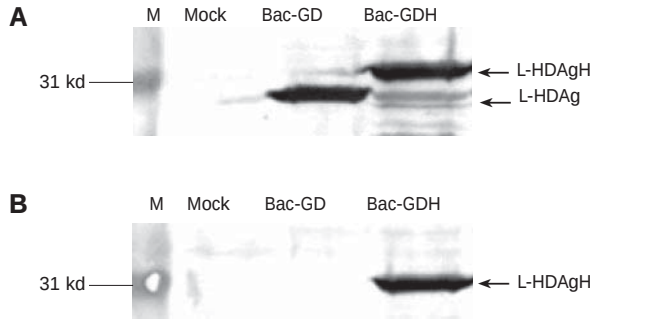
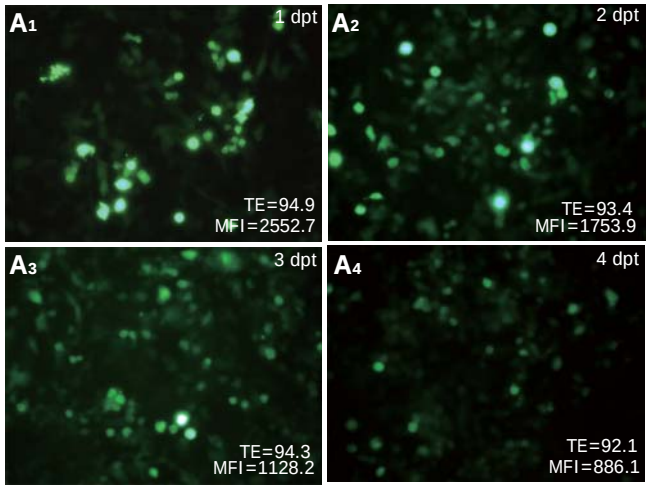


Figure 2 Western blot analysis of proteins expressed by mock-, Bac-GD- or Bac-GDH-transduced BHK cells. **A**: Anti-HDV sera; **B**: Anti-His$_6$ mAb.



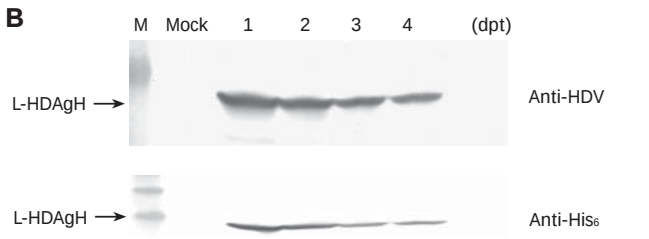


Figure 3 A: Variation of the transduction efficiency (TE) over time post-transduction. **B**: Time-course profile of L-HDAgH expression. Western blot using anti-HDV sera and anti-His6 mAb.

confirmed by analysis of the lysates using anti-His$_6$ mAb (Figure 2B). As expected, no His$_6$ tagged proteins were found in the mock- or Bac-GD-transduced cell lysates. In contrast, the 31 kd protein expressed by Bac-GDH was clearly visible. Figures 2A, B collectively confirme the expression of His$_6$ tagged L-HDAg in the BHK cells.

Transduction efficiency and time-course profile

To determine how efficiently the virus could transduce BHK cells and how the protein accumulation varied over time post-transduction, BHK cells were transduced and photographed using the fluorescence microscope. The percentages of GFP+ cells and the mean fluorescence intensities (MFI) were monitored over time by flow cytometry. As shown in Figure 3A, the percentage of GFP+ cells was up to 95 % at 1 dpt and remained higher than 90 % for 4 d, but the mean fluorescence intensities declined over time. Figure 3B shows the time-course profile of L-HDAgH expression and depicts that the expression reached the maximum rapidly at 1-2 dpt. The protein concentration declined slightly thereafter, but remained high at 4 dpt.

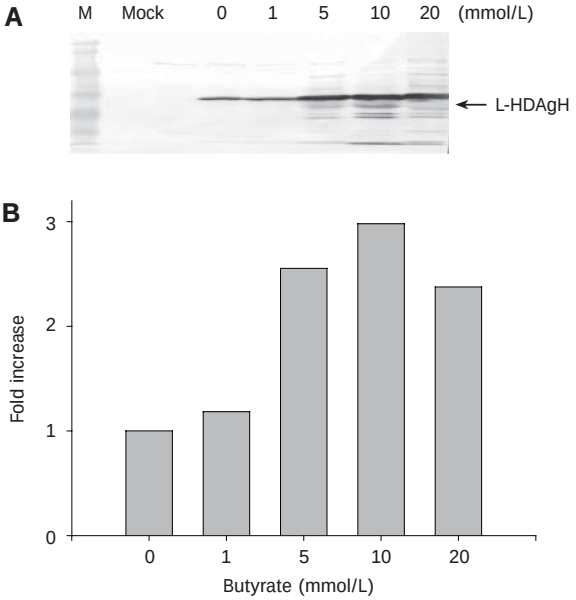


Figure 4 Effects of sodium butyrate on L-HDAgH expression. **A**. Western blot; **B**. The expression levels.

Enhancement of protein expression

To enhance the L-HDAgH expression, the cells were transduced on 10-cm dishes as described above, but varying concentrations of sodium butyrate, a histone deacetylase inhibitor shown to loosen the condensed chromosome and up-regulate the protein expression in mammalian cell system[20, 21], were supplemented to the medium after transduction. The cells were harvested at 2 dpt and the lysates were analyzed by Western blot. Figure 4A depicts a clear dependence of the L-HDAgH expression on the sodium butyrate concentrations. The scanning densitometry quantitatively revealed an about 3-fold enhancement in L-HDAgH expression by 10 mmol/L sodium butyrate, compared to the control without sodium butyrate (Figure 4B). The highest protein yield was roughly estimated to be about 19 pg/cell by scanning densitometry using serially diluted purified protein as the standard. At a higher concentration (i.e. 20 mmoL/L), the overall L-HDAgH expression dropped slightly probably due to high cytotoxicity associated with sodium butyrate[22].

Characterization of L-HDAgH

To determine whether baculovirus transduction and protein overexpression affected the cell physiology and deterred the correct protein localization, BHK cells were transduced by Bac-GDH, labeled by SYBR Green I and anti-HDAg mAb and examined by confocal microscopy. As shown, the nuclei were clearly marked by SYBR Green I (Figure 5A) while L-HDAgH was marked by clusters of

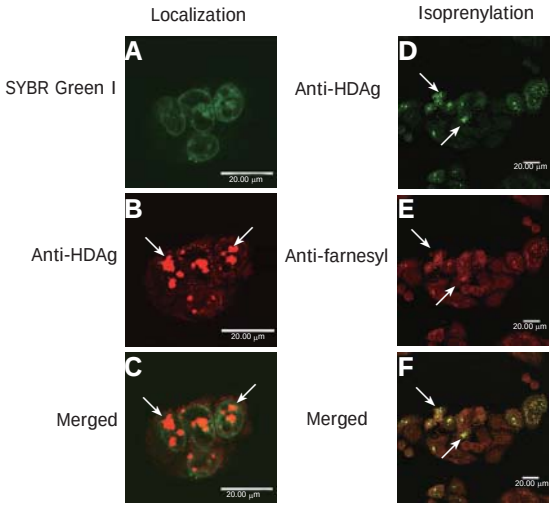


Figure 5 Subcellular localization and isoprenylation of L-HDAgH by immunofluorescence labeling. **A**: the presence of nuclei labeled by SYBR green I; **B**: L-HDAg detected using Cy3-conjugated goat anti-mouse IgG; **C**: merged photograph of A and B; **D**: the presence of L-HDAgH labeled by FITC-conjugated mAb; **E**: The presence of isoprenylation marked by TRITC-conjugated mAb; **F**: merged photograph of D and E. Bars, 20 μm.

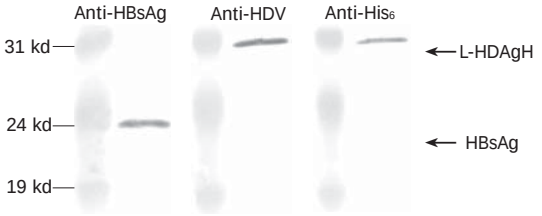


Figure 6 Western blot using anti-HBsAg, anti-HDAg and anti-His$_6$ antibodies.

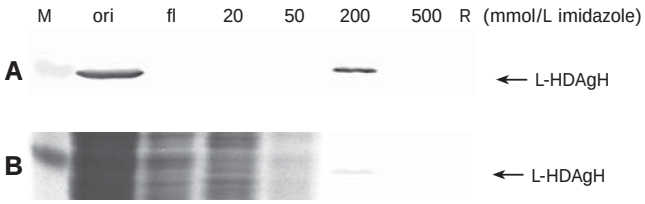


Figure 7 Western blot (**A**) and SDS-PAGE(**B**). M, marker; ori, original sample; fl, flow through; R, samples stuck in the resin. The numbers indicate the imidazole concentration at which the sample was collected.

red dots (Figure 5B). The merged photograph (Figure 5C) clearly illustrates the co-localization of L-HDAgH with the nuclei, confirming that the expressed L-HDAgH was localized within the nuclei.

To evaluate whether L-HDAgH was properly isoprenylated, Bac-GDH-transduced BHK cells were subject to immunofluorescence double labeling using anti-HDAg and anti-farnesyl mAb. Figure 5D shows the green dots marking the presence of L-HDAgH using anti-HDAg mAb, while Figure 5E illustrates the detection of isoprenylation using anti-farnesyl mAb as evidenced by the red dots. The merged photograph (Figure 5F) clearly illustrates the co-localization of both red and green dots, indicating that the baculovirus-expressed L-HDAgH was isoprenylated.

Since the assembly of HDV virions or virus-like particles (VLP) is an important function of L-HDAg, whether L-HDAgH remained capable of assembling into VLP was investigated. It has been shown that L-HDAg along with HBsAg was necessary and sufficient for the assembly of HDV VLP[13], thus BHK cells were co-transduced by Bac-GB and Bac-GDH. The conditioned medium was collected, concentrated and analyzed by Western blot. Figure 6 shows that the pellet contained proteins recognized by anti-HBsAg, anti-HDAg and anti-His$_6$ antibodies, confirming the simultaneous secretion of both HBsAg and L-HDAgH into the medium. Because L-HDAgH alone was localized within the nucleus, the co-secretion proved the assembly and secretion of HDV VLP[23-25].

Purification of L-HDAgH

Thanks to the fused His$_6$ tag, L-HDAgH was purified by IMAC. Under the native conditions, however, the protein binding to the resin was very weak (not shown). Therefore, the protein was denatured by 8 mol/L urea and purified under denaturing conditions. The Western blot (upper panel, Figure 7) shows that the flow through contained no L-HDAgH, indicating significantly improved binding. The proteins were then eluted with a mixture of buffers A and B with decreasing urea concentration and increasing imidazole concentration. The L-HDAgH was eluted by 200 mmol/L imidazole buffer (4.8 mmol/L urea). The purity was 7 % as revealed by SDS-PAGE (lower panel, Figure 7).

DISCUSSION

As mentioned above, L-HDAg may represent an excellent vaccine candidate, but characterization and evaluation of the immunological properties of L-HDAg have been impeded due to the lack of a proper method for efficient expression and purification. Although attempts to express L-HDAg using *E coli* or baculovirus/insect cell systems have been made, both systems are not suited for L-HDAg expression because the former lacks post-translational modification while the latter results in rapid degradation of L-HDAg after 2 d post-infection[26]. Besides, L-HDAg expressed in insect cells induces cell cycle arrest[27]. The integration of L-HDAg gene into the chromosome of mammalian cells for stable expression may offer another option, but has not been reported probably because L-HDAg is a nuclear protein, the accumulation of which can result in significant cytotoxicity. Transient expression in mammalian cells mediated by other virus systems (e.g. vaccinia virus) may be possible as well, but these viruses lead to cell death and lysis, thus potentially undermining the post-translational modification at later stage. Owing to these restrictions, L-HDAg expression is mainly achieved by transfecting plasmids expressing L-HDAg into hepatoma cells (e.g. Huh-7 and HepG2). The plasmid transfection into hepatocytes, however, is notorious for

its low efficiency and low expression level, consequently, highly sensitive detection methods such as isotopic labeling and chemiluminescence enhancement are required to detect the expression.

To express functional L-HDAg efficiently and rapidly for research purposes, the baculovirus/mammalian cell system was employed. Using PBS as the surrounding solution, the efficiency of baculovirus-mediated gene delivery into BHK cells was up to 94 % (Figure 3A), which far exceeded 20 %-30 % normally achieved by plasmid transfection. The detection of L-HDAgH using anti-HDV sera and anti-His_6 mAb (Figure 2) confirmed that the expressed L-HDAgH possessed the fused His_6 tag and was similar in size and antigenic properties to those of authentic proteins. The expression level was apparently higher than that can be obtained using plasmid transfection, as evidenced by the easy detection using BCIP/NBT as the color developing reagent, which was significantly less sensitive than chemiluminescence enhancement. Under the transcriptional control of CMV-IE promoter, the L-HDAgH expression peaked at day 1-2 and gradually declined thereafter, which probably stemmed from cell proliferation and attenuation of promoter activity. Albeit this, the expression level could be up-regulated by sodium butyrate and reached the maximum (at about 19 pg/cell) at 10 mmol/L sodium butyrate (Figure 4A).

Besides the high transduction efficiency and high expression level, Figures 5A-C illustrate that L-HDAgH formed speckles localized within the nuclei of BHK cells, which is the subcellular location of authentic L-HDAg[28]. The formation of nuclear speckles is a sign of isoprenylation[29] and could have stemmed from 1) the formation of dimmers or multimers of L-HDAg between the coiled-coiled domains or 2) the interaction of L-HDAg with host proteins such as nucleolin and delta-interacting protein A[30]. The formation of aggregates has been previously observed for L-HDAg expressed in hepatoma cell lines via plasmid transfection[28], and explains the low binding of L-HDAgH to Ni^{2+}-coupled resin under native conditions and the low purity in subsequent purification steps. Further improvement in the purification process is required and is currently underway. Furthermore, Figures 5D-5F revealed that L-HDAgH underwent proper isoprenylation, which is a prerequisite for the interaction of L-HDAg with HBsAg (and hence the virion formation). The Western blot analysis of concentrated pellets (Figure 6) further confirmed the assembly and secretion of HDV VLP. The retention of isoprenylation as well as formation of VLP implied that the biological function of L-HDAgH was not impaired by the His_6 tag[31, 32].

In summary, our data solidly confirm that the overexpression of His_6 tagged L-HDAgH in baculovirus-transduced mammalian cells does not hamper the proper localization and processing. The data also suggest the maintenance of accurate structure and appropriate biological functions and hence validate the use of baculovirus/mammalian cell system for the production of eukaryotic proteins requiring extensive post-translational modifications. This is partly attributed to the fact that baculovirus does not cause cell lysis or induce cytotoxicity. Additionally, the baculovirus system enables protein production in BHK cells which is one of the few cell lines approved for biopharmaceutical production. Moreover, the transduction protocol eliminates the need for virus concentration by ultracentrifugation so that transduction can be carried out in the bioreactor simply by incubating the virus solution/PBS with the cells, which otherwise is difficult for plasmid transfection. Since process scale-up of BHK culture has been well established, large scale production of L-HDAg can be made possible simply by transferring this process to a bioreactor (e.g. a packed bed bioreactor).

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BASIC RESEARCH

Relaxin prevents the development of severe acute pancreatitis

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Abstract

AIM: To investigate the severity of acute pancreatitis (AP) is associated to the intensity of leukocyte activation, inflammatory up-regulation and microcirculatory disruption associated to ischemia-reperfusion injury. Microvascular integrity and inhibition of pro-inflammatory mediators are key-factors in the evolution of AP. Relaxin is an insulin-like hormone that has been attributed vasorelaxant properties via the nitric oxide pathway while behaving as a glucocorticoid receptor agonist.

METHODS: AP was induced by the bilio-pancreatic duct-outlet-exclusion closed-duodenal-loops model. Treatment with relaxin was done at different time-points. Nitric oxide synthase inhibition by L-NAME and glucocorticoid receptor (GR) blockage by mifepristone was considered. AP severity was assessed by biochemical and histopathological analyses.

RESULTS: Treatment with relaxin reduced serum amylase, lipase, C-reactive protein, IL-6, IL-10, hsp72, LDH and 8-isoprostane as well as pancreatic and lung myeloperoxidase. Acinar and fat necrosis, hemorrhage and neutrophil infiltrate were also decreased. ATP depletion and ADP/ATP ratio were reduced while caspases 2-3-8 and 9 activities were increased. L-NAME and mifepristone decreased the efficiency of relaxin.

CONCLUSION: Relaxin resulted beneficial in the treatment of AP combining the properties of a GR agonist while preserving the microcirculation and favoring apoptosis over necrosis.

Key words: Acute pancreatitis; Relaxin; Nitric oxide; Glucocorticoid receptor; Necrosis; Apoptosis

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INTRODUCTION

Acute pancreatitis (AP) is an acute inflammatory disorder associated with premature activation and increase in pancreatic enzymes in blood and urine that may progress to implicate other organs such as kidney, lungs and liver[1]. Inflammatory mediators induce complications that can range from local to systemic evolving to a severe form with high morbi-mortality[2].

The peri-vaterian duodenum (PV-D) has an extremely rich autonomic duodenum-pancreatic innervation therefore endoscopic maneuvers, gallstones, biliary sludge and/or hemobilia migrating and impacting on the peri-vaterian duodenum stimulate primary sensory neurons to unchain autonomous-arc-reflexes (AARs) responsible for the initial acute pancreatic neurogenic inflammation that may range from local and edematous to regional and necrotizing pancreatitis[3-6]. These autonomic nerve fibers are the starting point of autonomic-arc-reflexes which may integrate in the celiac ganglia complex or in the bulbar-hypothalamic autonomous nucleus to end in the intrapancreatic ganglion[6]. Therefore irritative stimuli induce impulses that travel through thin, unmyelinated, capsaicin-sensitive (type C) nerve fibers to generate in the somata of the afferent neurons vasoactive peptides such as substance P, neurokinin-A, VIP, SOM and DYN[7]. These sensory peptides synthesized in the somata are then transported to the nerve peripheral endings where they are released. Substance P and other sensory peptides can activate mast-cells that will liberate histamine and pro-inflammatory factors thus recruiting leukocytes that will exacerbate the inflammatory response associated to vasodialtation, edema, increased vascular permeability and neutrophil infiltration associated to this neuroimmune-

inflammatory interaction observed in the neurogenic inflammation[7].

Over-stimulation of receptors in the peri-vaterian duodenum trigger autonomic-arc-reflexes that induce a sympathetic hypertone followed by cathecolaminic discharges from nerve endings in the pancreas that affect capillaries and blood vessels leading to microcirculatory disturbances responsible for ischemia/ reperfusion injury associated to synthesis and release of pro-inflammatory factors together with leukocyte recruitment and activation that correlate with intracellular enzyme activation[7]. Under these circumstances the systemic inflammatory response syndrome (SIRS) may end in a multiorgan dysfunction syndrome (MODS) characterized by metabolic and homodynamic alterations, multifocal organic dysfunction and pluriparenchymatose failure[8]. The bilio-pancreatic-duct outlet exclusion closed duodenal loops (BPDOE-CDLs) model, that we have previously described and validated, mimics these circumstances[6].

Relaxin is a peptide hormone of ovarian origin that belongs to the insulin superfamily[9]. It has been reported that relaxin offers protection for reperfusion-induced cardiac injury increasing coronary blood flow favoring blood supply to the ischemic myocardium while it exerts a relaxant action on smooth muscle cells[10]. It has been shown that relaxin up regulates nitric oxide synthase (NOS) II expression thus inducing nitric oxide mediated vasodilatation by a controlled activation of endogenous nitric oxide biosynthesis[11]. By regulating nitric oxide relaxin inhibits neutrophil extravasation, adhesion, recruitment and activation[12]; decreases the expression of chemokines and adhesion molecules[13]; inhibits histamine release by mast cells[14]; depresses platelet aggregation[15]; alters Ik-B phosphorylation and degradation diminishing NF-kB activation and binding to DNA and enhances the activation of the glucocorticoid receptor (GR)[16]. Therefore, via the nitric oxide pathway relaxin exerts vaso-relaxant, anti-thrombotic and anti-inflammatory properties.

Relaxin has also been shown to bind and stimulate the glucocorticoid receptor that when activated will translocate to the nucleus where it inhibits NF-kB and AP-1 by transrepression and stimulates transcription of glucocorticoid response elements (GREs) of DNA inducing transcription of responsive genes by transcativation[16] . As an agonist of the glucocorticoid receptor relaxin will then possess anti-inflammatory properties inhibiting pro-inflammatory molecules.

Our aim was to investigate the influence of relaxin on the evolution of acute pancreatitis considering the relevance of the nitric oxide pathway and glucocorticoid receptor stimulation. Pancreatic, metabolic, kidney, liver and severity indicators were assessed.

MATERIALS AND METHODS

General concepts

The Bilio-pancreatic-duct-outlet-exclusion closed-duodenal-loops model (BPDOE-CDL) model was carried out in male inbred adult Wistar rats, weighing 250 - 350 g, free of pathogens, under controlled environmental conditions (room-temperature, 20-25 ℃; 12:12 light-dark cycle), with free access to standard rodent chow and water. The study was performed in accordance with national guidelines for the use and care of laboratory animals and was approved by the local animal care and use committee. Rats were randomly assigned to one of the three series: basal, control or experimental. Each of these series was divided into groups of 16 rats.

The animal series comprised eleven groups. *Group 1* (reference baseline values), consisted of un-fasted, healthy animals that did not undergo any surgery or treatment. *Group 2* (surgery control), only underwent the surgical maneuvers involved in triggering AP. *Group 3* (AP, BPDOE-CDLs model) acute pancreatitis was triggered by filling the closed-duodenal-loops at constant pressure with 8% sodium taurocholate [SIGMA, USA] and 200 uL of methylene blue to corroborate duodenal-reflux absence[6]. *Group 4* (PBS vehicle control and BPDOE-CDLs) acute pancreatitis was induced as for Group 3 and 100 uL PBS were injected sc 1h before and 4 h after the surgery. *Group 5* (LN+RLX, non-selective NOS inhibitor and relaxin) L-NAME (N^G-nitro-L-arginine methyl ester [SIGMA, USA]) at 10 mg/kg and 5 mg/kg and relaxin [Phoenix Pharmaceuticals Inc, USA] at 2.5ug/kg were administered sc 1h before and 4h after triggering acute pancreatitis. *Group 6* (MIF+RLX, GC receptor antagonist and relaxin) received mifepristone [SIGMA, USA] at 3 mg/kg and relaxin at 2.5 ug/kg sc 1h before and 4h after unchaining acute pancreatitis. *Group 7* (LN+MIF+RLX, non-selective NOS inhibitor associated to GC receptor antagonist and relaxin) L-NAME at 10 mg/kg and 5 mg/kg, mifepristone at 3 mg/kg and relaxin at 2.5ug/kg were administered sc 1h before and 4h after triggering acute pancreatitis. *Group 8* (RLX 1h pre BPDOE-CDLs) received 5ug/kg sc of relaxin 1h before the surgical maneuvers. *Group 9* (RLX 1h post BPDOE-CDLs) was injected with 5 ug/kg sc of relaxin 1h after the procedure. *Group 10* (RLX 1h pre and 4h post BPDOE-CDLs) was administered Relaxin at 2.5 ug/kg sc 1h before and 4h after unchaining acute pancreatitis. *Group 11* (RLX 4h post BPDOE-CDLs) was treated with 5 ug/kg sc of relaxin 4h after triggering acute pancreatitis. L-NAME and mifepristone were dissolved in saline solution, relaxin was dissolved in PBS. *Group 12* (RLX 12h &1h pre and 4h post BPDOE-CDLs) received relaxin at 5 ug/kg sc 12 h before the surgery and 2.5 ug/kg sc 1h prior to the procedure and a second administration 4h after it.

Experimental procedures

Bilio-pancreatic-duct-outlet-exclusion closed-duodenal-loops model (BPDOE-CDLs) We described the BPDOE-CDLs model in detail Figure 1, (reproduced with permission granted by the Journal of Pancreatology and S. Karger AG, Basel)[6]. The normally fed animals were anesthetized with sodium pentothal (5 mg/100 g) (Thiopental, Abbott, USA). A median laparotomy was done to exteriorize the antrum-duodenum-pancreas, and a double-tube double-hole intra-duodenal catheter was inserted, through which saline solution (3 mL) was instilled. Two closed-duodenal-loops were prepared: first two proximal pre-pyloric ligatures and one post-pyloric just above the common-bilio-pancreatic duct outlet

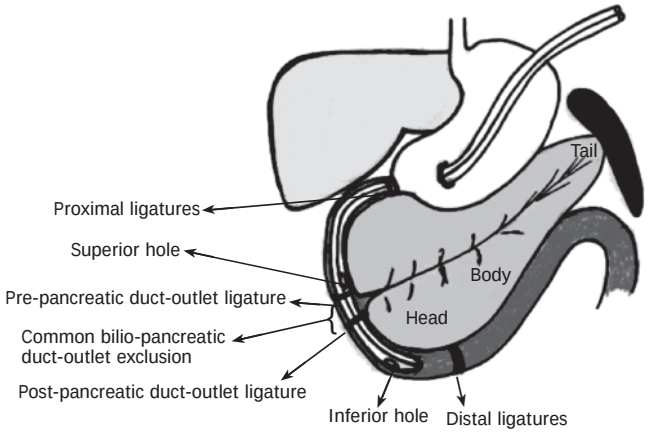

Figure 1 BPDOE-CDL model to trigger acute pancreatitis. Two closed-duodenal-loops were created in such a way that the entrance of the common bilio-pancreatic duct-outlet was absolutely excluded. To double check the absence of duodenal-reflux content or duodenal leakage, methylene blue was added to the sodium tauracholate which was instilled into the peri-vaterian duodenum through the superior and inferior holes (reproduced with permission granted by the Journal of Pancreatology and S. Karger AG, Basel)[6].

were placed. For the second loop, a ligature was placed just below the common-bilio-pancreatic-duct and two more ligatures were placed at the distal end of the peri-vaterian duodenum, thus excluding the common-bilio-pancreatic-duct outlet. Each closed-duodenal-loop was filled with 8% sodium taurocholate (1 mL) and 200 uL of methylene blue at constant pressure, following a 45-minute observation period. The closed-duodenal-loop content was aspirated, the ligatures removed and the laparotomy closed. The animal was then placed in an individual cage with microenvironmental temperature control and allowed to recover. After 12 h, the rat was exsanguinated under anesthesia by aortic puncture and a blood sample was obtained for biochemical tests. All procedures were done with aseptic techniques. If, despite exclusion of the common-bilio-pancreatic-duct, duodenal-content filtered through the ligatures and refluxed towards the bilio-pancreatic ductal tree, as evidenced from blue coloring of the pancreas, the rat was excluded from the study.

Macroscopic examination, dissection and weighing with a precision scale (Microgram-Series BH 300) of the pancreas were performed. The gland was subsequently divided into cephalic and splenic segments[6,17,18].

The liver, duodenum, kidneys, suprarenal glands, spleen and lungs were examined and dissected. Each organ was divided into smaller portions, one was fixed in formol-buffer and stained with hematoxylin-eosin, the others were wrapped individually in aluminum foil and preserved at -70 ℃. The cephalic and splenic segments of the pancreas and the lungs were studied in more detail.

Amylase and lipase activity measurement and protein quantification in pancreatic tissue Assays were done on each segment; the tissue was homogenized on ice in 2 mL of extraction buffer (20 mmol/L Tris-HCL pH 7.6-[Sigma, USA], 0.3 mol/L NaCl-[Merck, Germany], 0.5% Triton X-100-[Sigma, USA]; distilled water). Proteins were quantified in the homogenate according to the method described by Lowry[19]. The homogenate was centrifuged at 10000 r/min for 15 min at 4 ℃. The supernatant was assayed for amylase (kinetic method at 405 nm with defined substrate at 25 ℃-[Wiener, Arg.]) and lipase (turbidimetric method UV color at 25 ℃-[Randox, UK]).

Myeloperoxidase (MPO) activity in pancreas and lung tissue MPO, an enzyme found in neutrophils that represents the 5% of its dry weight, catalyzes the oxidation of electron donors (eg.halides) by hydrogen peroxide[20]. MPO was measured in both pancreatic segments and in lung tissue as a biochemical indicator of neutrophil infiltrate. To determine MPO activity the tissue was homogenized on ice with 1 mL hexadecyltrim ethylammonium bromide (HTAB) (0.5 % HTAB in 50 mmol/L phosphate buffer-pH 6.0). The homogenate was sonicated during 10 s at 200 W, and then centrifuged at 40000 r/min for 15 min at 4 ℃. MPO was assayed on the supernatant: 0.1 mL of the sample was combined with 2.9 mL of 5 mmol/L phosphate buffer, (pH 6.0, 0.167 mg/mL o-dianisidine hydrochloride and 0.0005% hydrogen peroxide). The change in absorbance at 460 nm was measured with a Clinicon 4010 spectrophotometer. One unit of MPO (UMPO) activity was defined as the amount that could degrade 1 μmol of peroxide per minute at 25 [21].

Caspase 2-3-8 and 9 activities in pancreas, liver and lung tissue Caspases 2-3-8 and 9 are early indicators of apoptosis in mammalian cells[22]. The CasPASE™ apoptosis activity assay kit [Genotech, MO-USA] is based on detecting the chromogenic and fluorogenic resulting molecule 7-amino-4-trifluromethyl cumarin (AFC) cleaved from the synthetic substrate, Z-VAD-fluoromethyl ketone (FMK) irreversibly and selectively inhibits the reaction. The excitation and emission wavelengths of AFC in a fluorometer are 360 nm and 510 nm respectively. Frozen tissues were homogenized at 4 ℃ in chilled lysis buffer and the lysates were then centrifuged at 15000 g for 30 min at 4 ℃, caspase activity was assessed on the supernatants. To calculate specific enzyme activity equal aliquots of supernatant were tested in presence of the Z-VAD-FMK inhibitor. Results were expressed as the specific cleavage of AFC per mg of protein per minute considering the nonspecific product formation coming from the substrate plus the inhibitor. All reagents were supplied by the kit and the assays were performed according to the manufacturer's instructions.

ATP and ADP/ATP ratio measurements in pancreas, liver and lung tissue ATP depletion allows to detect apoptosis, and when it is associated with the percentage of ATP loss and changes in the ADP/ATP ratio apoptosis can then be conveniently differentiated from necrosis[23]. Light formed from ATP and luciferin in the presence of luciferase can be measured at 560 nm. The tissue was homogenized in TCA (trichloroacetic acid) 6% for 1 min and centrifuged at 6000 g for 5 min at 4 ℃. The TCA in the supernatant was neutralized and diluted to a final concentration of 0.1% with Tris-Acetate pH 7.75 buffer. ATP concentration expressed as nmol/L per mg of protein and ADP/ATP concentration ratio were measured in the supernatant using the ENLITEN® ATP Assay System Bioluminescence Detection Kit [Promega, WI-USA] and ApoSENSOR™ ADP/ATP ratio assay kit (Alexis-Corp, Switzerland) respectively according to the manufacturer's instructions.

Extraction-purification-quantification of DNA The total mass of DNA in the pancreas, and lung tissue was used to standardize the units of enzymatic activity since it did not show significant quantitative variations in the BPDOE-CDL model.

The DNA was extracted and purified from tissue from both pancreatic segments and lung according to the manufacturer's instructions (Wizard Genomic- [Promega, USA]). Double stranded DNA concentration was measured at 260 nm.

Histologic evaluation Pancreatic tissue was graded on a scale of 0-4 each for: edema, hemorrhage, leukocyte infiltration, acinar necrosis and fat necrosis. Lung tissue was graded using a scale of 0-4 for leukocyte infiltration. Histologic changes were graded in a blinded manner by two independent observers counting frequency of foci per field seen at 40X (absent = 0, mild = 1, moderate = 2, severe = 3, overwhelming = 4).

Biochemical assays in blood samples *Loss of blood and hemoconcentration* were evaluated by the hematocrite. The *metabolic profile* consisted of glucose (enzymatic method in serum at 37 ℃-[Wiener, Arg.]), uric acid (enzymatic method in serum at 37 ℃-[Wiener, Arg]), proteins (enzymatic method in serum at 37 ℃-[Wiener, Arg.]) and calcium (direct colorimetric method in serum at 20 ℃-[Wiener, Arg.]). The *kidney profile* comprised urea (enzymatic method in serum at 37 ℃-[Wiener, Arg.]) and creatinine (end-point colorimetric method in serum at 37 ℃-[Wiener, Arg]). The *pancreatic profile* involved amylase (kinetic method at 405 nm with defined substrate in serum at 37 ℃-[Wiener, Arg.]) and lipase (UV turbidimetric method in serum at 37 ℃-[Randox, UK]). The *liver profile* consisted of AST and ALT (IFCC optimized UV method in serum at 37 ℃-[Wiener, Arg.]), bilirubin (colorimetric method to determine total and direct bilirubin in serum at 25 ℃, indirect bilirubin that results from the difference between them-[Wiener, Arg.]) and alkaline phosphatase (optimized DGKC-SSCC kinetic method at 405 nm in serum at 37 ℃-ALP 405-[Wiener, Arg.]) *Cellular injury* was assessed by LDH (DGKC optimized kinetic method in serum at 37 ℃-[Wiener, Arg.]). The *profile for cellular stress and inflammatory response associated to severity* involved leukocyte count, quantitative C reactive protein (immunoturbidimetric method in serum at 37 ℃ -PCR Latex Turbitest-[Wiener, Arg.]), heat shock protein (hsp) 72 (ELISA method for rat HSP 72 in plasma and serum-[StressGen Biotechnologies, USA)], IL-6 and IL-10 (ELISA technique in serum-[Assay Designs,USA]) and 8-Isoprostane (EIA method in plasma-[Cayman, USA]).

Statistical analysis

Biochemical assays in blood and organs The ANOVA test was used to establish differences between groups. The Dunnet test compared the difference between each group in relationship to group 1 (reference baseline level in control animals). The Student-Newman-Keuls test carried out after the ANOVA compared the groups between them and in relationship to group 1, evaluating the existence of homogeneous groups. Results are expressed as mean ± standard deviation (SD). Differences were considered statistically significant with $P < 0.05$.

Histopathological score The ANOVA test with two fixed factors (group and place: cephalic and splenic) was followed by the Dunnet test and Student-Newman-Keuls test. Results are expressed as mean ± SD. Differences were considered statistically significant with $P < 0.05$.

Correlation between MPO and leukocyte infiltrate In both pancreatic segments and in lung tissue, the Spearman correlation coefficient Rho was calculated to examine the correlation between MPO and leukocyte infiltrate[24].

RESULTS

Biochemical assays in blood and other tissues together with histopathological studies evaluated the influence of relaxin on the evolution of acute pancreatitis. Association with L-NAME and/or mifepristone pondered on the weight of the nitric oxide pathway and glucocorticoid receptor stimulation involvement in acute pancreatitis modulation by relaxin. These results are described in Tables 1-4.

Pancreatic and general biochemical assays in blood

To evaluate if relaxin helped to maintain pancreatic, metabolic, kidney and liver function after the initiation of acute pancreatitis, pancreatic and general biochemical assays in blood were performed (Table 1).

Cholestasis was absent in all groups. Uric acid, alkaline phosphatase, total - direct and indirect bilirubin showed no significant differences among the mean values for all groups, the rest of the assays did.

Amylase and lipase showed a significant increase for homogenous groups (3-4) that developed a severe acute pancreatitis, group 7 also exhibited a marked raise although not to the same extent and the values of the enzymes for homogenous groups (5-6) were slightly lower than for group 7. Relaxin treatment 12& 1h before and 4h after BPDOE-CDLs (group 12) was similar to the one corresponding to 1h pre and 4h post pancreatic maneuvers (group 10). These treatments resulted in the least increase in pancreatic enzymes. These same trend was observed for glucose, urea, creatinine, AST and ALT. The abnormal reduction profile in calcium and serum proteins presented the same course as the pathological augmentation observed for pancreatic enzymes. The hematocrite value, considering group 2 as the reference group due to blood loss during surgery, indicated that homogenous groups (10-12), developing mild acute pancreatitis did not manifest the hemoconcentration observed for homogenous groups (3-4).

2- Severity indicators in blood

We next wanted to assess if the severity of acute pancreatitis was ameliorated by relaxin treatment measuring blood parameters (Table 2 & Figure 2A). The raise in leukocyte count correlated ($R = 803$, $P < 0.05$) with the expression of inflammatory IL-6 and liver synthesized acute phase protein CRP. The significant increase observed for homogenous groups (3-4) was followed in order by that developed in group 7 and then by homogenous groups (5-6); these values were markedly diminished for homogenous groups (10-12). Inhibition of either the nitric oxide pathway or glucocorticoid receptor stimulation

Table 1 Pancreatic and general biochemical assays in blood (mean ± SD)

Groups	Amylase IU/L	Lipase IU/L	Glucose mg/dL	Urea mg/dL	Creatinine mg/dL	Calcium mg/dL	Proteins g/L	AST IU/L	ALT IU/L	Hto %
1 Basal	1103±108	85±18	92±13	29±3	0.35±0.04	13.48±0.39	85.5±1.2	177±25	88±19	39±5
2 Surgery control	1198±114	98±10	95±10	38±5	0.48±0.11	13.22±0.35	82.7±0.8	205±21	99±21	31±4
3 AP	7915±159	5619±194	338±24	219±19	1.82±0.21	8.92±0.16	61.1±1.3	589±26	295±28	49±9
4 AP+PBS	7921±137	5628±201	341±19	217±15	1.81±0.16	8.94±0.20	61.2±2.2	591±25	299±28	49±8
5 Rlx+LN	7154±165	4982±203	246±23	183±22	1.58±0.22	9.37±0.18	65.5±1.8	544±30	268±19	47±6
6 Rlx+MIF	7162±171	4997±207	249±27	185±24	1.60±0.25	9.35±0.14	65.1±1.9	552±28	270±22	47±8
7 Rlx+LN+MIF	7548±175	5327±214	262±33	194±21	1.69±0.22	9.11±0.21	62.4±1.4	563±34	281±27	48±5
8 Rlx 1h pre	6052±169	3618±155	190±16	125±11	1.13±0.14	11.19±0.22	74.5±2.3	405±24	196±23	40±4
9 Rlx 1h post	6427±185	3910±183	218±15	146±15	1.24±0.19	10.91±0.19	71.1±1.9	452±37	211±27	45±4
10 Rlx 1h pre+4h post	5589±129	3161±142	166±18	97±8	1.02±0.17	11.98±0.24	79.4±1.6	382±29	178±23	42±5
11 Rlx 4h post	6896±192	4533±178	235±29	165±19	1.39±0.20	10.21±0.19	69.9±2.1	497±33	235±25	46±5
12 Rlx 12h&1h pre+4h post	5535±144	3153±121	162±22	95±10	1.00±0.15	12.03±0.29	79.8±1.4	395±33	171±25	43±5

In relationship to Reference Basal-Group 1, BPDOE-CDLs AP-Group 3 presents a significant difference in all the assays evaluated $P<0.05$. The pancreatic and general biochemical profile significantly improved with relaxin treatment particularly for Groups 10 &12 that corresponded to administration times 12h and/or 1h pre and 4h post surgery $P<0.05$. Homogenous groups for amylase and lipase:1-2-(12-10)-8-9-11-(5-6-7)-(3-4), $P<0.05$. HTO: hematocrite, Rlx: relaxin, LN: L-NAME, MIF: mifepristone.

Table 2 Severity indicators assays in blood (mean ± SD)

Groups	Leukocytes nr/mL	CRP mg/dL	HSP 72 pg/mL	IL-6 pg/mL	IL-10 pg/mL	8-isoprostane pg/mL
1 Basal	6100±224	0±0	739±32	below detection limit	below detection limit	0.11±0.005
2 Surgery control	9856±272	3.3±1.02	1221±21	207±26	382±59	0.13±0.006
3 AP	26162±258	30.9±1.15	3396±64	1962±27	2216±94	0.55±0.011
4 AP+PBS	26145±294	30.6±1.21	3378±56	1956±32	2201±85	0.56±0.010
5 Rlx+LN	21223±207	21.7±1.24	2727±48	1792±28	2063±90	0.49±0.010
6 Rlx+MIF	21207±236	21.4±1.31	2765±52	1784±35	2052±78	0.48±0.009
7 Rlx+LN+MIF	23239±273	27.3±1.12	3023±39	1849±29	2113±122	0.50±0.008
8 Rlx 1h pre	17438±266	13.9±1.22	1924±49	1473±33	1695±72	0.37±0.010
9 Rlx 1h post	18776±248	16.4±1.52	2258±42	1601±25	1809±87	0.42±0.009
10 Rlx 1h pre+4h post	14527±285	10.8±1.44	1711±39	1098±32	1451±68	0.31±0.010
11 Rlx 4h post	19349±225	19.5±1.46	2506±38	1689±23	1975±84	0.46±0.008
12 Rlx 12h&1h pre+4h post	14542±295	10.9±1.27	1721±43	1105±28	1460±76	0.32±0.007

Severe AP was developed in groups 3 & 4. This severity was ameliorated by treatment with relaxin. In relationship to reference basal-group 1, BPDOE-CDLs AP in group 3 presented a significant difference in all the assays evaluated while treated groups 10 & 12 were the ones to exhibit the best prognosis with a significant improvement for all markers $P<0.05$.Homogenous groups for severity indicators :1-2-(12-10)-8-9-11-(5-6-7)-(3-4), $P<0.05$. Rlx: relaxin, LN: L-NAME, MIF: mifepristone.

reduced the efficiency of relaxin treatment; inhibiting simultaneously both of them although leading to a more severe manifestation of the disease still showed better prognosis than that observed for homogenous groups (3-4) suggesting that there may be another mechanism by which relaxin may be also acting. Treatment with a single dose of relaxin 1h before, 1h after or 4h after unchaining acute pancreatitis although still beneficial was not as effective. Pro-inflammatory IL-6 and anti-inflammatory IL-10 exhibited the same profile as a consequence of the induction of the inflammatory response and attempt to counter-act it. Lipid peroxidation leading to elevated 8-isoprostane and synthesis of hsp72 aiming to protect from ongoing cellular stress followed the same trend.

Assays in pancreatic and lung tissue

We then wanted to investigate the enzymatic and inflammatory pancreatic and lung response (Table 3).

There were no significant differences between the mean value of the groups for DNA of the lung and the same pancreatic segment, but the mean values for the cephalic and splenic segments for each group were significantly different (data shown previously[6]).

The decrease in pancreatic amylase and lipase was inverse to their increase in blood. Pancreatic proteins showed a similar expression profile to that of pancreatic enzymes, the elevation for group 2 may result from the synthesis of acute phase pancreatic proteins.

Pancreatic and lung MPO activity reflecting neutrophil

Table 3 Assays in pancreatic and lung tissue (mean ± SD)

	Panc. Amylase IU/mg DNA ds		Panc. Lipase IU/mg DNA ds		Panc. Proteins mg/mg DNA ds		Pancreatic MPO UMPO/mg DNA ds		Lung MPO UMPO/mg DNA ds	Panc. Wet Weight mg/100 g body wt
Groups	H	B&T	H	B&T	H	B&T	H	B&T		
1 Basal	995±24	603±27	98±10	75±11	185±17	103±12	0±0	0±0	0±0	1310±19
2 Surgery control	927±19	576±22	81±11	66±10	206±19	122±14	0±0	0±0	0±0	1385±24
3 AP	263±17	181±14	26±9	16±8	115±10	57±10	10.64±0.39	7.15±0.11	3.85±0.13	2335±38
4 AP+PBS	261±20	185±26	25±9	15±9	114±11	58±10	10.59±0.31	7.22±0.09	3.83±0.12	2321±43
5 Rlx+LN	392±18	216±21	34±5	24±6	129±14	64±8	8.12±0.32	5.21±0.12	2.93±0.17	2159±40
6 Rlx+MIF	389±19	219±23	35±7	23±5	130±12	64±13	8.09±0.36	5.27±0.15	2.96±0.15	2142±48
7 Rlx+LN+MIF	315±15	202±12	32±8	19±4	121±10	60±8	9.01±0.42	6.16±0.10	3.10±0.14	2198±39
8 Rlx 1h pre	590±29	342±16	50±12	37±7	154±18	81±16	5.94±0.22	3.68±0.08	2.21±0.08	1798±33
9 Rlx 1h post	505±33	307±35	45±10	32±4	147±20	70±10	6.23±0.25	3.95±0.11	2.43±0.09	1967±39
10 Rlx 1h pre+4h post	679±31	387±18	60±9	43±8	165±23	90±11	5.79±0.19	3.59±0.09	2.06±0.10	1688±29
11 Rlx 4h post	439±22	273±24	39±9	27±9	135±15	66±7	7.05±0.28	4.45±0.09	2.64±0.14	2087±42
12 Rlx 12h&1h pre+4h post	683±26	392±17	59±8	44±9	167±21	89±10	5.78±0.21	3.54±0.03	2.07±0.09	1693±25

Pancreatic amylase and lipase decreased significantly in severe AP group 3, $P<0.05$. Treatment with relaxin induced a significant improvement with an increase in pancreatic enzymes and proteins $P<0.05$, while there was a decrease in UMPO. This amelioration of the disease was even more remarkable for groups 10 & 12 indicating that treatment 12 and/or 1h pre and 4h post BPDOE-CDLs procedure had a protective role. The increase in pancreatic wet weight in groups with severe AP as a result of edema was significant $P<0.01$. Homogenous groups for pancreatic amylase-lipase and MPO: :1-2-(12-10)-8-9-11-(5-6-7)-(3-4), $P<0.05$; lung MPO: :1-2-(12-10)-8-9-11-(5-6-7)-(3-4), $P<0.05$. Rlx: relaxin, LN: L-NAME, MIF: mifepristone.

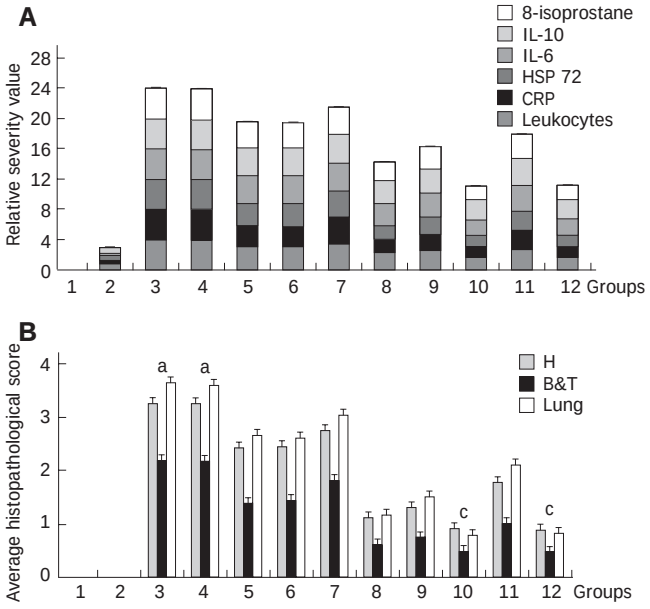

Figure 2 Relative severity value in blood and average histopathological score. **A**: Relative severity value in blood. To visualize the severity degree based on biochemical assays in blood, we arbitrarily established a single "relative severity value" of zero to group 1 (reference baseline value) and of four to group 3 (AP) per assay. To calculate the single "relative severity value" for the rest of the groups we used the following formula: "relative severity value" = [(X – reference baseline value) / (AP value – reference baseline value)] x 4. Introducing each mean value from table 2 (referred to in the above formula as X) we obtained six different single "relative severity values" for each group, whose contribution to the total "relative severity value" is shown in this graph. For those values in table 2 that were below the detection limit, the value assigned to X corresponded to that of the assay's sensitivity (IL-6: 112 pg/mL; IL-10: 12 pg/mL). Homogenous Groups are 1-2-(12-10)-8-9-11-(5-6-7)-(3-4), $P<0.05$. Results are shown as mean ± SD. **B**: Average histopathological score. To visualize the severity degree based on the score-associated to histopathological lesions, we averaged the means of the six parameters used in table 4 for each group. Homogenous groups are 1-2-(12-10)-8-9-11-(5-6-7)-(3-4), $P<0.05$. There was a significant increase in the assessed parameters for Groups 3&4 that developed severe AP $^{a}P<0.03$ while groups 10& 12 exhibited a significant improvement $^{c}P<0.05$ Results shown as mean ± SD.

infiltrate, was significantly higher for homogenous groups (3-4) while homogenous Groups (10-12) that had been treated with relaxin before and after triggering acute pancreatitis presented lesser UMPO values. The Spearman Correlation Coefficient between MPO activity and leukocyte infiltrate was significant for the pancreatic cephalic segment ($R=0.943$, $P<0.01$); for the pancreatic splenic segment ($R=0.831$, $P<0.001$); and for the lung ($R=0.871$, $P<0.01$).

The increase in pancreatic wet weight due to edema was more significant for homogenous groups (5-6)-7-(3-4) than for homogenous groups (10-12).

Histopathological scores in pancreas and lung tissue

Homogenous groups (10-12) that developed a mild acute pancreatitis exhibited edema as the main histopathological feature and a lower value for pancreatic and lung leukocyte infiltrate that correlated with MPO activity. Homogenous groups (5-6)-7-(3-4) were characterized by high scores for leukocyte infiltrate that correlated with MPO activity, pancreatic hemorrhage as well as acinar and fat necrosis. Necrotic foci presented a spotted pattern with higher frequency in the cephalic segment. group 11 that received relaxin 4h after triggering acute pancreatitis were characterized by hemorrhage. The average of pancreatic histopathological scores presented a significant lower value for homogenous groups (10-12) when comparing them to the average values obtained for homogenous groups (5-6)-7-(3-4). These results are described in Table 4 and Figure 2B.

General AP histopathology Groups with severe AP presented parenchymal necrosis with neutrophil infiltrate, fat necrosis, congestive blood vessels in peri-pancreatic fat, and intense hemorrhage. The duodenum exhibited lysis in the mucosa, subserosal blood vessel congestion and severe neutrophil infiltrate but there was no evidence of possible leakage towards the cephalic segment. These groups exhibited alterations in the liver, lung, kidneys, spleen and suprarenal glands. The liver showed necrosis, hemorrhage, blood vessel congestion, neutrophil infiltrate and steatosis.

Table 4 Histopathological scores in pancreas and lung tissue (mean ± SD)

	Pancreatic Edema		Pancreatic Hemorrhage		Panc. Leuk. Infiltrate		Panc. Acinar Necrosis		Panc. Fat Necrosis		Lung Leuk
	H	B&T	H	B&T	H	B&T	H	B&T	H	B&T	Infiltrate
Groups											
1 Basal	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
2 Surgery control	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
3 AP	1.31±0.09	1.14±0.08	3.58±0.08	1.84±0.06	3.61±0.10	2.47±0.08	3.87±0.10	2.90±0.08	3.98±0.12	2.60±0.08	3.65±0.15
4 AP+PBS	1.33±0.07	1.12±0.10	3.55±0.10	1.82±0.07	3.64±0.09	2.46±0.11	3.83±0.09	2.86±0.12	3.95±0.10	2.66±0.11	3.61±0.10
5 Rlx+LN	1.01±0.06	0.73±0.10	2.46±0.10	1.08±0.06	2.93±0.14	1.53±0.07	2.96±0.10	2.03±0.05	2.84±0.11	1.57±0.08	2.66±0.10
6 Rlx+MIF	0.99±0.10	0.75±0.09	2.49±0.06	1.27±0.05	2.97±0.11	1.58±0.09	2.99±0.09	2.00±0.07	2.80±0.07	1.61±0.09	2.62±0.07
7 Rlx+LN+MIF	1.26±0.10	0.92±0.08	2.97±0.09	1.42±0.05	3.23±0.08	2.25±0.06	3.35±0.05	2.45±0.08	3.00±0.09	2.08±0.10	3.04±0.08
8 Rlx 1h pre	0.51±0.08	0.42±0.08	1.37±0.07	0.59±0.05	0.86±0.08	0.45±0.05	1.39±0.11	0.85±0.08	1.43±0.10	0.75±0.08	1.17±0.09
9 Rlx 1h post	0.62±0.07	0.50±0.08	1.50±0.08	0.71±0.08	1.03±0.03	0.62±0.06	1.64±0.07	1.01±0.06	1.71±0.08	0.98±0.05	1.52±0.10
10 Rlx 1h pre+4h post	0.42±0.07	0.37±0.06	1.26±0.10	0.43±0.07	0.72±0.07	0.37±0.09	0.92±0.09	0.59±0.05	1.19±0.09	0.63±0.09	0.80±0.08
11 Rlx 4h post	0.77±0.08	0.59±0.07	1.97±0.05	0.89±0.09	1.67±0.05	0.89±0.08	2.28±0.13	1.45±0.08	2.23±0.09	1.19±0.07	2.11±0.05
12 Rlx 12h&1h pre+4h post	0.45±0.05	0.36±0.05	1.24±0.08	0.45±0.04	0.70±0.05	0.38±0.06	0.90±0.05	0.60±0.05	1.20±0.05	0.61±0.07	0.83±0.06

Groups 3&4 that developed severe AP presented as main features leukocyte infiltrate, hemorrhage and acinar and fat necrosis and the histopathological scores were higher. Groups 10 & 12 that received relaxin 12 and/or 1h pre and 4h post surgery developed a mild AP and exhibited the lowest scores being the main feature edema. Homogenous groups for pancreatic edema, hemorrhage, acinar and fat necrosis and pancreatic and lung leukocyte infiltrate: :1-2-(12-10)-8-9-11-(5-6-7)-(3-4), $P<0.05$. Rlx: relaxin, LN: L-NAME, MIF: mifepristone.

The lungs were highly compromised exhibiting congestion of big blood vessels and alveolar capillaries with neutrophil infiltrate; the bronchial wall had reactive lymphoid hyperplasia and aspirative hemorrhage. The main features observed in the kidneys were congestion of cortical and medullar blood vessels and glomerular capillaries jointly with tubular and medullar acute necrosis. Suprarenal glands were characterized by edema and partial lipid loss while the spleen presented edema.

Caspases activity (2-3-8-9)

We next examined if treatment of pancreatic injury was associated to the activation of caspases 2-3-8-9 in the pancreas as primary site of injury and in the lung and the liver as relevant affected distant organs. Homogenous groups (10-12) that developed a mild acute pancreatitis manifested a greater activation of caspases 2-3-8-9 suggesting that the activity of both the initiator caspase 8 and the executor caspase 3 together with caspases 9 and 2 involved in the apoptosome or intrinsic pathways are stimulated by relaxin treatment suggesting that apoptosis is more significant in these groups than in homogenous groups (5-6)-7-(3-4) that developed a severe necrotizing acute pancreatitis (Figure 3).

Death due to apoptosis or necrosis

The next step was to elucidate whether relaxin treatment favored death by apoptosis over necrosis. Blood lactate dehydrogenase (LDH) is a marker of cellular injury and death. The raise in blood LDH due to acute pancreatitis was ameliorated in homogenous groups (10-12) (Figure 4A). To evaluate if the undergoing death process in the pancreas as primary site of injury and in the lung and the liver as relevant affected distant organs corresponded to apoptosis or necrosis we measured ATP concentration (Figure 4B) and then calculated the % of loss of ATP (Figure 4C) given the fact that the death process involves ATP consumption being an 85% or more ATP depletion associated to necrosis. Also the ADP/ATP ratio (Figure 4D) helped to differentiate between these two death mechanisms as a higher increase is indicative of necrosis while a lower one points towards apoptosis.

ATP depletion for homogenous groups (5-6)-7-(3-4) associated to a higher % of ATP loss and ADP/ATP ratio correlated ($R=0.842$; $P<0.05$) with the higher frequency observed in necrotic foci and severity indicators for these groups together with the elevated LDH value suggesting that for groups undergoing severe acute pancreatitis necrosis was the main form of cell death.

Homogenous Groups (10-12) that manifested a mild acute pancreatitis evidenced lesser ATP depletion with a % of ATP loss lower than 40% together with a lower ADP/ATP ratio. These results correlated with lower values for LDH ($R=0.798$, $P<0.05$) while edema was the principal histopathological feature. This suggested that for these groups apoptosis was the main form of cell death resulting in a more controlled process with less pro-inflammatory stimulation ameliorating the aggressiveness of the pathology.

Increased degree of severity

For each variable with significant differences between the groups' mean values, the relationship of these results were compared to the reference baseline level established in group 1. We then integrated this information to rank the groups in order of "increased degree of severity" (Table 5) showing that the severe acute pancreatitis developed by homogenous groups (3-4) was significantly ameliorated for homogenous groups (10-12).

DISCUSSION

According to the severity degree exhibited by acute pancreatitis will be the complexity of the pathology involving other organs apart from the pancreas that will lead to metabolic, liver, kidney and lung alterations

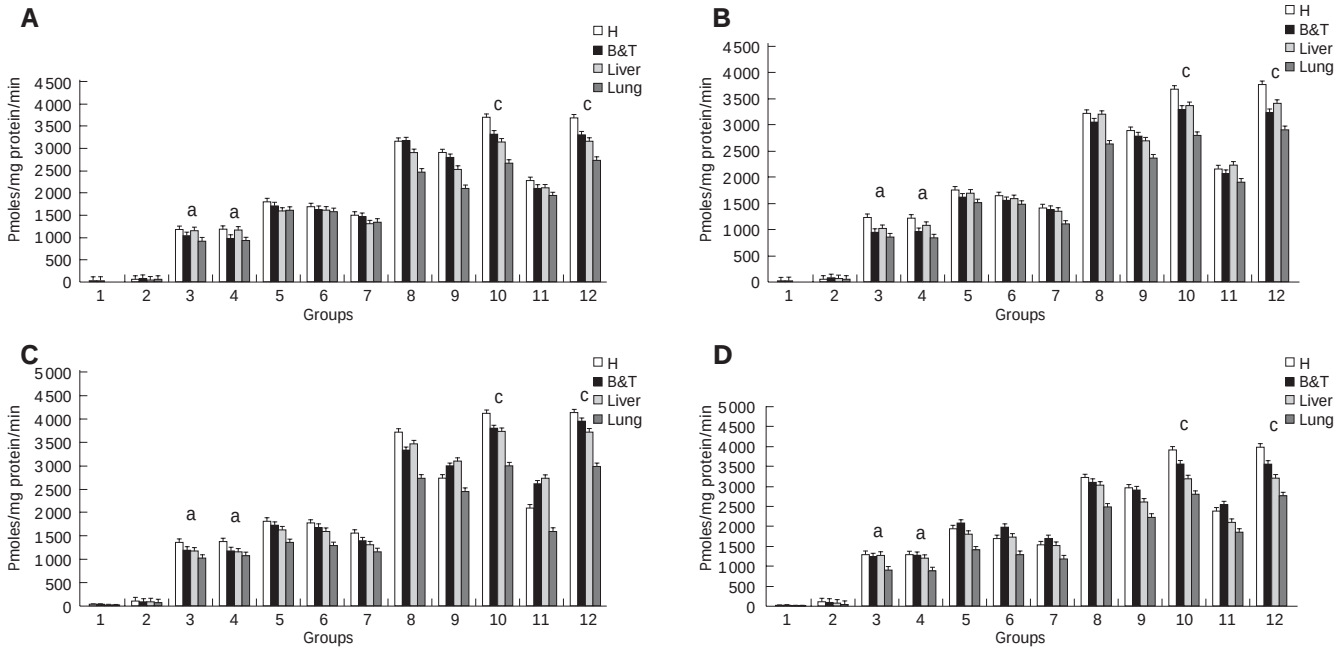

Figure 3 Caspase Activities. **A**: *Caspase 2 activity* was measured to evaluate the upstream activation of the intrinsic apoptotic pathway. Groups 10&12 presented a significant activation $^{c}P<0.05$ which was reduced for groups 3&4 $^{a}P<0.05$. Homogenous groups for caspase 2 activity are: 1-2-(12-10)-8-9-11-(5-6-7)-(3-4), $P<0.05$. H: pancreatic head, B&T: pancreatic body and tail. **B**: *Caspase 3 Activity* was measured to evaluate the activation of the executor caspase 3. Groups 10&12 presented a significant activation $^{c}P<0.05$ which was reduced for groups 3&4 $^{a}P<0.05$. Homogenous groups for caspase 2 activity are: 1-2-(12-10)-8-9-11-(5-6-7)-(3-4), $P<0.05$. H: pancreatic head, B&T: pancreatic body and tail. **C**: *Caspase 8 Activity* was measured to evaluate the activation of the initiator caspase 8. groups 10&12 presented a significant activation $^{c}P<0.05$ which was reduced for groups 3&4 $^{a}P<0.05$. Homogenous groups for caspase 8 activity are: 1-2-(12-10)-8-9-11-(5-6-7)-(3-4), $P<0.05$. H: pancreatic head, B&T: pancreatic body and tail. Results shown as mean±SD. **D**: *Caspase 9 Activity* was measured to evaluate the activation of the intrinsic pathway .Groups 10&12 presented a significant activation $^{c}P<0.05$ which was reduced for groups 3&4 $^{a}P<0.05$. Homogenous groups for caspase 9 activity are: 1-2-(12-10)-8-9-11-(5-6-7)-(3-4), $P<0.05$. H: pancreatic head, B&T: pancreatic body and tail. Results are shown as mean±SD.

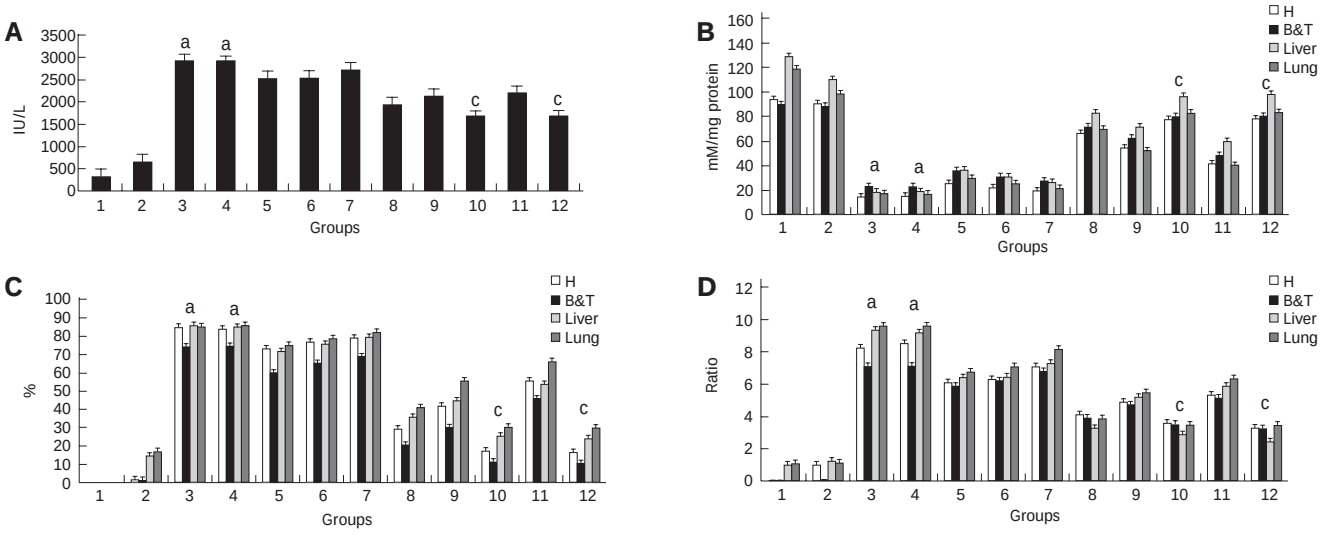

Figure 4 Apoptosis versus necrosis. **A**: Blood lactate dehydrogenase. As a marker of cellular injury and death significant high blood levels of LDH for groups 3&4 $^{a}P<0.005$ reflected the undergoing severe AP while groups 10&12 presented a significant reduction in the enzyme value $^{c}P<0.005$. Homogenous. Groups for LDH are:1-2-(12-10)-8-9-11-(5-6-7)-(3-4), $P<0.005$. **B**-**D**: ATP concentration. The significant decrease in ATP concentration that surpassed the 80% loss for groups 3&4 $^{a}P<0.005$,is the hallmark of the necrotic process that is being developed while groups 10&12 that underwent apoptosis did not manifest such extreme values $^{c}P<0.005$. This expression in the nucleotide levels is translated into the ADP/ATP ratio that is significantly increased $^{a}P<0.01$ for groups 3&4 that underwent necrosis while groups 10&12 that underwent apoptosis had significantly lower values $^{c}P<0.05$. Homogenous Groups are: 1-2-(12-10)-8-9-11-(5-6-7)-(3-4), $P<0.005$. Results are shown as mean±SD.

reflected in the histopathology and numerous biochemical parameters that require to be assessed in order to arrive to a proper classification of the disease.

It has been reported that relaxin can stimulate the nitric oxide pathway or the glucocorticoid receptor[7,14]. In our work we have evaluated the combined vasorelaxing and anti-inflammatory properties of relaxin on the evolution of acute pancreatitis, our results show that relaxin plays a highly beneficial role when administered within the first hour of the initiation of the noxa being this even more pronounced when the drug was given 1h pre and 4h post triggering acute pancreatitis, this would be particularly relevant for the prevention of post-ERCP pancreatitis providing basis for future studies where combined drug

Table 5 Order of groups according to the increase in the severity degree

GROUP NR.	GROUP DESCRIPTION
1	Basal Reference Control
2	Surgery Control
12	Relaxin administered 12h & 1h pre- BPDOE-CDLs and 4h post-BPDOE-CDLs
10	Relaxin administered 1h pre- BPDOE-CDLs and 4h post-BPDOE-CDLs
8	Relaxin administered 1h pre-BPDOE-CDLs
9	Relaxin administeres 1h post-BPDOE-CDLs
11	Relaxin administered 4h post-BPDOE-CDLs
5	Relaxin and L-NAME administered 1h pre- BPDOE-CDLs and 4h post-BPDOE-CDLs
6	Relaxin and Mifepristone administered 1h pre- BPDOE-CDLs and 4h post-BPDOE-CDLs
7	Relaxin, L-NAME & Mifepristone administered 1h pre- BPDOE-CDLs and 4h post-BPDOE-CDLs
3	BPDOE-CDLs AP model
4	Vehicle (PBS) control

The BPDOE-CDLs model induces a severe AP (group 3).Prophylactic treatment with relaxin, as shown for groups10&12, markedly reduces the severity of the pathology, being still useful although in a lesser degree even after AP was triggered.

treatments can be considered. When relaxin was received 4h after unchaining acute pancreatitis although still beneficial the efficiency of the treatment was reduced the reason probably being that additional supplemental therapies are required at this stage in order to restore the already altered hemodynamic and metabolic balance. The positive effect exerted by relaxin was significantly diminished by L-NAME and by mifepristone indicating that the nitric oxide pathway and glucocorticoid receptor stimulation are involved. The association of both inhibitors produced an even stronger reduction in the effect induced by relaxin however this group still presented less severe results than those observed for non-treated acute pancreatitis suggesting that there may be other pathways implicated.

While *apoptosis* is a programmed and controlled mechanism of cell death that is associated to reduced inflammation, *necrosis*, caused by irreparable cell injury causing membrane alterations that favor the release of enzymes that are responsible for degrading cellular structures and thus activating the inflammatory response[25]. Pancreatic hemorrhage and necrosis characterize severe acute pancreatitis being the consequence of dysfunctional tissue irrigation due to an initial hypoxia leading to an ischemia-reperfusion process due to alternative vasoconstriction followed by vasodilatation. These microcirculatory disturbances are linked to neutrophil activation and inflammation together with cellular stress that will convert cell death by apoptosis to necrosis, downregulating these factors by preserving pancreatic microvascular blood flow to ensure adequate tissue oxygenation and inhibiting pro-inflammatory mediators reduces the risk of necrotizing pancreatitis by favoring cell death by apoptosis over necrosis.

The fact that the severity of acute pancreatitis is inversely related to the degree of apoptosis suggests that this type of cell death may be a beneficial response to acinar cell injury particularly in acute pancreatitis. Clearly, the degree of inflammation and, therefore, the severity of pancreatitis are less in situations associated with acinar cell apoptosis than under conditions associated with acinar cell necrosis. The group of Steer[25] emphasizes then the notion that medications or other interventions that favor the development of apoptosis may minimize the severity of pancreatitis, and they could, therefore be of substantial clinical value. Together with Jones and Gores[26] they remark the concept that pharmacological induction of pancreatic apoptosis during the early stages of acute pancreatitis provides a relevant therapeutic strategy for the treatment of this disease.

The high density of neurons embedded in the pancreatic cephalic segment associated to the intimate relation between the peri-vaterian duodenum and the pancreas linked by the gastroduodenal plexus[27-29] emphasizes the relevance of the rich population of receptors in the peri-vaterian duodenum that when activated by irritative stimuli such as endoscopic maneuvers will unchain duodenopancreatic and duodenogastric autonomic-arc-reflexes. The BPDOE-CDLs model mimics this situation in which severe acute pancreatitis results from a sympathetic neurogenic overstimlaution that causes adrenergic hyper-reactivity responsible for the initial vasoconstriction and the subsequent ischemia/reperfusion injury. We propose that relaxin would, in first instance, counteract vasoconstriction arising from sympathetic-adrenergic stimulation by inducing vasodilatation through the nitric oxide pathway and exert anti-inflammatory properties by glucocorticoid receptor stimulation while favoring apoptosis over necrosis.

Severe acute pancreatitis induced by the BPDOE-CDLs model manifested kidney failure translated by the significant increase in urea and creatinine in blood associated to acute tubular necrosis; metabolic and liver alterations. The raise in severity factors indicating an intense inflammatory response defined by the increase in CRP and leukocyte count that correlated with neutrophil infiltrate in pancreatic and lung tissue associated to high activity values for MPO in these tissues suggested a SIRS in which the activation of neutrophils converted cellular death by apoptosis to necrosis as suggested by the high necrosis score in pancreatic tissue and the elevation of blood LDH describing organ injury. For this group caspases 2-3-8 and 9 activities were significantly reduced when comparing them to treated homogenous groups

(10-12). The dramatic depletion in ATP concentration, correlated with a loss of ATP that surpassed the 80 % thus inhibiting the initiation of the apoptotic pathway, and the increase in ADP/ATP ratio would be indicating a strong necrotic mechanism associated to a pro-inflammatory process as corroborated by the histopathology.

Groups that were treated with relaxin showed an improvement in blood metabolic parameters with reduced values for leukocyte count and CRP together with lower leukocyte infiltrate and MPO activity in pancreatic and lung tissue pointing to a milder inflammatory response. The increase in the activity of caspases 2-3-8 and 9 manifesting that both the intrinsic and the effector apoptotic pathways are activated is associated to a less drastic depletion of ATP that would not interfere with apoptosis and thus exhibit a reduced inflammatory reaction. Treatment with relaxin 4h after unchaining acute pancreatitis was beneficial however the best results were observed when relaxin was given within the first hour being optimal when administered 1h before and 4h after triggering acute pancreatitis as quite probably in this circumstance it could preserve microcirculation ensuring tissue oxygenation while inhibiting the expression of pro-inflammatory mediators associated with pro-apoptotic molecules, situation that is kept-up by the second dose, these results are relevant for the prevention of post-ERCP acute pancreatitis. There was no significant difference between homogenous groups 10 and 12 implying that starting treatment 12h or 1h prior to triggering acute pancreatitis exerts the same effect.

To evaluate the pathways involved in treatment with relaxin we associated the administration of relaxin 1h pre and 4h post BPDOE-CDLs procedure with L-NAME (NOS inhibitor) and/or mifepristone (glucocorticoid recptor antagonist). The inhibition of either the nitric oxide pathway or glucocorticoid receptor stimulation significantly reduced the positive effects described previously for homogenous groups (10-12). Surprisingly for group 7 when we associated both inhibitors the severity that was developed although greater than for homogenous groups (6-7) was still less than that displayed by homogenous groups (3-4) suggesting that there is another pathway involved in the treatment with relaxin.

We therefore conclude that relaxin plays a beneficial role in the evolution of acute pancreatitis triggered by the BPDOE-CDLs model as it induces an anti-inflammatory response via glucocorticoid receptor stimulation while favoring apoptosis over necrosis; simultaneously it helps to preserve pancreatic microcirculation by the nitric oxide pathway ensuring an appropriate blood flow and tissue oxygenation thus avoiding ischemia and the consequent ischemia/reperfusion injury while it reduces the expression and activation of pro-inflammatory factors and decreases neutrophil infiltration of the pancreas.

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BASIC RESEARCH

Cis-hydroxyproline-induced inhibition of pancreatic cancer cell growth is mediated by endoplasmic reticulum stress

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Abstract

AIM: To investigate the biological effects of cis-hydroxyproline (CHP) on the rat pancreatic carcinoma cell line DSL6A, and to examine the underlying molecular mechanisms.

METHODS: The effect of CHP on DSL6A cell proliferation was assessed by using BrdU incorporation. The expression of focal adhesion kinase (FAK) was characterized by Western blotting and immunofluorescence. Induction of endoplasmic reticulum (ER) stress was investigated by using RT-PCR and Western blotting for the glucose-related protein-78 (GRP78) and growth arrest and DNA inducible gene (GADD153). Cell viability was determined through measuring the metabolic activity based on the reduction potential of DSL6A cells. Apoptosis was analyzed by detection of caspase-3 activation and cleavage of poly(ADP-ribose) polymerase (PARP) as well as DNA laddering.

RESULTS: In addition to inhibition of proliferation, incubation with CHP induced proteolytic cleavage of FAK and a delocalisation of the enzyme from focal adhesions, followed by a loss of cell adherence. Simultaneously, we could show an increased expression of GRP78 and GADD153, indicating a CHP-mediated activation of the ER stress cascade in the DSL6A cell line. Prolonged incubation of DSL6A cells with CHP finally resulted in apoptotic cell death. Beside L-proline, the inhibition of intracellular proteolysis by addition of a broad spectrum protease inhibitor could abolish the effects of CHP on cellular functions and the molecular processes. In contrast, impeding the activity of apoptosis-executing caspases had no influence on CHP-mediated cell damage.

CONCLUSION: Our data suggest that the initiation of ER stress machinery by CHP leads to an activation of intracellular proteolytic processes, including caspase-independent FAK degradation, resulting in damaging pancreatic carcinoma cells.

Key words: Cis-hydroxyproline; Pancreatic cancer cell; endoplasmic reticulum stress; FAK; Caspase-3

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INTRODUCTION

The prognosis of pancreatic carcinoma is uniformly fatal. Conventional cytostatic treatments for inoperable pancreatic cancer make little impact on the median survival. Therefore, the development of novel therapeutic concepts that activate proliferation-independent cell death are needed[1]. Cell damage by targeting protein biosynthesis may be a promising strategy for cancer therapy[2,3]. Previous studies have shown that cis-4-hydroxy-L-proline (CHP) as an analogue of L-proline inhibits the collagen synthesis and its extracellular deposition[4,5]. Furthermore, there are reports describing inhibitory effects of CHP on proliferation, adhesion, and migration of various cell types like fibroblasts, epithelial cells and tumor cells[5-8]. These data pointed to additional and/or other proteins whose synthesis might be affected by incorporation of the incorrect amino acid leading to disturbances of cellular mechanisms.

Based on the CHP-induced inhibition of cell adhesion, we hypothesized that the focal adhesion kinase (FAK) may be involved in the CHP-induced signal transduction. FAK is a 125 ku non-receptor tyrosin kinase that mediates different cellular responses to adhesion after clustering of transmembrane integrins at contact sites between the cell and the extracellular matrix (ECM) termed focal adhesions[9]. Frisch *et al*[10] have shown that constitutively activated FAK conferred protection against anoikis of epithelial cells. Consequently, the inhibition

of FAK resulted in apoptosis of fibroblasts as well as in detachment and cell death of human breast tumor cells, suggesting a pivotal role of FAK in the transmission of anti-apoptotic signals[11,12].

The incorporation of amino acid analogues into newly synthesized proteins has been shown to interfere with the correct protein folding leading to an accumulation of misfolded proteins in the endoplasmic reticulum (ER)[13]. The ER has emerged as an organelle that is exquisitely sensitive to alterations in homeostasis that initiates a diversity of molecular defence mechanisms referred to as 'ER stress'[14]. The complex ER stress response to a variety of different stimuli results eventually in adaptation for survival or induction of apoptosis[14]. Mechanisms activated upon accumulation of unfolded protein in the ER should ensure that only properly folded proteins abandon the ER lumen[15]. This quality control system includes the unfolded protein response (UPR) which is characterized by an up-regulation of chaperons, such as the glucose-regulated protein-78 (GRP)78, as well as by an induction of transcription factors like the pro-apoptotic growth arrest and DNA gene product (GADD)153[16].

The aim of this study was to characterize the biological effects of CHP on the rat pancreatic tumor cell line DSL6A. Addressing the question of the underlying molecular mechanisms, we investigated the participation of ER stress and FAK in the CHP effect on cell proliferation, adhesion, survival and apoptosis.

MATERIALS AND METHODS

Reagents

Dulbecco's minimal essential medium (DMEM) and fetal calf serum (FCS) were purchased from Biochrom (Berlin, Germany). Iscove's modified Dulbecco medium (IMDM) was purchased from PAA Laboratories (Cölbe, Germany). Nonessential amino acids, penicillin, streptomycin, trypsin, and all reagents for reverse transcription [Superscript RT, oligo(dT)$_{12-18}$, dNTP] were purchased from Invitrogen (Karlsruhe, Germany). Cis-4-hydroxy-L-proline were from Riemser Arzneimittel AG (Greifswald, Germany). Colorimetric 5-bromo-2'-deoxyuridine (BrdU)-labelling cell proliferation ELISA and apoptotic DNA ladder kit were obtained from Roche (Mannheim, Germany). CellTiter AQ$_{ueous}$ kit from Promega (Mannheim, Germany), RNeasy Mini RNA extraction kit and Taq polymerase from Qiagen (Hilden, Germany) were used. Primers for PCR were generated by using NCBI GenBank as the source for any sequences and were synthesized by BioTez (Berlin, Germany). Mouse monoclonal anti-FAK$^{(N354-534)}$ was purchased from Transduction, mouse anti-GRP78 from BD Biosciences, anti-β-actin from Sigma (Taufkirchen, Germany), anti-cleaved caspase-3 from anti-poly-(ADP ribose) polymerase (PARP), anti-cyclinD1 from Santa Cruz. Secondary horseradish peroxidase-labelled anti-mouse or anti-rabbit Ig antibodies, nitrocellulose membranes, ECL plus kit and hyperfilm ECL were purchased from Amersham (Freiburg, Germany). Alexa Fluor® 488 goat anti-mouse IgG was purchased from MoBiTec (Göttingen, Germany). Caspase-3 inhibitor Z-DEVD-FMK, general caspase inhibitor Z-VAD-FMK and the protease arrest reagent were purchased from Calbiochem (Schwalbach, Germany).

Cell culture

The rat pancreas carcinoma cell line DSL6A was cultured in DMEM supplemented with 100 mL/L FCS, 100 U/mL penicillin and 100 μg/mL streptomycin or in IMDM containing non-essential amino acids in addition to 100 mL/L FCS and antibiotics.

In contrast to IMDM, DMEM did not contain L-proline. To prevent cell adhesion, culture plates were coated with 20 g/L agarose as previously described[17].

Cell proliferation and cell survival

Cell proliferation was assessed using the colorimetric 5-bromo-2´-deoxyuridine (BrdU) DNA incorporation assay. Cells suspended in DMEM supplemented with 100 mL/L FCS were seeded into 96-well plates ($1x10^4$ cells/well). After becoming adherent, the cells were treated for 24 h and 48 h with different CHP concentrations dissolved in serum-free DMEM. BrdU labelling was performed during a further 20-h incubation. The BrdU incorporation was quantified using the ELISA kit according to the manufacturer's instructions[18].

For the detection of alive cells, the metabolic activity was measured by a colorimetric method based on the conversion of the tetrazolium salt MTS into formazan using the CellTiter AQ$_{ueous}$ kit according to the manufacturer's instructions. Cell cultivation and incubation was performed as described for BrdU labelling.

Apoptosis

Apoptosis was detected by the cleaved caspase-3 as well as the degradation of poly(ADP-ribose) polymerase (PARP) using the immunoblot technique. To show DNA laddering, cells were seeded into 12-well plates. Adherent cells were cultured in serum-free DMEM for 24 h and 48 h with or without 10 mmol/L CHP. At the end of the incubation, cells were harvested and DNA was extracted using the apoptotic DNA-Ladder kit according to the manufacturer' s instructions. Isolated DNA was electrophoretically separated on 15 g/L agarose gel containing ethidium bromide. The ethidium bromide fluorescence was visualised using an electronic camera.

RT-PCR

Total RNA was isolated using the RNeasy extraction kit. Three microgram of total RNA was reversed transcribed into cDNA as previously described[19]. Quantification of mRNA expression was preformed by using competitive PCR for the housekeeping gene β-actin and co-amplification with a defined concentration of a synthetic DNA fragment as internal standard[19]. On this basis, various cDNA samples were adjusted to equal input concentrations and electrophoretically separated on 18 g/L agarose gel containing ethidium bromide. Visualisation and measurement of resulting bands were performed with an electronic camera analysing the data with the EASY program (Herolab, Wiesloch, Germany). The primers for RT-PCR were as follows: for GRP78, GACATTTGCCCCAGAAGAAA (sense) and

ATGACCCGCTGATCAAAGTC (antisense), product size 375 bp; for GADD153, AGCTGAGTCTCTGCCTTTCG (sense) and TGTGGTCTC TACCTCCCTGG (antisense), product size 456 bp[20].

Western blot analysis

Cells were seeded into 60-mm dishes and grown to confluence. Protein extracts of DSL6A cells were prepared as previously described[18]. Proteins were electrophoretically separated on an 80 g/L SDS-polyacrylamide gel and blotted onto nitrocellulose membrane. Blots were incubated with the respective primary antibody for 2 h at room temperature. For visualization of the antibody binding, filters were exposed to a horseradish peroxidase (POD)-labelled anti-rabbit or anti-mouse Ig antibody and developed using the ECL Plus kit. The following primary antibody dilutions were used: FAK 1:1000, GRP78 1:500, cleaved caspase-3 1:500, PARP 1:1000, β-actin 1:1000. For re-probing with additional antibodies, membranes were stripped of bound antibodies by treatment with stripping buffer containing 62.5 mmol/L Tris (pH 6.7), 2 g/L SDS, 7 mL/L 2-mercaptoethanol for 30 min at 50 ℃.

Immunofluorescence

Cells were cultivated on glass cover slips as previously described[18]. For immunofluorescence staining, cells were fixed with ice-cold methanol, followed by incubation with a mouse monoclonal antibody (mAB) detecting FAK. Binding of the specific mAB was determined by Alexa Fluor anti-mouse IgG and visualized using the Confocal LASER scanning microscope (Leica TCS SP2 AOBS).

Statistical analysis

Results were expressed as mean ± SE. Data were analyzed using Wilcoxon's rank sum test. $P < 0.05$ was considered statistically significant.

RESULTS

CHP-induced morphological appearance of DSL6A cells

The incubation with 10 mmol/L CHP induced rounding up of adherently growing DSL6A cells resulting after 24 h in a complete detachment (Figure 1B). The CHP antagonist L-proline circumvented this CHP-induced loss of adherence (Figure 1C). Moreover, cells which had been rounded up through CHP treatment, spread out again after L-proline addition, forming finally a normal monolayer (Figure 1D).

Effects of CHP on cell proliferation and viability

Figure 2A shows the influence of CHP on the S phase of the cell cycle as measured by BrdU incorporation into the DNA of DSL6A cells. We observed CHP significantly inhibited the proliferation in a time- and dose-dependent manner. The determination of metabolically active DSL6A cells (Figure 2B) revealed a discrepancy between the efficiency of CHP in regard to cell proliferation and viability. While the BrdU incorporation after treatment with 10 mmol/L CHP for 24 h averaged only 20% of the controls (Figure 2A), cell viability was more than 50% (Figure 2B). The results obtained by BrdU incorporation

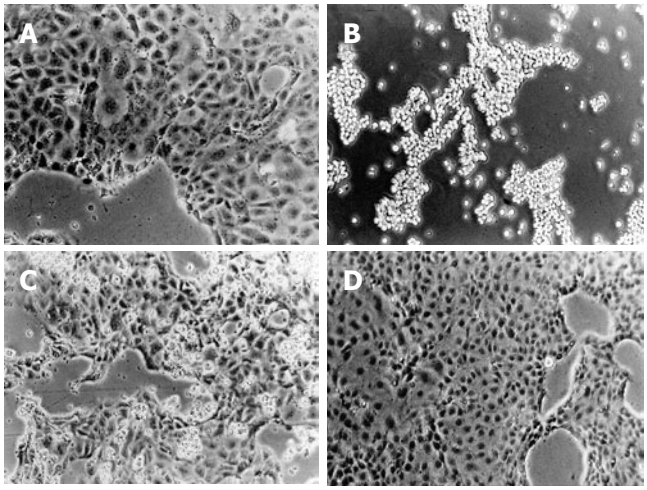


Figure 1 Effect of CHP on morphological appearance of DSL6A cell (Phase contrast microscopy, original magnification x200). A: Adherently growing controls; B: Incubation with 10 mmol/L CHP for 24 h caused rounding, followed by detachment of DSL cells; C: Addition of 20 mmol/L L-proline abolished the effects of CHP; D: CHP-treated cells cultured for further 48 h with 20 mmol/L L-proline, resulting in the re-adherence of the cells.

have been reflected on the molecular level analysing cyclin D1, a molecule substantially involved in the cell cycle progression[21]. As shown by immunoblotting, CHP triggered a decline of the cyclin D1 protein (Figure 2C). The CHP-caused growth inhibition could be prevented through addition of L-proline, indicating a competitive replacement of CHP by the correct amino acid (Figures 2A and 2B).

To examine whether the impaired cell functions could be restored, L-proline was added to the CHP-containing culture medium 24 h after beginning of the incubation with CHP. Cells were allowed to grow for further 24 h, followed by the measurement of BrdU incorporation. As shown in Figure 2D, the addition of L-proline induced a re-increase in BrdU incorporation.

In order to exclude the possibility that the restoring of proliferation was due to residual intact cells, we measured the BrdU uptake of cells remaining in the culture plates after removal of cells detached by CHP treatment. The BrdU incorporation was not significantly higher than immediately after CHP incubation (data not shown). These results suggested that CHP-induced DSL6A cell detachment is a reversible process.

Influence of CHP on FAK

The strong inhibition of cell adherence by CHP pointed to an involvement of the focal adhesion kinase (FAK) in the CHP-mediated molecular mechanisms[10]. Analysis of FAK using the Western blot technique revealed the expected 125 ku protein (Figure 3A). Treatment of the cells with CHP caused a time-dependent decline of the FAK signal and a simultaneous appearance of fragments of about 80 ku, suggesting a proteolytic degradation of the FAK molecule leading to a complete fragmentation during 48 h (Figure 3A).

Based on the hypothesis that CHP brought about FAK inactivation by proteolytic cleavage of the enzyme, we tested the influence of a broad spectrum protease inhibi-

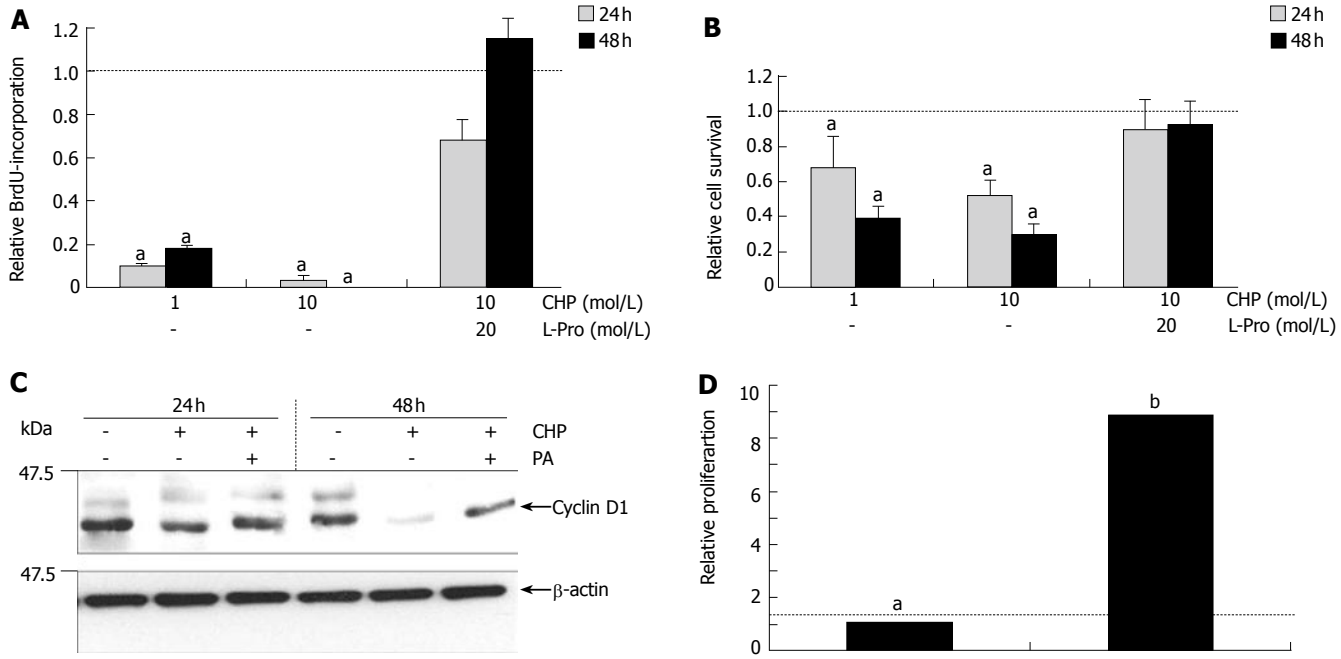
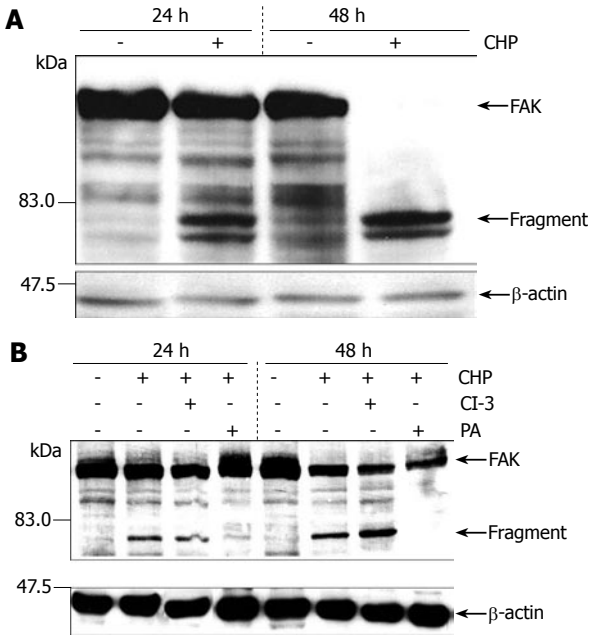


Figure 2 Inhibition of cell proliferation and survival by CHP. A: After the addition of BrdU, cells were cultured for further 20 h, followed by quantification of BrdU incorporation; B: Four hours after the addition of CellTiter AQueous solution, conversation of the MTT salt was measured. CHP-caused decrease of proliferation (A) and metabolically active cells (B) was prevented by the addition of L-proline. Results are expressed as mean±SE (n = 6, $^{a}P \leqslant 0.05$). C: Immunoblotting of total cell extracts from DSL6A cells using an anti-cyclin D1 antibody. Results are representative for three independent experiments. D: DSL6A cells were treated with 10 mmol/l CHP for 24 h before culture medium was (a) replaced by DMEM without CHP or (b) 20 mmol/L L-proline was added to the CHP-containing medium.



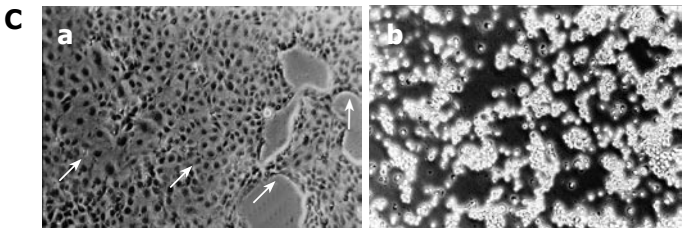


Figure 3 FAK fragmentations in DSL6A cells induced by CHP. A Immunoblotting of protein extracted from DSL6A cells after being incubated for 16 h and 48 h with or without 10 mmol/L CHP. CHP induced a time-dependent degradation of the 125 ku FAK protein, resulting in fragments of about 80 ku. B Simultaneous addition of the Protease Arrest Reagent (PI) to the CHP-treated cells attenuated fragmentation of FAK; in contrast, the caspase-3 inhibitor z-DEVD-fmk (CI-3, 100 μmol/L) was not able to abolish the CHP-induced FAK degradation. To show equal protein loading, the membranes were stripped and re-probed using an anti-β-actin specific antibody. C Effect of CHP on morphological appearance of DSL6A cells; (a) simultaneous addition of 10 mmol/L CHP and a broad spectrum protease inhibitor (PI, Protease Arrest Reagent) abolished the CHP-induced loss of adherence; (b) In contrast to the broad spectrum protease inhibitor, the caspase-3 inhibitor z-DEVD-fmk (CI-3, 100 μmol/L) had no effect on the CHP-induced loss of adherence (Phase contrast microscopy, original magnification x 200).

tor [protease arrest (PA) reagent]. The repression of the intracellular proteolytic activity was able to abolish the CHP-induced FAK degradation (Figure 3B). In addition, pre-incubation of DSL6A cell cultures with the protease inhibitor prevented the CHP-caused cell detachment (Figure 3Ca). Moreover, the cyclin D reduction in DSL6A cells treated for 48 h with CHP was attenuated by the PA reagent (Figure 2C)

In contrast, the inhibition of caspase-3 activity by Z-DEVD-FMK (CI-3) had neither an effect on CHP-mediated FAK degradation (Figure 3B) nor on cell detachment (Figure 3Cb). In addition, treatment with Z-VAD-FMK, a general caspase inhibitor including caspase-3, 7, 8, 10, could not abolish CHP-caused influences on DSL6A cells (data not shown).

Since FAK is localized in focal adhesions while the cell is in contact with extracellular matrix, we, using immunofluorescence staining, analysed the influence of CHP on the intracellular assembly of the enzyme. Results were visualized by laser scanning microscopy (Figure 4). We clearly observed the focal adhesions in adherently growing control cells (Figure 4A), whereas the CHP-caused cell

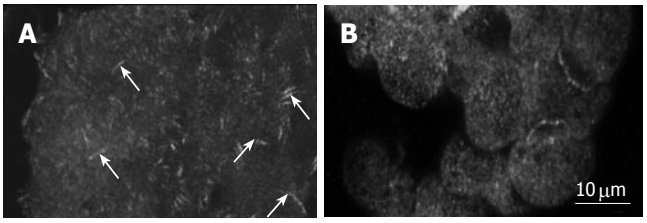


Figure 4 Immunocytochemical localisation of FAK. DSL6A cells grown on glass cover slips were cultured for 24 h without (A) or with 10 mmol/L CHP (B). Cell staining was performed by incubation with a mouse anti-FAK specific antibody. Binding of the primary antibody was detected by an AlexaFluorTM-labelled anti-mouse antibody. Arrows: focal adhesions; Bar: 10 μm.

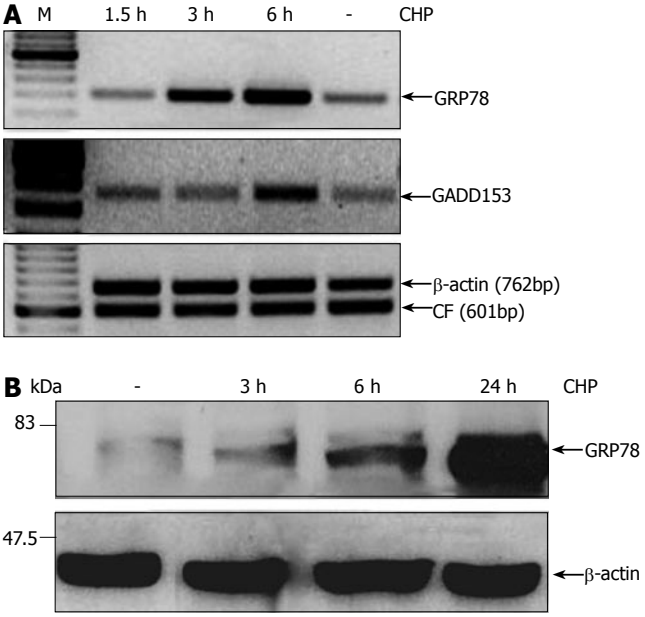


Figure 5 Influence of CHP on expression of GRP78 and GADD153. A: RT-PCR; M = 100-bp molecular weight marker; B: Western blot.

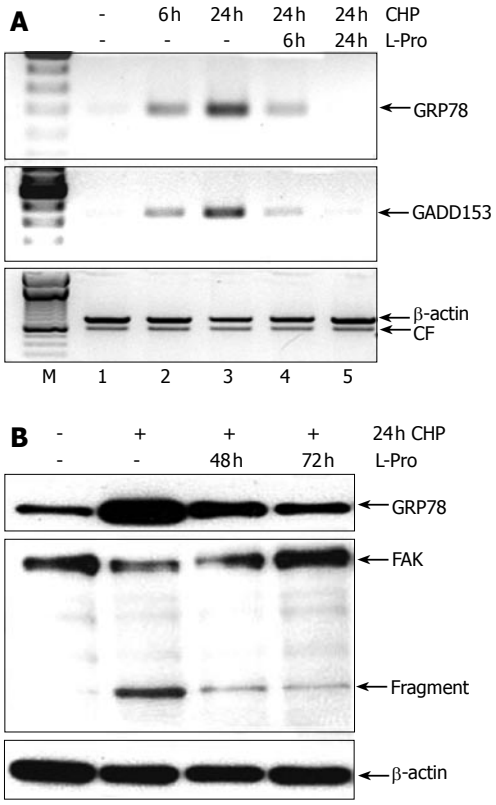


Figure 6 Reversion of CHP-mediated molecular effects by L-proline. A: RT-PCR analysis of mRNA expressions of GRP78 and GADD153. Lane 1: DSL6A cells cultured without 10 mmol/L CHP for 6 h; lane 2: DSL6A cells cultured with 10 mmol/L CHP for 6 h; lane 3: DSL6A cells cultured with 10 mmol/L CHP for 24 h; lane 4: DSL6A cells treated for 24 h with CHP after the addition of 20 mol/L L-proline for further 6 h; lane 5: DSL6A cells treated for 24 h with CHP after the addition of 20 mol/L L-proline for further 24 h. B: Western blot analysis of GRP78 and FAK protein. Results are representative of at least three independent experiments.

rounding resulted in a loss of focal adhesions and a diffuse distribution of the FAK protein (Figure 4B).

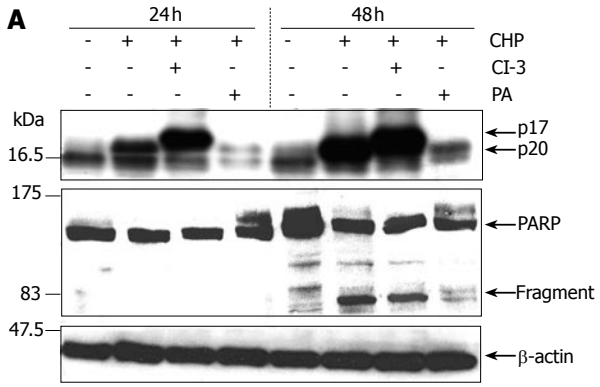
Influence on the expression of ER stress markers GRP78 and GADD153

CHP was earlier described as an inhibitor of normal collagen folding[22], a condition that inevitably affects the homeostasis of the endoplasmic reticulum (ER) referred to as ER stress[15]. In order to test whether CHP effects are mediated by ER stress, we analyzed the expression of GRP78 and GADD153 proteins whose up-regulation was defined as a component of the unfolded protein response (UPR)[18,15]. We found that CHP induced the mRNA expression of the both ER stress markers (Figure 5A). GRP78 transcript levels were found to be increased 3 h after the addition of CHP, while the up-regulation of GADD153 was detected only after 6 h. The CHP-induced GRP78 expression was confirmed on protein level by immunoblotting. A modest increase of the UPR component could be detected 3 h after the addition of CHP. The GRP78 protein levels in the cells increased during prolonged incubation with CHP reaching a maximum after 24 h (Figure 5B).

To test whether the L-proline-caused restoration of cell functions (Figure 2) was reflected on the molecular level, we investigated the time course of the expression of GRP78 and GADD157 during incubation with L-proline in CHP-treated cells. Six hours after the addition of L-proline, the transcript levels of both UPR components were reduced, reaching normal values during 24 h (Figure 6A). In contrast, the decrease of GRP78 protein levels could be detected only after 48 h (Figure 6B). Furthermore, L-proline-induced reversion of the CHP-mediated impairment of cell functions was accompanied by a time-dependent reconstitution of the FAK molecule associated with a reduction of the protein fragments (Figure 6B).

Impact of CHP on apoptosis

The reduction in cell viability pointed to the induction of apoptosis by CHP. Using the Western blot technique, we analyzed the processing of caspase-3 and poly-(ADP ribose) polymerase (PARP) to verify programmed cell death. The activation of apoptosis-executing caspase-3 occurs in a two-step proteolytic process. Initially, the procaspase-3 is cleaved into the subunits p12 and p20, followed by an autocatalytic fragmentation of the latter one, resulting in the fully active p17 enzyme[23]. Treatment of



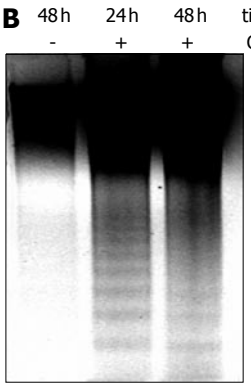
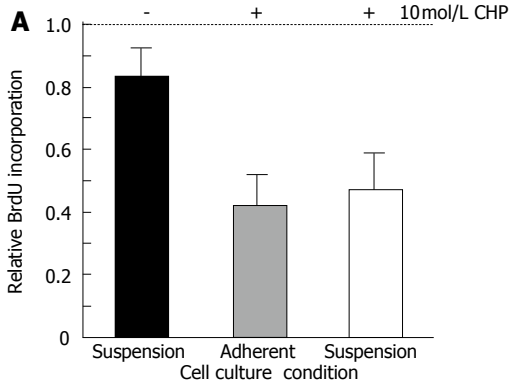


Figure 7 Induction of apoptosis by CHP. A: Immunoblotting using specific antibodies for cleaved caspase-3 or poly (ADP) ribose polymerase (PARP). B: DNA laddering induced by CHP. Results are representative of at least three independent experiments.



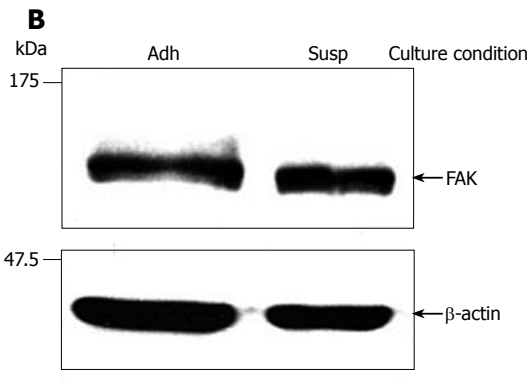


Figure 8 Survival of DSL6A cells cultured in suspension. A: Assessment of cell viability using the CellTiter AQueous assay. Adherent cells (adh.) and cells in suspension (susp.) were incubated for 24 h with or without 10 mmol/L CHP. Results are expressed as a percentage of living cells with regard to adherently growing, untreated control. Data are expressed as mean±SE (n=6). B: Western blot analysis of FAK protein levels.

DSL6A cells with CHP caused a time-dependent activation of caspase-3 represented by the p17 molecule (Figure 7A). Similarly, the cleaved PARP protein of 85 ku occurred during CHP treatment (Figure 7A). Intriguingly, the CHP-induced apoptotic DSL6A cell death, determined by the activation of caspase-3 and PARP, could be prevented by pre-treatment of the cells with the broad spectrum protease inhibitor PA (Figure 7A). These results together with the prevention of CHP-induced FAK degradation (Figure 3B) as well as cyclin D1 reduction (Figure 2C) by inhibition of intracellular proteolysis indicate a superior role of proteases in the CHP-mediated effects.

The caspase-3-specific inhibitor Z-DVED-FMK (CI-3) did not prevent the initial cleavage of procaspase-3 into the p20 fragment. Therefore, corresponding with the blockade of the subsequent autocatalytic processing generating the active p17 enzyme, the level of the p20 protein increased under the treatment with Z-DVED-FMK (Figure 7A).

Another characteristic sign of apoptosis is the degradation of genomic DNA into multiple fragments referred to as DNA laddering. Electrophoretical separation of DNA isolated from DSL6A cells treated for 24 h and 48 h with CHP exhibited the typical DNA ladder, whereas DNA degradation was not detected in untreated controls (Figure 4C). Interestingly, there was no effect of CHP on the anti-apoptotic factor bcl-2 (data not shown).

Loss of adherence and survival

To verify the hypothesis that CHP-induced cell death was not simply the consequence of the loss of adherence, cell survival was quantified in suspension cultures compared with adherently growing DSL6A cells. Indeed, the rate of metabolically active cells (17%) in suspension was lower than that in the adherent counterparts (Figure 8A). However, the portion of dead cells in monolayer cultures incubated with CHP for 24 h increased to about 60% as compared to the untreated controls. Moreover, there were no significant differences between adherent and suspension cultures in CHP-treated cells (Figure 8A). Interestingly, the FAK molecule in DSL6A cells bared from adhering remained intact (Figure 8B). Taken together, our data suggest that the CHP-mediated damage of DSL6A cell functions, including detachment, growth inhibition and apoptosis, is a consequence of molecular mechanisms initiated by intracellular proteolytic processes.

DISCUSSION

In this study, we have shown that cis-hydroxyproline initiates a stress machinery in the rat pancreatic cancer cell line DSL6A, leading sequentially to growth inhibition, loss of adherence and apoptosis. The capacity of CHP to inhibit cell proliferation is consistent with the results for fibroblasts, mammary carcinoma and retinal epithelium cells[6-8].

The CHP-induced damage of DSL6A cells was associated with proteolytic fragmentation and inactivation of FAK. Various studies have suggested that FAK is essentially implicated in integrin-mediated transfer of extracellular stimuli from the cell surface across the plasma membrane, resulting in the activation of several intracellular signalling pathways[22-24]. FAK activation by phosphorylation has been shown in a variety of cell types to be dependent on integrins binding to their extracellular ligands[27]. In addition, there are several reports regarding the regulation of FAK activity by limited cleavage of the

enzyme protein. Using human platelets, Cooray *et al*[28] could identify calpain as the FAK-hydrolysing protease. In a model of oncogenic cell transformation by retroviral gene transfer of v-src, Carragher *et al*[29] showed that cell detachment is associated with calpain-dependent FAK degradation. Furthermore, focal loss of adhesion followed by apoptosis after switching off v-src is accompanied by caspase-mediated FAK proteolysis[29]. The data and the different effects of specific protease inhibitors let the authors conclude that proteolysis of FAK might be a general phenomenon associated with disassembly of focal adhesions, but can be mediated by distinct proteases under different biological conditions[29]. We have shown that in addition to FAK cleavage, the CHP-induced cell detachment, cyclin D1 decline, and caspase-3 activation in DSL6A cells could be attenuated by proteolysis inhibition. The circumvention of the different molecular and functional effects by inhibition of intracellular proteolysis suggests that protease(s) might play a superior role in the execution of CHP-mediated cell toxicity. Furthermore, CHP induced the expression of GRP78 and GADD153, both of which have been defined to indicate the UPR, a pathway of the ER stress machinery[15].

As early as 1975, Uitto *et al*[22] found that non-helical CHP-containing pro-collagen is retained in the ER of chick embryo tendon cells. However, the molecular consequences of these observations could not fully be realised at this time. The CHP-initiated impairment of DSL6A cell functions might be likely attributed to more comprehensive cellular mechanisms rather than exclusively to the disturbance of collagen synthesis, especially because DSL6A cells do not express collagen type I but only traces of collagen type IV-specific transcripts (data not shown). Thus, an interference of CHP with the synthesis of proline-rich proteins involved in striking regulatory cell functions can be assumed[31].

L-proline is able to abolish CHP-induced disturbances of DSL6A cell functions. The mutual competitive replacement of the isomeric compounds proline and CHP has to be expected[5,8]. More interestingly, the addition of L-proline could arrest and even reverse the ongoing process of the CHP-induced ER stress. The restoration is accompanied by a decrease of the expression of the stress proteins, indicating the reversibility of the UPR. GRP78 transcript levels could be achieved during about 6 h of the starting status, while the normalization of the protein concentration takes more than 2 d. According to reports by Liu *et al*[32], the delayed protein decline is probably attributed to its half life time of more than 36 h.

Intriguingly, the L-proline-induced reversion of the CHP-mediated impairment of cell functions is accompanied by a time-dependent reconstitution of the FAK molecule. Furthermore, the survival of DSL6A cells maintained in suspension cultures suggests that the proteolytic FAK degradation is not resulted from apoptosis, but occurs downstream or even constitutes a component of the ER stress cascade. In addition to the proteolytic fragmentation of FAK shown by several studies[30,29], there are reports describing ER stress-mediated activation of caspases, leading to apoptotic cell death[33]. In contrast to these data, we showed that CHP-caused DSL6A cell injury could not be influenced by inhibition of caspases, suggesting that other and/or additional protease(s) trigger CHP-induced proteolytic activity. In this context, reports should be noted which suggest the existence of a physiological role of caspases, apart from that of death executioners[33].

Based on our data, we hypothesize that CHP initiates a signal cascade including sequentially the induction of ER stress, activation of protease(s) that catalyse the caspase-independent FAK fragmentation, followed by cell detachment and finally leading to apoptosis. Further investigations are necessary to identify proteases involved in the CHP-mediated mechanisms.

Interestingly, CHP-caused FAK inactivation leading to detachment of DSL6A cells is not directly associated with apoptosis, in general the cellular response to the loss of anchorage referred to as anoikis[34]. This anoikis-resistance of the DSL6A cells can be attributed most likely to their malignant phenotype[35].

Taken together, we have shown, probably for the first time, an ER stress-mediated cytotoxic effect exerted by CHP in pancreatic cancer cells. Since the molecular equipment may vary between different cell populations, further studies are needed to characterize the susceptibility of other cell types to CHP. Lastly, in order to test the practicability of CHP in cancer therapy, studies on animal models are under way.

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BASIC RESEARCH

Expression and location of Smad2, 4 mRNAs during and after liver fibrogenesis of rats

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Abstract

AIM: To investigate the location alteration of Smad2 and Smad4 mRNAs in the liver during and after fibrogenesis in rats.

METHODS: Eighty male Wistar rats weighing approximately 200 g each were used. The rat models of experimental hepatic fibrosis were established by injection with carbon tetrachloride (CCl_4), normal rats and rats were injected with olive oil and served as control groups. *In situ* hybridization(ISH) was used to detect the Smad2 and Smad4 mRNA in liver.

RESULTS: *In situ* hybridization showed Smad2 and Smad4 mRNA expressions in the cytoplasm of hepatic stellate cells (HSC), fibroblasts and myofibroblasts around the central vein and hepatic sinus during and after fibrogenesis. Expression of Smad2, 4 mRNA was higher than that in normal and control rats.

CONCLUSION: In the process of and after hepatic fibrosis formation, HSC, fibroblasts and myofibroblasts are the major cells that express Smad2 and Smad4. The more serious the hepatic fibrosis is in the injured liver, the higher the level of Smad2 and Smad4 gene expression is during and after fibrogenesis respectively.

Key words: Smad2; Smad4; mRNA; Liver fibrogenesis

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INTRODUCTION

Fibrosis is associated with many liver diseases, including hepatitis C virus infection, iron deposition, alcohol consumption, and nonalcoholic fatty liver disease. Hepatic fibrosis results from a net increased synthesis and decreased degradation of extracellular matrix (ECM) proteins. Type Ⅰ collagen is the most prevalent ECM protein deposited[1], with activated hepatic stellate cells(HSCs) serving as the primary source. Following a fibrogenic stimulus, HSCs are activated from their normal quiescent state, whereby they increase synthesis of procollagen type Ⅰ protein[1,2], and increase cellular proliferation, migration, and contractility[3,4]. Excess ECM accumulation results in scarring within the tissue[5].

In the progression of HSCs activation, many kinds of cytokines play important roles, including transforming growth factor-beta (TGF-β)[6]. TGF-β is a multifunctional cytokine with diverse effects on development, growth, and homeostasis in most tissues[7]. Signaling by TGF-β occurs through type Ⅰ and type Ⅱ transmembrane serine/threonine kinase receptors and intracellular Smad transduction molecules. The TGF-β ligand binds to its type II receptor, either directly or via coreceptors. Once activated by TGF-β, type II receptors recruit, bind, and transphosphorylate type I receptors, thereby stimulating protein kinase activity of the later. The activated type I receptors phosphorylate Smad2 or Smad3, which then bind to Smad4. The resulting Smad complex then moves into the nucleus, where it interacts in a cell-specific manner with various transcription factors to regulate the transcription of target genes[8].

It has been reported that there is a correlation between TGF-β and the formation of liver fibrosis[9,10]. TGF-β has a very important role in ECM production by HSC and many investigations have been done[11,12]. Antagonism of cytokines as a route to antifibrotic therapy, by blocking the interaction between TGF-β and its receptors, and the signal transduction mechanism for TGF-β, has been reported for the treatment of fibrosis[13,14]. Thus the investigation of the Smads signal transduction is indispensable for the study

of fibrosis and the development of its therapy. Until now, there have been no reports on the location and expression alteration of Smad2,4 during and after fibrogenesis. In this article, the expression and location of Smad2,4 mRNA were measured in rat livers with cRNA probes of Smad2,4 by *in situ* hybridization.

MATERIALS AND METHODS

Animal experiments

Eighty male Wistar rats weighing approximately 200 g each were used. Chronic liver injury was produced in 10 rats in each group by subcutaneous injections of 50% CCl_4 in olive oil at a volume of 2 ml/kg body weight twice weekly for 3, 6, 9, 12, 15, and 18 wk. Ten control rats similarly received olive oil alone as control group, and 10 other rats were not treated as normal group. All rats were maintained on a standard diet with water *ad libitum*. On the sixth day after the last treatment, 10 rats in each group were decapitated. Their livers were removed immediately, fixed in 4% paraformaldehyde at room temperature and snap-frozen in liquid nitrogen. The tissue specimens were stored at -80 ℃ until analysis.

Cloning of Smad2 and Smad4 cDNAs

Smad2 and Smad4 cDNAs were cloned in order to synthesize ISH riboprobes. The two cDNAs were obtained from rat liver total RNA by reverse transcription coupled to PCR by the use of the primers Smad2-up (5' GGTGGATCCGTGTCTCATCGGAAAGGG-3'), Smad2-GTGAATTCTGGAATGGAGTGGGTATAG-3') and Smad4-up (5'-AACCGGATCCATCTTCAGC ACCACCC-3'), Smad4-down (5'CGAATTCTTTGCCTA TGTGCAACCTTGC-3'), respectively. The two resulting fragments were digested with *BamH* Ⅰ and *Eco*R Ⅰ and cloned into the pSPT18 vector (Boehringer Mannheim) to obtain the pSPT18+/Smad2 and pSPT18+/Smad4 plasmids.

ISH riboprobe synthesis

The cDNA-containing plasmids were linearized as follows: pSPT18+/Smad2 and pSPT18+/Smad4 with *BamH* Ⅰ and *Eco*R Ⅰ. The linearized plasmids were then gel-isolated (Hua Shun Corp., China) and used as templates for anti-sense and sense digoxigenin (DIG)-labeled riboprobe synthesis(Boehringer Mannheim). The transcription mixture (20 μL) included 1 μg of linearized template cDNA , ATP, GTP and CTP at 1 mmol/L each, UTP 0.7 mmol/L, DIG-UTP 0.3 mmol/L, DTT 10 mmol/L, RNase inhibitor (1 U/μL of transcription mix), and T3 or T7 RNA polymerase (1 U/μL of transcription mix). Transcription was performed for at least 2 hr at 37 ℃. The template cDNAs were then digested by RNase-free DNase (2 μL at 1 U/μL, 15 min at 37 ℃), and all reactions were stopped by adjusting the reaction volume to 100 μL with EDTA (pH8.0). The riboprobes were then purified through two precipitation steps by addition of 100 μL LiCl (4 mmol/L) and 500 μL EtOH (100%), and centrifugation for 30 min at 4 ℃ in a microfuge. The pellet was resuspended in 100 μL DECP-treated water.

In situ hybridization

The serial paraffin sections (thickness 4 μm) were dried at 55 ℃, then deparaffinized by xylene and rehydrated in graded ethanol, incubated for 2×5 min in TBS containing 0.1% active DEPC (Fluka), acidified in 0.2 N HCl for 20 min at room temperature, and incubated for 2×5 min in TBS before digestion with proteinase K for 15 min, and then incubated for 2×5 min in TBS. After postfixation in 4% paraformaldehyde-PBS, sections were incubated for 2 ×5 min in TBS. And then sections were dehydrated with graded ethanol. After prehybridization at 42 ℃ for 2 h, the labeled RNA probes were added to the hybridization mix (Smad2 3.7 ng/μL, Smad4 2.3 ng/μL). The hybridization was carried out at 42 ℃ for 18 h with 20μL of hybridization mix on each section. The sections were washed with 2×SSC, 1×SSC, and TBST(TBS containing Tween20) respectively. After washed, the sections were incubated for 15 min at room temperature with Buffer I containing 1% blocking reagent (Boehringer Mannheim), and then at 37 ℃ with alkaline phosphatase-coupled anti-digoxigenin antibody(Boehringer Mannheim) diluted at 1:50 in Buffer I. Excess antibody was removed by 5×4 min washes in TBST, and the sections were equilibrated for 2× 5 min in Buffer I(Tris-HCl 100mmol/L, NaCl 100mmol/L, and $MgCl_2$ 100mmol/L, pH9.5). Color development was performed at 37 ℃ for 8 h in Buffer I containing NBT and BCIP(Promega). Staining was stopped by a 10 min washing in tap water, and non-special staining was removed in EtOH 95% with gentle agitation. Then the sections were rehydrated through successive baths of EtOH (70, 95, and 100%) and xylol (2×15 min each) and mounted in gum for microscopic examination and photography. Blank control: Smad2 and Smad4 cRNA probes for positive hepatic tissues were replaced by prehybridization solution. Negative control: *in situ* hybridization was performed in normal liver tissues. False positive control: *in situ* hybridization was performed in liver tissues injected with olive oil.

Pathology observation

The whole liver sections were stained with hematoxylin and eosin, and examined under microscope.

Statistic analysis

The *t* test was used to determine statistical significance between groups. *P* value of less than 0.05 was considered statistically significant.

RESULTS

Pathologic findings

During the hepatic fibrosis formation in the experimental rats, in liver tissues at 3 wk, infiltration of inflammatory cells was found around the portal area and central vein; at 6 wk, fatty degeneration and infiltration of inflammatory cells was detected; at 9 wk, hyperplasia of the lattice fibers and collagenous fibers was observed in portal area; at 12 wk, hyperplasia of the lattice fibers and collagenous fibers was observed in portal area and extended outwards, hyperplasia surrounding the central vein observed was

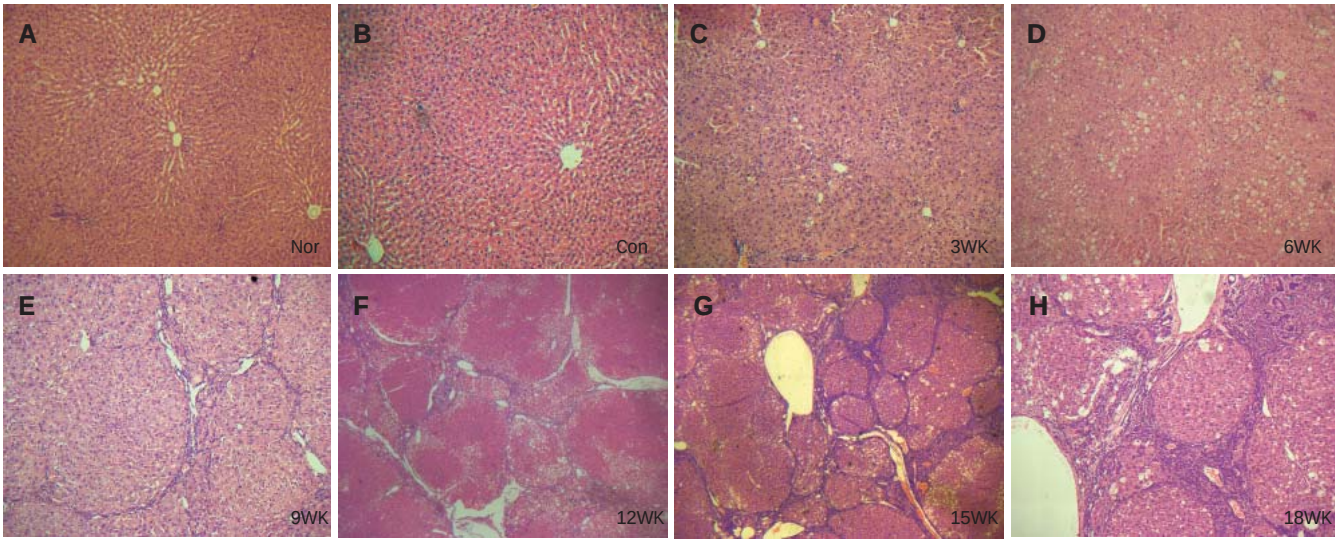

Figure 1 Pathology observation of the experimental rat liver sections stained with hematoxylin and eosin (×100). **A**: normal rats; **B**: control rats; **C**: CCl_4 treated rats at 3 wk; **D**: CCl_4 treated rats at 6 wk; **E**: CCl_4 treated rats at 9 wk; **F**: CCl_4 treated rats at 12 wk; **G**: CCl_4 treated rats at 15 wk; **H**: CCl_4 treated rats at 18 wk.

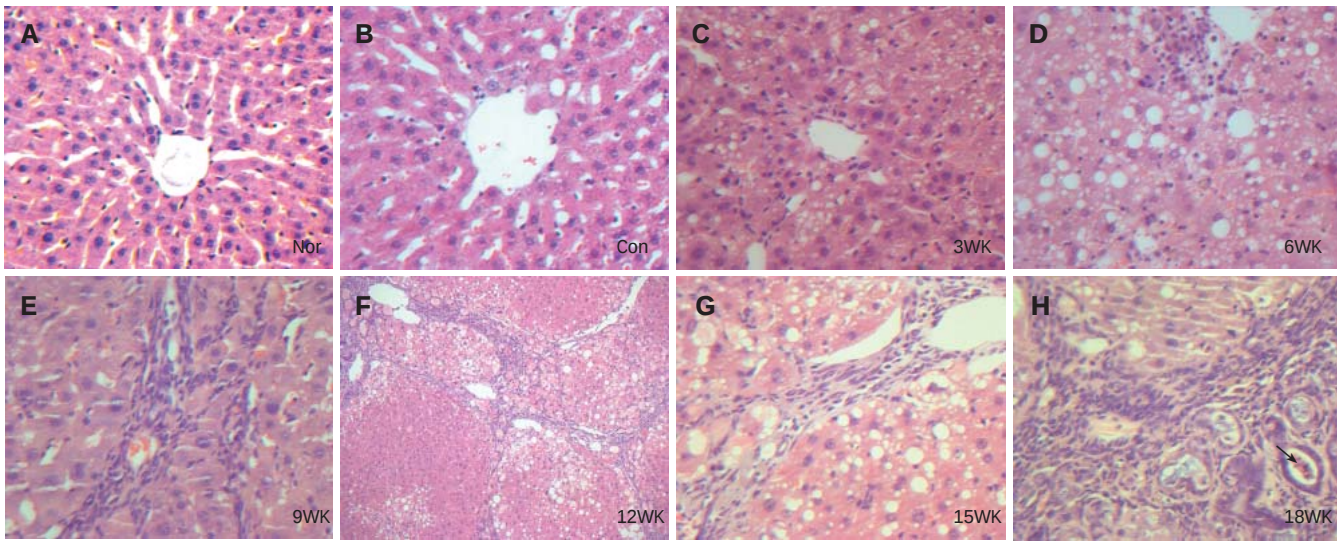

Figure 2 Pathology observation of the experimental rat liver sections stained with hematoxylin and eosin (×400). The arrow shows atypical hyperplasia in the epithelium of bile capillary. **A**, **B**, **C**, **D**, **E**, **F**, **G**, **H**, see Figure 1.

distributed along hepatic sinus and connected with each other, the hepatic lobules were encysted and separated by collagen bundles, the normal structure of lobules was destroyed, and pseudolobules formed, infiltration of inflammatory cells was found around the portal area and central vein; at 15 and 18 wk, excluding the findings of 12 wk, atypical hyperplasia was found in the epithelium of biliary capillary. The structure of liver tissues was normal in normal and control groups (Figures 1 and 2).

Smad2 and Smad4 location

Smad2 and Smad4 mRNAs in liver from experimental rats were detected mainly in the HSCs, fibroblasts and myofibroblasts around the portal area and central vein. Expressions of Smad2 and Smad4 mRNAs exhibited as blue particles in cytoplasm. No positive expression was found in nuclei. There was only mild positive expression in normal and control groups. Image pattern analysis showed that the expression in the experimental group was much stronger than that in the normal and control groups (Table 1, Figures 3 and 4).

DISCUSSION

To establish the model of liver fibrosis successfully is the key for detection of Smad2 and Smad4 mRNAs in the liver tissue during and after fibrogenesis. Researches for establishing the model of liver fibrosis with CCl_4 began in 1936. After that many methods to establish the model of liver fibrosis have been tried. Among them, liver fibrosis models induced by immune response or CCl_4 have been generally accepted mainly because they are more close to the disease characteristics in terms of the distinct stages of the disease of human body and a low mortality. In this study, the method of injecting CCl_4 was used to establish the model of liver fibrosis, and it was proved successful

Table 1 Expression of Smad2 and Smad4 mRNA during and after liver fibrogenesis (mean ± SD)

Group	*n*	Nor	Con	3wk	6wk	9wk	12wk	15wk	18w
Smad2	80	120.20±9.00	113.30±15.18	150.50±8.13^{ce}	213.40±13.92^{ace}	310.30±25.43^{ace}	510.80±7.53^{ace}	523.50±17.35^{ace}	568.40±7.64^{ace}
Smad4	80	99.40±3.65	105.40±10.89	143.50±6.47^{ce}	165.00±32.10^{ace}	305.10±18.53^{ace}	485.00±4.08^{ace}	500.40±10.94^{ace}	529.00±6.78^{ace}

$^{a}P<0.05$ *vs* a previous time point, $^{c}P<0.05$ *vs* normal group, $^{e}P<0.05$ *vs* control group.

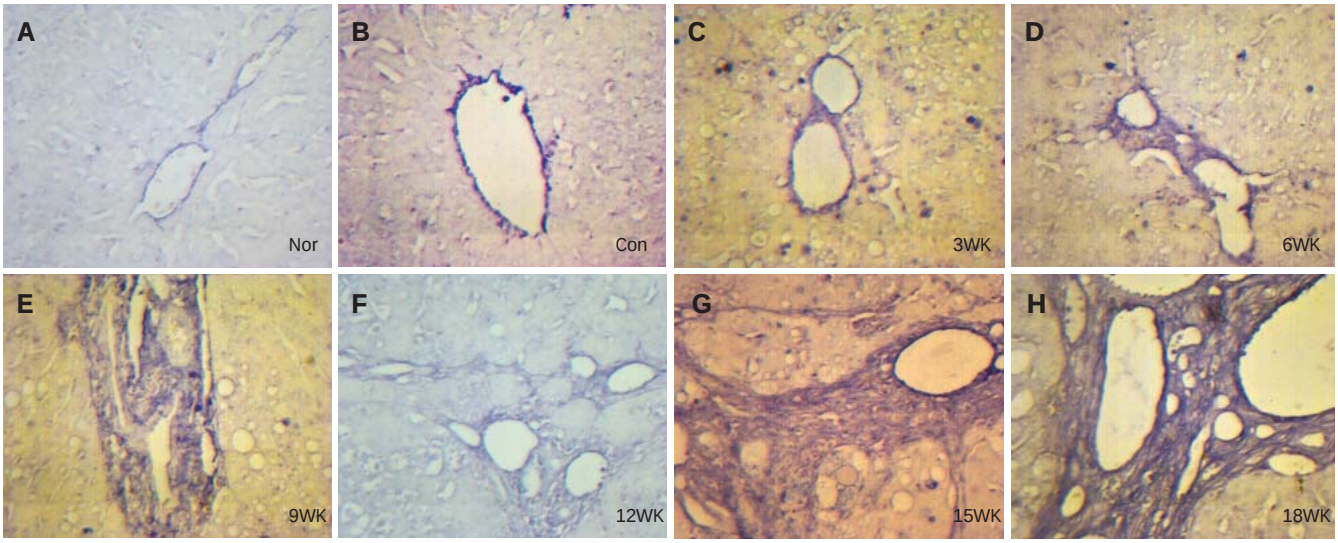

Figure 3 Expression of Smad2 mRNA in experimental rats liver sections during and after fibrosis (*in situ* hybridization ×400). **A**, **B**, **C**, **D**, **E**, **F**, **G**, **H**, see Figure 1.

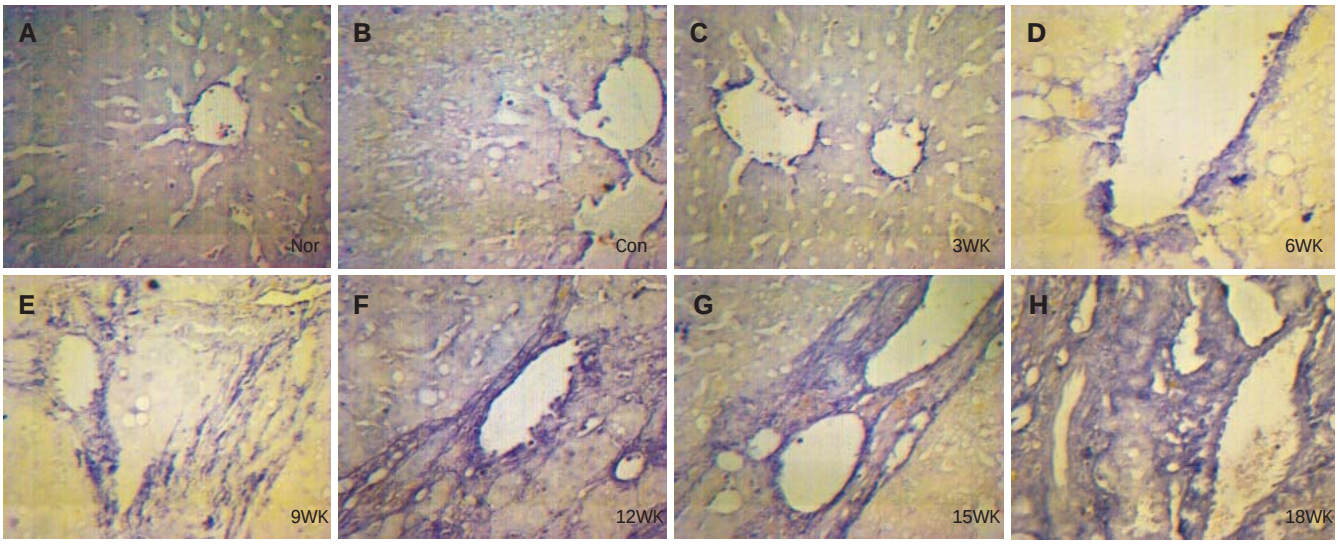

Figure 4 Expression of Smad4 mRNA in experimental rats liver sections during and after fibrosis (*in situ* hybridization ×400). **A**, **B**, **C**, **D**, **E**, **F**, **G**, **H**, see Figure 1.

by histochemical analysis of the sections stained with hematoxylin and eosin. According to the pathologic findings of this study, the exact time for establishment of rat hepatic cirrhosis by injecting CCl_4 may be at 12 wk. From 15 and 18 wk on, atypical hyperplasia was found in the epithelium of bile capillary. It may indicate that injecting CCl_4 may cause hepatic carcinoma.

TGF-β consists of a big molecular family that has a common structure including BMP and activin. These proteins have many roles in the cell structure and function, such as cell growth, differentiation, apoptosis and regulation of ECM production[15,16]. The main functions of the TGF-β super family are morphogenesis, inflammation, tissue recovery and oncogenesis[17]. Smads are intracellular signal transductive molecules of the TGF-β super family. These molecules were discovered as Mothers against dpp (Mad)[18,19], or sma[20] and named Smad in 1996[21]. According to the differences of structure and function, nine Smads have been reported and classified into three groups[7,22]. Smads 2 and 3 are named R-Smads in the

pathway and Smad 4 a Co-Smads for all these pathways. Smads 6, 7, 8 and Xsmad 8 are inhibitory factors of these Smads. When TGF-β binds to its receptor, Smad 2/3 is phosphorylated and binds with Smad 4, together they move into the nucleus for translation and expression of the target gene[7,23]. These mechanisms are thought to play a role in the process of liver damage, recovery, as well as liver fibrosis. In a damaged liver including chronic inflammation, in hepatitis B, TGF-β signaling was reported[24]. Not only Kupffer cells but also the HSC produce TGF-β by the autocrine system[25-28]. TGF-β1 stimulation evokes ECM production, which develops into liver fibrosis.

In this study, Smad2 and Smad4 mRNAs were investigated in rat liver during and after fibrogenesis caused by CCl_4 *in vivo*. With the method of *in situ* hybridization, we found Smad2 and Smad4 mRNAs were expressed obviously in HSC, myofibroblasts and fibroblasts of livers of liver fibrosis group, mostly in portal area and fibrous septum, while very mildly in vascular endothelial cells of livers from normal and control groups. While with the development of liver fibrosis, expressions of Smad2 and Smad4 mRNAs were increased also. These results indicate that Smad2 and Smad4 play an important role in the progression of liver fibrosis. In the sections of liver fibrosis at 15 and 18 wk, expressions of Smad2 and Smad4 mRNAs were higher in the epithelium of bile capillary than in normal and control groups. At this stage, there was atypical hyperplasia in the epithelium of bile capillary, indicating early stage of carcinoma. Therefore, Smad2 and Smad4 may be associated with cancerogenesis.

After stimulation of TGF-β, Smad2, 3 and 4 proteins translocate from cytoplasm to nucleus in MV1Lu cells, analyzed by immunofluorescence using specific antiserum[22]. Moreover, Smad3 seems to play the most important role in liver fibrosis, because Smad3 interacts with SP1 which increases expression of ECM by regulating COL1A2 gene[29]. By transfection of Smad3 expression plasmid in CF37 cells, ECM also increases by regulating COL1A2 gene. Nevertheless, COL1A2 transcription in CF37 cells is markedly increased by TGF-β after transfection with a Smad2 expression plasmid[30]. So Smad2 also plays an important role in liver fibrosis. Our findings are consistent with the above results. The difference is perhaps due to use of different cell lines or experimental conditions. Further researches are needed to confirm the results.

As the liver fibrosis is a complex process, involving many cytokines, cells, and signal transduction pathways, the mechanism cannot be fully explained by these experiments. However, it is clear that TGF/Smads signal transduction pathway plays an important role in liver fibrosis which has been proved by many researches. In our study, Smad2 and Smad4 mRNAs were detected in the plasma of HSC, fibroblasts and myofibroblasts around the portal area and central vein by *in situ* hybridization. Expressions of Smad2 and Smad4 mRNAs were higher during and after liver fibrogenesis than normal and control groups, indicating that they play an important role in liver fibrosis.

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CLINICAL RESEARCH

Expression of *c-kit* receptor in human cholangiocarcinoma and *in vivo* treatment with imatinib mesilate in chimeric mice

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Abstract

AIM: To investigate the *c-kit* expression in biliary tract cancer cell lines and histological sections from patients with extrahepatic cholangiocarcinoma (CC) and to evaluate the efficacy of *in vitro* and *in vitro* treatment with imatinib mesilate.

METHODS: The protein expression of *c-kit* in the human biliary tract cancer cell lines Mz-ChA-2 and EGI-1 and histological sections from 19 patients with extrahepatic CC was assessed by immunoblotting, immunocytochemistry, and immunohistochemistry. The anti-proliferative effect of imatinib mesilate on biliary tract cancer cell lines Mz-ChA-2 and EGI-1 was studied *in vitro* by automated cell counting. In addition, immunodeficient NMRI mice (Taconic™) were subcutaneously injected with 5 x 10^6 cells of cell lines MzChA-2 and EGI-1. After having reached a tumour volume of 200 mm^3, daily treatment was started intraperitoneally with imatinib mesilate at a dose of 50 mg/kg or normal saline (NS). Tumor volume was calculated with a Vernier caliper. After 14 d, mice were sacrificed with tumors excised and tumor mass determined.

RESULTS: Immunoblotting revealed presence of *c-kit* in Mz-ChA-2 and absence in EGI-1 cells. Immunocytochemistry with *c-kit* antibodies displayed a cytoplasmatic and membraneous localization of receptor protein in Mz-ChA-2 cells and absence of *c-kit* in EGI-1 cells. *c-kit* was expressed in 7 of 19 (37%) extrahepatic human CC tissue samples, 2 showed a moderate and 5 a rather weak immunostaining. Imatinib mesilate at a low concentration of 5 μmol/L caused a significant growth inhibition in the *c-kit* positive cell line Mz-ChA-2 (31%), but not in the *c-kit* negative cell line EGI-1 (0%) ($P < 0.05$). Imatinib mesilate at an intermediate concentration of 10 μmol/L inhibited cellular growth of both cell lines (51% *vs* 57%). Imatinib mesilate at a higher concentration of 20 μmol/L seemed to have a general toxic effect on both cell lines. The IC_{50} values were 9.7 μmol/L and 11 μmol/L, respectively. After 14 d of *in vitro* treatment with imatinib mesilate, using the chimeric mouse model, *c-kit* positive Mz-ChA-2 tumors had a significantly reduced volume and mass as compared to NS treatment ($P < 0.05$). In contrast to that, treatment of mice bearing *c-kit* negative EGI-1 tumors did not result in any change of tumor volume and mass as compared to NS treatment.

CONCLUSION: *c-kit* expression is detectable at a moderate to low protein level in biliary tract cancer. Imatinib mesilate exerts marked effects on tumor growth *in vitro* and *in vitro* dependent on the level of *c-kit* expression.

Key words: Cholangiocarcinoma; Imatinib; Tyrosine kinase inhibitor; c-kit; Chimeric mice

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INTRODUCTION

Protein kinases are functionally involved in a broad variety of human cancer[1,2]. In tumor cells, it is common that key tyrosine kinases are no longer adequately controlled, and excessive phosphorylation sustains signal transduction pathways in an activated state. Receptor tyrosine kinases (RTKs) are membrane bound proteins, consisting of a ligand-binding domain at the extracellular surface, a single transmembrane segment, and a cytoplasmic part harboring the protein tyrosine kinase activity. Ligand-induced dimerization, resulting in autophosphorylation of their cytoplasmic domains, is the major mode of activation of RTKs[3]. Many oncogenic mutations in RTKs involve either point mutations leading to constitutive dimerization of the receptors or translocations causing fusion of oligomeriza-

tion motifs to RTKs. Overexpression, probably caused by mutations in gene regulatory sequences or gene amplification, may lead to constitutive dimerization of RTKs. Moreover, mislocalization of RTKs due to fusion to other proteins may contribute to their oncogenic phenotype. Phosphorylated tyrosine residues in the receptor tails then function as recruitment sites for downstream signalling proteins containing phosphotyrosine-recognition domains, such as the SRC homology 2 (SH2) domain or the phosphotyrosine-binding (PTB) domain. These molecules act as relay points for a complex network of independent signalling molecules that ultimately affect gene transcription within the nucleus. *c-kit* (CD-117) is a transmembrane tyrosine kinase receptor in which the extracellular protein binds a ligand known as stem-cell factor (also known as Steel factor) and the intracellular portion contains the actual kinase enzymatic domain[4-6]. *c-kit* is similar in structure to several other RTKs with oncogenic capabilities, including platelet-derived growth factor receptor (PDGF-R) A and B, colony stimulating factor 1 receptor (CSF1-R), and fms-related tyrosine kinase 3 receptor (FLT3-R). *c-kit* is expressed at high levels in hematopoietic stem cells, mast cells, melanocytic cells, germ cells, and the interstitial cells of Cajal (ICC)[7-10]. The receptor forms homodimers upon ligand binding leading to receptor activation. This triggers activation of critical downstream signalling pathways, such as Ras/Raf/mitogen-activated protein kinase kinase (MAPKK)/mitogen-activated protein kinase (MAPK) (cell proliferation) and phosphatidylinositol 3-kinase (PI3K)/AKT (cell survival)[11,12]. There are receptor activating oncogenic *KIT* mutations which involve the extracellular (exon 9), juxtamembrane (exon 11) and kinase domains (exons 13 and 17). As a consequence, in hematologic neoplasms, the exact signalling pathways activated by the mutant *KIT* differ from those activated by normal *KIT*[13,14].

The clinical development of targeted tyrosine kinase inhibitors represents a breakthrough for cancer treatment. They are designed to take advantage of the molecular differences specific to tumor cells compared with normal tissues. The goal is to achieve tumor responses with better safety profiles than those associated with cytotoxic chemotherapies that exhibit narrow therapeutic indices and little selectivity for cancer cells over normal proliferating cells. Imatinib mesilate (STI-571, Gleevec™, Novartis), a 2-phenylaminopyrimidine, was primarily designed to treat chronic myeloid leukemia (CML) by inhibiting bcr-abl fusion protein, created by the t(9; 22) chromosomal translocation, which generates the distinctive Philadelphia (Ph) chromosome[15]. However, imatinib mesilate is not a specific bcr-abl inhibitor, its action extends to c-abl receptor, PDGF-R, and *c-kit*-R[15]. Moreover, it promotes NK cell activation[16] and downregulates telomerase activity[17]. Therefore, imatinib mesilate has been evaluated for the therapy of gastrointestinal stromal tumors (GISTs), because most of them characteristically show *c-kit* overexpression. For metastatic GISTs, imatinib mesilate is nowadays the therapeutical standard, based on a phase II study, including 147 patients with unresectable GISTs, which reported a partial response rate of 54 % and a rate of stable disease of 28 %[18]. A closer inspection of the data clearly displayed that *KIT* mutational status correlated with response to imatinib mesilate. Thus patients with activating *KIT* mutations in exon 11 had a partial response of close to 80%. In contrast, patients whose tumors expressed wild-type *KIT*, with no mutations, had a response rate of only 18%[19]. However, there are only a few published phase I and II studies investigating the effect of imatinib mesilate on gastrointestinal tumors, others than GISTs. Whereas three studies detected a modest level of biological effect of imatinib in hepatocellular carcinoma (HCC) and carcinoid tumor, there was no effect for the treatment of pancreatic carcinoma in two other studies[20-24]. In this study we investigated *c-kit* protein expression in biliary tract cancer cell lines and histological sections from 19 patients with extrahepatic cholangiocarcinoma (CC) and evaluated the efficacy of *in vitro* and *in vivo* treatment with imatinib mesilate.

MATERIALS AND METHODS

Materials

Human gallbladder cancer cell line Mz-ChA-2[25], human extrahepatic cholangiocarcinoma cell line EGI-1[26], and colorectal carcinoma cell line HT-29[27] were cultured as mono-layers in Dulbecco`s modified Eagle`s medium (DMEM, Invitrogen GmbH Karlsruhe, Germany) supplemented with 100 mL/L fetal bovine serum (FBS, Invitrogen GmbH Karlsruhe, Germany), 100 ku penicillin and 100 g/L streptomycin in humidified atmosphere of 900 mL/L air and 100 mL/L CO_2 at 37 ℃. Small cell lung cancer (SCLC) cell line NCI-H69[28] was kept in RPMI 1640 medium (Invitrogen GmbH Karlsruhe, Germany) containing 100 mL/L FBS and antibiotics in humidified atmosphere of 950mL/L air and 50mL/L CO_2 at 37 ℃. The 2-phenylaminopyrimidine derivative STI-571 (imatinib mesilate, Gleevec™) was provided by Novartis Pharma AG (Basel, Switzerland). Stock solution (10 g/L) was prepared in normal saline (NS) and stored at -20 ℃. Hoechst dye was purchased from Sigma (Sigma-Aldrich Chemie GmbH Munich, Germany), rabbit polyclonal c-kit antibody (CD117 Ab-6) from Neomarkers (Dunn Labortechnik GmbH, Asbach, Germany), and Cy™3-conjugated donkey anti-rabbit antibody from Jackson ImmunoResearch (Jackson ImmunoResearch Europe Ltd., Cambridgeshire, United Kingdom). Six to eight-week-old female athymic NMRI nude mice were supplied from Taconic (Taconic Europe, Ry, Denmark) and held under pathogen-free conditions. Human care was administered, and study protocols complied with the institutional guidelines.

Immunoblotting

Cell culture mono-layers were washed twice with ice-cold PBS and lysed with buffer containing Tris-HCl (20 mmol/L, pH 7.5), NP-40 (10 g/L), Triton-X (5 g/L), NaCl (250 mmol/L), EDTA (1 mmol/L), 100 ml/L glycerol, and one tablet of complete mini-EDTA-free protease inhibitor cocktail (Boehringer, Mannheim, Germany) (in 10 mL buffer). Protein concentration was determined by the Bradford protein assay (Bio-Rad, Munich, Germany). 30 μg of cell lysates were separated on SDS-polyacrylamide gels and electroblotted onto poly-

vinylidene difluoride membranes (Amersham Pharmacia Biotech, Freiburg, Germany). Membranes were then incubated in blocking solution (50 g/L BSA in 10 mmol/L Tris-HCl, 140 mmol/L NaCl, 1 g/L Tween-20) (TBS-T), followed by incubation with the primary antibody at 4 ℃ overnight. The membranes were then washed in TBS-T and incubated with HRPO-conjugated secondary antibodies for 1 h at room temperature. Antibody detection was performed with an enhanced chemoluminescence reaction.

Immunocytochemistry

For immunocytochemistry 1 x 10^5 tumor cells were seeded on coverslips of 12-well plates and incubated for one day. Then, medium was removed, cells were washed with PBS and fixed with 8 g/L formaldehyde in PBS for 2 h, washed again, and then permeabilized in 3 g/L Triton-X and 10 g/L DMSO in PBS for another 10 min. After nonspecific antibody binding was blocked by incubation with 50 ml/L donkey serum in 3 g/L Triton-X and 10 g/L DMSO in PBS at room temperature for 2 h, the primary antibody was added, followed by an incubation at 4 ℃ overnight. The omission of primary antibody served as negative control. The next day, cells were washed again and probed with Cy^{Tm}3-conjugated donkey anti-rabbit antibody at room temperature for 2 h. Then, extensive washing and nuclear staining was performed using Hoechst dye according to manufacturers instructions. Finally, coverslips were mounted on slides with 900 ml/L glycerol and slides were analyzed with a Zeiss LSM 510 laser scanning microscope (Carl Zeiss Jena GmbH, Jena, Germany).

Immunohistochemical staining

Paraffin-embedded tissue sections from resection specimens of 19 patients with extrahepatic CC were de-waxed, hydrated through graded alcohol, and immunostained with antibodies to c-kit using the avidin-biotin horseradish peroxidase method as previously described[29]. Immunoreactivity was revealed using True Blue^{TM} peroxidase substrate (KPL, Gaithersburg, USA). The sections were counterstained with hematoxilin and mounted with Permount (Fisher, Pittsburg, USA). For the tumor tissues, the percentage of positive cells was estimated and the staining intensity was semiquantitatively recorded as 1+, 2+, or 3+. For statistical analyses, the staining results were categorized into four groups according to Went *et al*[30]. Tumors without any staining were considered negative. Tumors with 1+ staining intensity in less than 60% of cells and 2+ intensity in less than 30% of cells were considered weakly positive. Tumors with 1+ staining intensity in ⩾ 60% of cells, 2+ intensity in 30% to 79%, or 3+ intensity in less than 30% were considered moderately positive. Tumors with 2+ intensity in ⩾ 80% or 3+ intensity in ⩾ 30% of cells were considered strongly positive. Only membranous or membranous plus cytoplasmic staining was considered for analysis because cytoplasmic staining alone proved to be false-positive in all preabsorption control experiments.

Inhibition of cell growth

The effect of imatinib treatment on cellular proliferation was assessed by automated cell counting (Schaerfe Casy 2.0 Cell Counter, Reutlingen, Germany) according to the manufacturers instructions. Briefly, 2 × 10^5 cells were seeded in T-25 cell culture flasks. Twenty-four hours after incubation, cells were treated with imatinib mesilate at 5 different concentrations (0, 1, 5, 10, and 20 μmol/L, respectively). After 6 d of incubation, cells were trypsinized, washed, and analyzed in triplicates by automated cell counting.

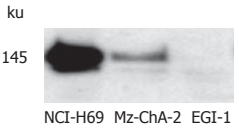

Figure 1 c-kit protein expression in biliary tract cancer cell lines.

Animal studies

Tumors were induced by injecting 5×10^6 Mz-ChA-2 or EGI-1 cells in 200 μL PBS sc into the flank region of NMRI nude mice (n = 40). Treatment was started when an average tumor volume of 200 mm³ was reached (usually after 2 wk). The verum group (n = 2×10) received imatinib mesilate, dissolved in normal saline (NS), ip once a day at 50 mg/kg, whereas the control group (n = 2×10) received NS only. Treatment was continued for 14 consecutive days, tumors were daily measured with a Vernier caliper and tumor volumes were calculated using the formula tumor volume = 0.5 × L × W^2, where L represents the length and W the width of the tumor. When treatment was finished, animals were sacrificed with tumors excised and weighed.

Statistical analysis

Statistical calculations were performed using SPSS, version 10.0 (SPSS Inc., Chicago, USA). Numeric data were presented as mean value with standard deviation (SD). Inter-group comparisons were performed with the Student t test. P< 0.05 was considered significant.

RESULTS

c-kit protein expression in biliary tract cancer cell lines

The expression of *c-kit* in both human biliary tract cancer cell lines Mz-ChA-2 and EGI-1 was assessed by immunoblotting. Cell lysate of SCLC cell line NCI-H69, which has been reported previously to express *c-kit*[28], served as positive control and showed an intense band at about 145 kDa, corresponding to *c-kit* receptor (Figure 1). In accordance with our previous study of *c-kit* m-RNA expression[31], immunoblotting of Mz-ChA-2 cell lysate demonstrated *c-kit* protein expression in this cell line as well. In contrast, *c-kit* expression was not detected in the m-RNA negative cell line EGI-1 (Figure 1). To further analyze *c-kit* expression in these cell lines, immunocytochemistry was performed. In this experiment, colorectal cancer cell line HT-29, which is known to express *c-kit*, served as positive control[27]. Immunocytochemical analysis showed a strong membraneous staining of the receptor protein in this cell line (Figure 2). In the tested biliary tract cancer cell lines, staining of *c-kit* revealed both cytoplasmatic and membraneous localization of receptor protein in Mz-

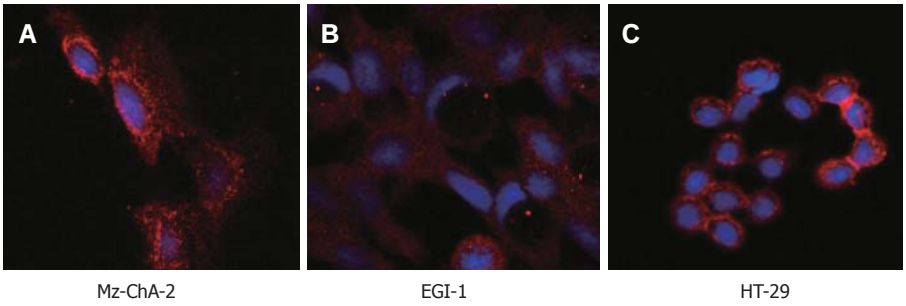

Figure 2 Immunocytochemistry in biliary tract cancer cell lines of Mz-ChA-2 (**A**), EGI-1 (**B**) and HT-29 (**C**).

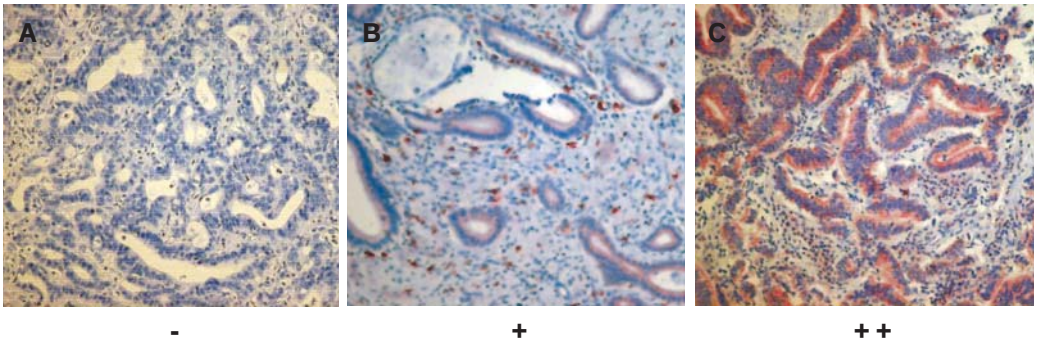

Figure 3 c-kit protein expression in 12 (**A**), 5 (**B**) and 2 (**C**) human biliary tract cancer tissue samples (SABC x 200).

ChA-2 cells and absence of *c-kit* in EGI-1 cells (Figure 2).

c-kit protein expression in human biliary tract cancer tissue

In order to analyze the expression of c-kit in human biliary tract cancer, paraffin-embedded tissue sections from 19 patients suffering from extrahepatic hilar CC were immunostained with c-kit-antibodies using the avidin-biotin horseradish peroxidase method. The clinical characteristics of the study population were as follows: median age of patients was 61 years (range, 39-74 years); UICC IA, IB, IIA, IIB, III, and IV tumor stage (UICC 2002)[32] had 1, 3, 7, 6, 1, and 1 patients, respectively; Bismuth-Corlette[33] types I, II, IIIA, IIIB, and IV were found in 1, 1, 8, 2, and 7 patients, respectively; and 16 patients had well or moderately differentiated tumors. As a result, 2 of the 19 tissues samples displayed a moderate immunostaining (++), 5 a rather weak immunostaining (+), and 12 were negative (-). Thus, 7 of 19 (37%) of the histological sections of extrahepatic hilar CC tested showed moderate to weak expression of c-kit. The immunoreactivity of c-kit was found mainly in the cell membrane (Figure 3). Highly-differentiated tumors seemed to show a higher level of c-kit expression than low-differentiated tumors.

Inhibition of cellular growth by imatinib mesilate

To assess the effect of imatinib mesilate treatment on cellular proliferation of both biliary tract cancer cell lines, automated cell counting was performed, using the colorectal carcinoma cell line HT-29 as a positive control[27]. After 6 d of incubation, imatinib mesilate at a low concentration of 5 μmol/L caused a significant growth inhibition in *c-kit* positive cell line Mz-ChA-2 (31%±7%), a moderate growth inhibition in *c-kit* positive cell line HT-29 (7%±9%), and no growth inhibition in *c-kit* negative cell line EGI-1 (0%±10%) ($P<0.05$, Mz-ChA-2 *vs* EGI-1). Imatinib mesilate at an intermediate concentration of 10 μmol/L inhibited cellular growth of both biliary tract cancer cell lines significantly (51%±4% *vs* 57%±4%, Mz-ChA-2 *vs* EGI-1). However, this effect was even stronger for HT-29 (91%±19%) ($P<0.05$, HT-29 *vs* Mz-ChA-2 and EGI-1). Imatinib mesilate at a higher concentration of 20 μmol/L seemed to have a general toxic effect in all cell lines (99%±1% *vs* 99%±1% *vs* 100%, Mz-ChA-2 *vs* EGI-1 *vs* HT-29) (Figure 4). The resulting IC_{50} values were 9.7 μmol/L, 11 μmol/L, and 9.5 μmol/L, respectively.

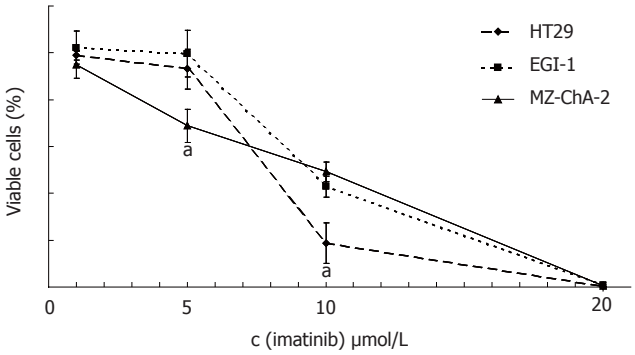

Figure 4 Inhibition of imatinib on cell growth ($^aP<0.05$).

Inhibition of tumor cell growth by imatinib mesilate in nude mice

To assess the antitumor activity of imatinib mesilate *in vivo*, tumors were induced in nude mice by subcutaneous injection of either Mz-ChA-2 or EGI-1 cells into the flanks of the animals ($n=20$ for each cell line). Treatment of mice consisted of daily ip injections of 50 mg/kg BW imatinib mesilate, and injection of NS served as control.

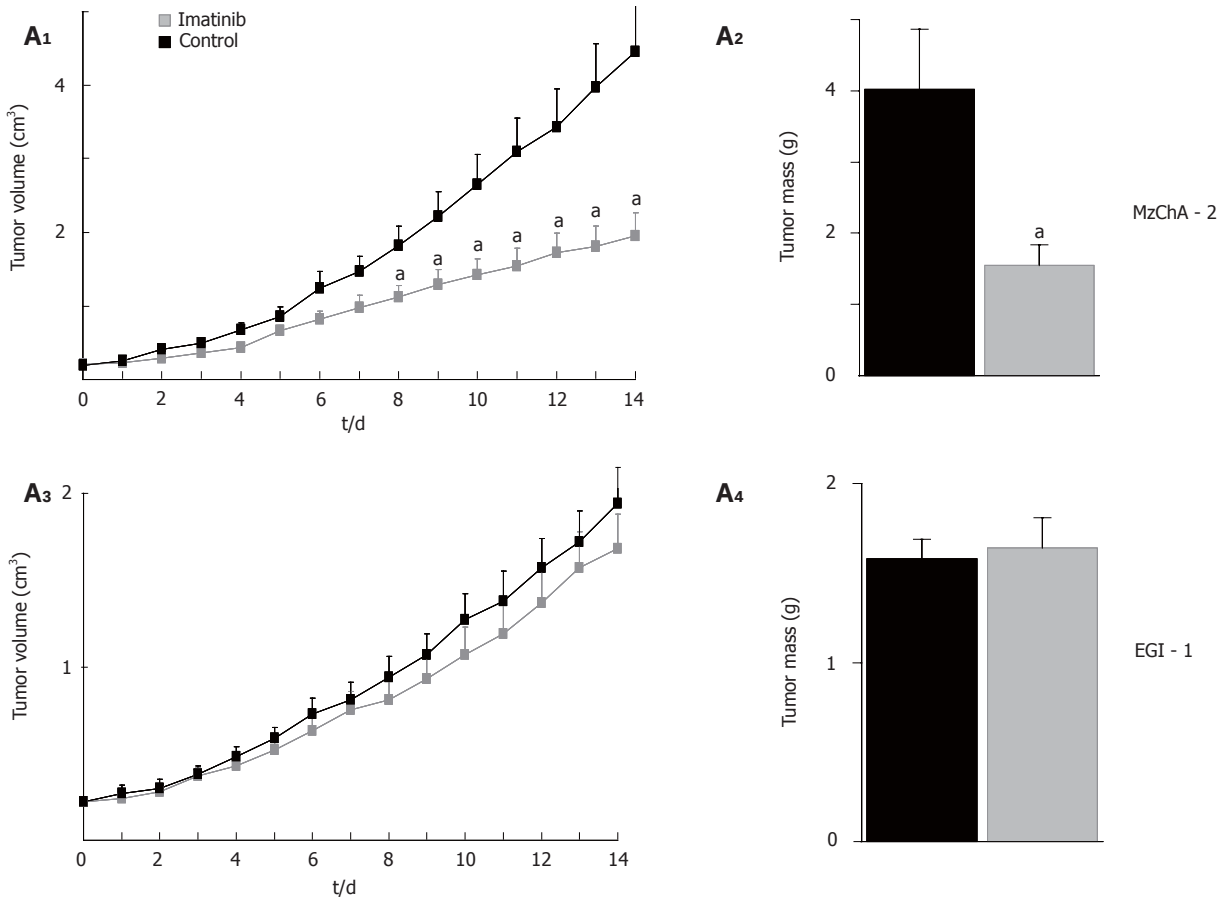

Figure 5 *In vivo* treatment with imatinib mesilate in chimeric mice; **A**: effect on tumor volume and tumor mass ($^aP<0.05$, imatinib *vs* control); **B**: hematoxilin-eosin stained paraffin-embedded tissue sections.

Eight to 14 d after beginning of treatment, Mz-ChA-2 cell tumors had a significantly reduced volume in comparison to control ($P<0.05$). At the end of the experiment (d 14) tumor mass was significantly diminished as compared to control ($P<0.05$). In contrast to that, imatinib mesilate treatment of mice bearing EGI-1 cell tumors did not result in any change of tumor growth compared to control (Figure 5A). Hematoxilin-eosin stained paraffin-embedded tissue sections from mouse tumors showed the typical features of low-differentiated adenocarcinoma (Figure 5B).

DISCUSSION

Non-resectable biliary tract cancer is associated with a poor prognosis due to wide resistance to chemotherapeutic agents and radiotherapy. It is therefore essential to search for new therapeutical approaches. Imatinib mesilate (STI571, Gleevec™), which was found to inhibit the *bcr-abl* tyrosine-kinase resulting from the translocation t(9;22) in chronic myeloid leukemia (CML), as well as *c-kit* and PDGF-R tyrosine kinases, may be an alternative[34]. We have previously shown expression of *c-kit* and PDGF-R m-RNA in 50% and 75% of 12 biliary tract cancer cell lines[31]. Activating mutations of *KIT* were not found, which is consistent with the data of Sihto *et al*[35]. This group screened 334 human cancers (32 histologic tumor types) for mutations with sensitive denaturing high-performance liquid chromatography (DHPLC) and found *KIT* mutations only in GIST tumors. In our study, *c-kit* receptor ligand SCF was present in all cells lines, respectively. We reasoned that the proliferation of these cells might be stimulated by an autocrine mechanism. In order

to test this hypothesis, we treated cell lines with different concentrations of imatinib mesilate. Imatinib mesilate at an intermediate concentration of 10 μmol/L and a higher concentration of 20 μmol/L reduced survival in all cell lines examined by manual cell counting after staining with trypan blue. The reduction was statistically significant higher in *c-kit* positive cell lines ($P < 0.02$) and was independent from PDGF-R status. At 50 μmol/L, a general non-specific toxic effect occurred in all cell lines. The average IC_{50} for growth inhibition of *c-kit* positive cell lines was estimated to be 10 μmol/L. 5-Fluorouracil (5-FU) alone (0.1 μg/mL) reduced cell growth by 40 % - 50 %, while the combination with imatinib mesilate reduced cell growth by another 20% (1 μmol/L) ($P = 0.008$) and 30% (10 μmol/L) ($P = 0.0001$), respectively. Additionally, treatment of cells with imatinib mesilate ± 5-FU had no modulating effects on S-phase fraction, but was associated with a significant induction of apoptosis[31].

However, at that time it was unclear whether *c-kit* could be detected at the protein level and how its distribution whithin the tumor cells was. Moreover, while this work was in progress, Chiorean *et al* were unable to detect *c-kit* at protein levels in human cholangiocarcinoma cell lines KMCH-1 and Mz-CHA-1[36]. In the present study, immunoblotting confirmed the results of our previous m-RNA analysis for Mz-ChA-2 and EGI-1 cells. Immunocytochemistry with *c-kit* antibodies displayed a cytoplasmatic and membraneous localization of receptor protein in *c-kit* positive Mz-ChA-2 cells, indicating a steady receptor turnover. Cell lysates of SCLC cell line NCI-H69 and colorectal cancer cell line HT-29 served as positive controls for immunoblotting and immunocytochemistry. Both cell lines displayed a stronger protein expression that might correlate with a higher sensitivity for imatinib mesilate treatment as shown in previous studies[27,28,37,38]. In order to verify that *c-kit* protein is not only expressed in cholangiocarcinoma cell lines, but also in human biliary tract cancer tissue, paraffin-embedded tissue sections from 19 patients suffering from extrahepatic hilar CC were immunostained with *c-kit*-antibodies using avidin-biotin horseradish peroxidase method. *c-kit* was expressed in 7 of 19 (37%) extrahepatic human CC tissue samples, 2 showed a moderate and 5 a rather weak immunostaining. This result is comparable to a previous analysis of 13 patients with intrahepatic cholangiocarcinoma which demonstrated an expression level of 31%[39]. In contrast, a study from Argentina detected only 6% *c-kit* expression in 50 specimens from gallbladder carcinoma[40]. Moreover, a weak to moderate *c-kit* expression was shown in 7 of 12 (58%) bile duct and gallbladder adenocarcinomas by Aswad *et al*[41]. Finally, a recent multitumor tissue microarray (TMA) analysis by Went *et al*, containing more than 3500 paraffin-embedded tumor samples representing more than 120 tumor types and subtypes, and a recent TMA analysis by Sihto *et al* showed negative results for gallbladder carcinoma ($n = 27$) and cholangiocarcinoma ($n = 3$)[30,35]. These controversial data could be explained by different antibodies used or different tissue selections. Using automated cell counting, we tried to confirm our manual cell counting data for the *c-kit* positive cell line Mz-ChA-2 and the *c-kit* negative cell line EGI-1 after treatment with imatinib mesilate. Imatinib mesilate at a low concentration of 5 μmol/L caused a significant growth inhibition in *c-kit* positive cell line Mz-ChA-2 (31%), but not in *c-kit* negative cell line EGI-1 (0%) ($P < 0.05$). Imatinib mesilate at an intermediate concentration of 10 μmol/L inhibited cellular growth of both cell lines (51% *vs* 57%), but was most effective in colorectal carcinoma cell line HT-29 (91%). Imatinib mesilate at a higher concentration of 20 μmol/L seemed to have a general toxic effect in both cell lines. The results for these particular cell lines indicate that: (1) at a concentration of 5 μmol/L imatinib mesilate seems to exhibit a *c-kit* receptor dependent cell growth inhibition. The different IC_{50} values of 9.5 μmol/L for HT-29, 9.7 μmol/L for Mz-ChA-2, and 11 μmol/L for EGI-1 may therefore be influenced by the different level *of c-kit* expression; (2) at a concentration of 10 and 20 μmol/L the inhibition by imatinib mesilate seems to be no longer *c-kit* receptor dependent. A possible explanation may be a significant decrease in epidermal growth factor receptor (EGF-R) and focal adhesion kinase (FAK) phosphorylation at these concentrations, as observed by Chiorean *et al* for cholangiocarcinoma cell line KMCH-1 which was associated with a reduction in Akt activity resulting in loss of Mcl-1, a potent anti-apoptotic Bcl-2 family member[36]. Whether an additional dose dependent inhibition of telomerase activity, as observed for *c-kit* positive Ewing sarcoma, melanoma, myeloma, breast cancer, Fanconi anemia, and *c-kit* negative murine pro-B cells, plays a role in cholangiocarcinoma has not been evaluated yet[17]; (3) the assumed general toxic effect of imatinib mesilate at a concentration of 20 μmol/L indicates that even a *c-kit* and PDGF-R negative cell line like EGI-1 can sometimes be destroyed at this concentration whereas our previous meta-analysis of 6 *c-kit* m-RNA negative biliary tract cancer cell lines demonstrated a general toxic effect only at 50 μmol/L with a potential inhibition at 20 μmol/L[31].

Even more important are therefore the results from our chimeric mouse model since so far there has been no animal model published for *in vivo* treatment with imatinib mesilate in biliary tract cancer. At the end of a 14 d treatment period with imatinib mesilate, *c-kit* positive Mz-ChA-2 tumors had a significantly reduced volume and mass as compared to NS treatment ($P < 0.05$). In contrast to that, treatment of mice bearing *c-kit* negative EGI-1 tumors did not result in any change of tumor volume and mass as compared to NS treatment. These results support the notion of a *c-kit* receptor expression dependency of sensitivity to imatinib mesilate *in vivo*. At the selected dose of 50 mg/kg, which was chosen according to a study by Druker *et al*[42], no toxic side effects occurred. As discussed by Druker *et al*[43], at steady state, a once-daily administration of 400 mg imatinib mesilate, the commonly used oral dose, translates into a mean maximal serum concentration of 4.6 μmol/L. Therefore, a daily oral dose of 600 mg may be reasonable for a study with patients suffering from biliary tract cancer. Drug-related adverse effects of imatinib mesilate include nausea, vomiting, myalgias, edema, diarrhea, and, less commonly, anemia, thrombocytopenia, neutropenia, and myelosuppression[43-45]. Moreover, there are concerns regarding the drugs liver toxicity in biliary tract cancer patients since it is primarily metabolized in the liver and tumor infiltration of the liver may reduce liver

function. Therefore, Eckel *et al* studied pharmacokinetics of imatinib mesilate in 17 patients with hepatocellular carcinoma and Child A-liver cirrhosis and compared the data with the data of 6 patients with CML and normal liver function[46]. As a result, they found no changes in pharmacokinetics in the first group of patients, thus letting assume the drugs safety in patients with hepatocellular carcinoma and Child A-liver cirrhosis. It is likely that these data can be transferred to patients with invasive biliary tract cancer. However, a phase I/II study is now required to further evaluate our preliminary in vitro and in vivo data. Therefore, our group has designed a clinical study for patients with biliary tract cancer that is now in progress.

In conclusion, *c-kit* expression is detectable by immunoblotting, immunocytochemistry, and immunohistochemistry at a moderate to low protein level in biliary tract cancer. Imatinib mesilate exerts marked effects on tumor growth *in vitro* and *in vivo* dependent on the level of *c-kit* expression. A phase I/II study has been designed to further study the role of imatinib mesilate treatment in patients with biliary tract cancer.

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CLINICAL RESEARCH

Effect of 5-HT$_1$ agonist (sumatriptan) on anorectal function in irritable bowel syndrome patients

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Abstract

AIM: To evaluate the effect of sumatriptan, a selective 5-HT$_1$ agonist, on anorectal function in irritable bowel syndrome (IBS) patients.

METHODS: Twenty-two IBS patients selected according to the Rome II criteria (F 15, M 7; mean age 29.3±6.8, range 22-44 years) were examined. The study was blind, randomized and placebo-controlled with a crossover design. Anorectal manometry and rectal balloon distension test were performed before and after the administration of placebo and sumatriptan.

RESULTS: The administration of sumatriptan caused a significant increase in the resting anal canal pressure from 9.2±2.0 kPa to 13.1±3.3 kPa (P<0.0001) connected with the increase in the anal sphincter length and high pressure zone. After sumatriptan injection a remarkable increase in the threshold for the first sensation from 27±9 mL to 34±12 mL (P<0.05) and urge sensation from 61±19 mL to 68±18 mL (P<0.01) was observed. Sumatriptan did not affect either the volume evoking the rectoanal inhibitory reflex or the results of the straining test.

CONCLUSION: 5-HT$_1$ receptors participate in the regulation of anorectal function. Elucidation of the role of 5-HT$_1$ receptors in the pathophysiological mechanisms of IBS may have some therapeutic implications.

Key words: Sumatriptan; 5-HT receptors; Irritable bowel syndrome, Anorectal function

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INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) is one of the main neurotransmitters involved in the control of the gastrointestinal tract function and plays an important role in the pathophysiology of irritable bowel syndrome (IBS)[1,2]. A number of abnormal motor and sensory patterns reported in IBS patients is supposed to be a result of disturbances in serotonergic mechanisms. Different 5-HT receptor subtypes are involved in the regulation of enteric reflexes and signaling to the central nervous system[3]. Pharmacological data confirm that the specific 5-HT$_3$ antagonists and 5-HT$_4$ agonists are beneficial in the management of IBS[4-6]. Among new emerging serotonergic agents for functional gastrointestinal disorders the role of 5-HT$_1$ agonists was discussed[7]. Many IBS patients complain of various non-gastrointestinal symptoms and disorders such as headache, migraine, non-cardiac chest pain, back pain, dysuria, sleeping difficulties, chronic fatigue syndrome, fibromyalgia syndrome, anxiety and depression. From the clinical point of view frequent coexistence of IBS with headache reported by 23%-45% of IBS patients may also indicate the common pathogenetic and therapeutic link for these diseases, possibly related to the serotonin-dependent processes[8].

Sumatriptan is a selective 5-HT$_{1B/D}$ agonist used for migraine treatment. Recent research results on sumatriptan effects on the gastrointestinal tract function have shown that the drug alters oesophageal motility, favours transient lower oesophageal sphincter relaxations despite the increase in the lower oesophageal sphincter pressure, prolongs postprandial fundic relaxation delaying gastric emptying, and induces a premature intestinal third phase of the migrating motor complex in the jejunum. Encouraging results have been obtained in patients with dyspepsia and impaired postprandial gastric relaxation treated with sumatriptan[9,10]. A therapeutic potential for 5-HT$_1$ agonists in IBS patients has been also suggested according to the observation that sumatriptan causes a significant relaxation of the descending colon influencing the perception of colonic distension.

Anorectal function in IBS draws attention due to commonly reported symptoms by patients with this disorder including urgency, excessive straining or feeling of incomplete evacuation. Up to now, consistent association

between the above symptoms and anorectal dysfunction has not been clearly established. In some IBS patients anorectal manometry is performed to exclude abnormalities that may coexist with or imitate IBS. Moreover, assessment of anorectal function in IBS patients, particularly by use of anorectal manometry, presents an easily accessible way to evaluate the effects of drugs on the lower gastrointestinal tract function[11].

The aim of the present study was to test the hypothesis that sumatriptan influences anorectal function in patients with IBS.

MATERIALS AND METHODS

Subjects

Twenty-two IBS patients selected according to the Rome II criteria (15 women (F), 7 men (M); mean age 29.3± 6.8, range 22-44 years) participated in the study. Based on the bowel pattern, three subgroups of IBS patients were distinguished – constipation predominant IBS (C-IBS: 5 F, 0 M), diarrhea predominant IBS (D-IBS: 3 F, 4 M), and alternating IBS (A-IBS: 7 F, 3 M).

The organic causes for the symptoms were excluded by the evaluation of detailed medical history and physical examination, basic laboratory tests and normal colonoscopy within the five years preceding inclusion in the study. Patients who previously underwent abdominal or gastrointestinal surgery, except for appendectomy, were not included. Patients with ischemic heart disease, arterial hypertension, after myocardial infarction or cerebral stroke, and over 45 years of age were not included in the study, either. Blood pressure and heart rate values in all subjects were within the normal range. All patients had a normal electrocardiogram performed just before the study. The study was approved by the Ethics Committee of the Medical University Hospital and written informed consent was obtained from all subjects.

Recording methods

Anorectal manometry and rectal balloon distension were performed using a four lumen water-perfused catheter with a polyethylene balloon attached to the tip (Zinetics Manometric Catheter, Medtronic). Pressure was sensed by external pressure transducer connected to an analogue-digital converter (PC Polygraph, Synectics Medical, Synecpol). The system was calibrated at 0 and 6.67 kPa at the beginning of each study. The following measurements were derived from the manomertic recordings: the maximal anal resting pressure (MRP) and the maximal anal squeeze pressure (MSP); the anal sphincter length (SL) and high pressure zone (HPZ) within the anal canal, both during rest and squeeze. Moreover, strain, squeeze and cough tests as well as volume initiating the internal anal sphincter relaxation (rectoanal inhibitory reflex, RAIR) and volume required to elicit first sensation, urgency and discomfort or pain were evaluated.

The maximal resting and squeeze pressure along the anal canal was measured from the rectum to the anal margin by the dynamic pull-through technique. The procedure was performed both in the normal relaxed state and in the voluntary squeeze state by pulling a catheter through the sphincters at a speed of 10 mm/s. Three pulls in both states were performed. Evaluation of the strain and cough tests were carried out using the stationary pull-through technique. RAIR and sensory thresholds were measured by the intermittent, phasic rectal distension, starting with 10 mL of air and increasing in steps of 10 mL. The rectal balloon was emptied after each distension. The duration of the distension amounted to 60 s, with 40-60 s intervals between each inflation. During distensions subjects were asked to report and classify their sensation in terms of the first sensation, urge sensation and the maximal tolerable volume due to discomfort or pain. Whenever unbearable discomfort or painful sensation were experienced during any level of distension the experiment was immediately suspended.

Study protocol

Patients examined after an overnight fast without a cleansing enema in the left lateral position were allowed to acclimate to the assembly for 20 min. Patients were instructed not to take any analgesic, spasmolytic, anxiolytic, antidepressant, or anti-inflammatory agents within 72 h before the examination. Women participating in the study were examined within the first part of the menstrual cycle. The study was blind, randomized and placebo-controlled with a crossover design, in which each subject received placebo and drug on two separate days at least one week apart. After the baseline recordings sumatriptan 6 mg sc (Imigran, Glaxo Wellcome Group) or saline 0.5 mL sc was given in a random fashion. The second recordings were performed 30 min after the administration of the drug or placebo. Therefore, four recordings in each subject were performed (recording before and after sumatriptan injection, and recording before and after placebo injection). Blood pressure and heart rate were monitored during the study. All adverse events were noted.

Pressure data converted by PC polygraph were displayed, recorded, and analyzed on an IBM-compatible computer using Polygram software (Synectics Medical). Baselines were automatically set by the computer program. The amplitude was measured from the baseline to the peak of the resting and squeeze pressure area. The maximal resting anal pressure and the maximal squeeze anal pressure were defined as the mean of the three highest values observed at any site in the anal canal during three pulls in the relaxed state, and in the squeeze state, respectively. Each increase in the pressure during pull was later marked as an event in the tracing. The position of the event marked the position of the anal sphincter. The anal sphincter length is a product of the applied catheter pulling speed and time of the pressure increase during the pull-through procedure. The length of the zone with a pressure higher than the half of the maximal anal resting pressure value was defined as the high pressure zone.

The cough reflex was evaluated by measuring the mean of the three highest increments in the anal canal pressure during cough. The anal canal pressure during attempts to defecate was evaluated to assess the presence of pelvic floor dysynergia. RAIR was defined as a 20% reduction or more in the resting anal pressure in response to the rectal distension. The perception corresponding to each distend-

Table 1 Effect of sumatriptan on manometric and volumetric anorectal parameters in IBS patients (mean ± SD)

Anorectal parameter	Placebo	Sumatriptan
Maximal resting pressure	9.2±2.0 kPa	13.1±3.3 kPa[b]
Sphincter length during rest	4.0±0.5 cm	4.5±0.4 cm[b]
High pressure zone during rest	2.6±0.3 cm	3.1±0.5 cm[b]
Sphincter length during squeeze	5.1±0.4 cm	5.4±0.5 cm[b]
Urge sensation threshold	61±19 mL	68±18 mL[b]
First sensation threshold	27±9 mL	34±12 mL[c]
High pressure zone during squeeze	2.8±0.6 cm	3.0±0.7 cm[c]
Pain threshold: ≤100mL/>100mL (number of patients)	13/9	8/14
Rectoanal inhibitory reflex (RAIR)	21±8 mL	21±9 mL
Straining test results:		
Proper relaxation/lack of relaxation/ contraction (number of patients)	14/7/1	13/8/1
Maximal squeeze pressure	25.5±8.5 kPa	27.2±8.4 kPa
Maximal cough pressure/maximal resting pressure	2.9±0.8	3.1±1.1

[b]P<0.01, [c]P<0.05, *vs* Placebo.

Paired Student's t test was used in all cases except for analysis of straining test results and pain thresholds where chi-square test was used.

ing volume up to 100 mL was recorded.

Statistical analysis

Data are expressed as mean±SD unless otherwise stated. Statistical analysis was performed using paired Student's t test and the chi-square test, as well as EPIINFO statistical package ver. 3.2. Differences were taken to be significant for values of P<0.05.

RESULTS

Influence of placebo on anorectal function

The effect of placebo on the anal sphincter function and rectal perception thresholds was assessed in all 22 IBS patients participating in the study. Administration of placebo had no significant influence on the manometric parameters or on visceral perception thresholds. No changes in the distending volumes inducing the first rectal perception, urgency or pain sensations were observed. Lowered pain threshold (distending volume ⩽ 100 mL), considered as a sign of visceral hypersensitivity in IBS patients, was reported before and after the placebo administration in 12 and 13 patients (55% and 59%), respectively.

Effect of sumatriptan on the anal sphincter function

As compared with placebo, sumatriptan considerably increased the maximal resting pressure (MRP) from 9.2±2.0 kPa to 13.1±3.3 kPa (P<0.0001). The rise in the maximal squeeze pressure (MSP) was not statistically significant (P=0.0634, Table 1). After sumatriptan injection the increase in the anal canal pressure in response to cough (maximal cough pressure, MCP) was higher than after placebo injection. However, the increase in the MCP was proportional to the corresponding resting pressure, as the ratio MCP/MRP after the drug injection was not altered (P=0.245, Table 1). Manometric evaluation of the anal canal pressure during attempts to defecate revealed improper response in 9 patients (41%). Signs of pelvic floor dyssynergia appeared as paradoxical contractions of the anal sphincters in one subject and as a failure to relax the pelvic floor in 8 patients. The administration of the drug, as in the placebo case, did not change the results of the straining test. The anal sphincter length as well as high pressure zone of sphincter pressure evaluated by the pull-through technique during both rest and squeeze remarkably increased after sumatriptan injection (Table 1).

In comparison of the volume evoking the rectoanal inhibitory reflex (RAIR) after placebo and sumatriptan administration, which were respectively 21±8 mL and 21±9 mL, no significant difference was shown (P=0.665).

Effect of sumatriptan on visceral perception

According to the conscious rectal sensitivity thresholds, 30 min after sumatriptan injection significant increases in the thresholds for the first sensation from 27±9 mL to 34±12 mL (P< 0.05) and urgency from 61±19 mL to 68±18 mL (P< 0.01) were observed. During the baseline recordings lowered rectal pain sensation evoked with the distending volume up to 100 mL was observed in 13 IBS patients (59%) including 5 out of 7 D-IBS patients, 8 out of 10 A-IBS patients, and none of C-IBS patients. After the administration of the drug rectal pain threshold increased in 5 out of 13 patients, but in 8 (36%) it was still evoked by the distending volume up to 100 mL. The increase in the pain threshold was not significant (P=0.227).

Adverse events

After placebo administration in 5 out of 22 patients (23%) transient burning sensation in the place of injection was reported. No other adverse events were observed, except for one patient who complained of mild but long-lasting headache (about 7 d). A variety of side effects after the drug administration was reported in 20 out the 22 patients enrolled (91%). The majority of the adverse events were mild to moderate in severity. They began at a mean of 4 min (range 2-8 min) after sumatriptan injection and lasted for a mean of 12.5 min (range 1-25 min). The most commonly reported adverse events after sumatriptan injection were: burning sensation in the place of injection in 45% of patients; headache and throat tightness in 36%; weakness in 32%; tingling of lower and upper jaw or temple and formication of the head or face skin in 27%; flare in the place of injection, heaviness in chest, limbs, and in head in 23%; nose tightness, dyspnoea, bodily warmth, acroparesthesia, abdominal pain or discomfort in 9%; and nausea, drowsiness as well as total numbness in 4.5%. Many side effects occurred simultaneously, but the typical triad of symptoms, called "triptan sensations", i.e. throat tightness, heaviness in chest and bodily warmth, was not observed in any patient. In two patients after sumatriptan administration short-lasting proctalgia (about 1 min) occurred.

DISCUSSION

Based on the manometric evaluation, anorectal dysfunction in IBS patients included the signs of pelvic floor dysynergia observed in 41% of patients and lowered visceral pain thresholds in 59% of patients, particularly in

those with diarhoea predominant IBS and with alternating bowel pattern. According to the literature data an obstructive pattern of defecation is exhibited in about 20% of healthy subjects. Two times higher prevalence of improper response to the straining test in IBS patients, whether constipated or not, may suggest general changes in pelvic floor mobility in IBS. The presented results have shown that sumatriptan, a selective 5-HT$_{1B/D}$ agonist, noticeably affects anorectal function in IBS patients.

The drug administration in a standard therapeutic dose of 6 mg sc induces a considerable increase in the maximal anal resting pressure, and much less significant increase in the maximal anal squeeze pressure. The increase in the anal canal resting pressure observed after sumatriptan administration reflects mainly the internal anal sphincter contraction. On the contrary, squeeze pressure depends mostly on the external anal sphincter. Similarly, other manometric parameters characterising anorectal function and being connected with the anal canal pressure, such as the functional sphincter length and high pressure zone, increased as well. The maximal cough pressure after the drug injection was proportional to the corresponding resting pressure. Sumatriptan did not have any impact on the straining test results.

Interestingly, sumatriptan has also revealed its influence on visceral sensation thresholds. The increases in the first sensation and urge sensation thresholds were proved to be statistically important. During evaluation of rectal sensation, lowered pain threshold with the distending volume up to 100 mL was evoked in 59% IBS patients, which is in accordance with the literature data[12]. Visceral hypersensitivity was common in D-IBS and A-IBS, but not in C-IBS. After sumatriptan administration a tendency to lower visceral hypersensitivity in IBS patients was observed. The statistical analysis, however, did not confirm the significance of the increase in the pain threshold probably due to methodological limitations as with the maximal distending volume amounting to 100 mL not in all IBS patients pain or discomfort sensation was evoked. To evaluate precisely the influence of sumatriptan on rectal pain threshold another study would be needed, in which higher distending volumes and optimally a barostat or tensostat would be used. Regarding the results of the recent studies which have shown that sumatriptan affects the discomfort threshold during gastric and colonic distension, similar observation may be expected in the rectum. The influence of sumatriptan on colonic motility is important for further evaluation.

For better understanding of the role of 5-HT$_1$ receptors, the effect of sumatriptan on lower gastrointestinal tract function in the different subgroups of IBS patients as well as in healthy subjects should be investigated. From the methodological point of view, a crossover design of the study enabled us to avoid considerable interindividual variability common in functional testing such as anorectal manometry. However, an intraindividual variability could still have some impact on the manometric and volumetric parameters. Foster and colleagues used a similar protocol of the study evaluating the effect of sumatriptan on the oesophageal motility. All the women participating in the study were investigated in the follicular phase, as it has been shown that the menstrual cycle affects rectal sensitivity in IBS patients but not healthy volunteers[13]. Patients were examined after an overnight fast to eliminate the gastrointestinal reflexes and the postprandial increase in visceral sensitivity[14,15].

The majority of the adverse events were mild to moderate in severity and short-lasting. However, it cannot be totally excluded that they might stress the patients, and in this way affect the results to some extent.

Several recent studies dealt with the effect of sumatriptan on the upper part of the gastrointestinal tract[9]. Two observations were particularly important from the clinical point of view. The first one concerned chest symptoms, which occurred in 3-5% of patients using sumatriptan in the migraine treatment. Foster *et al* in the study on 16 healthy subjects have shown that a therapeutic dose of sumatriptan (6 mg, sc) altered oesophageal motility without affecting the ECG, supporting at the same time an oesophageal rather than cardiac cause for the sumatriptan-induced chest pain. However, contrary to what happened in healthy subjects, the drug failed to modify either the wave's amplitude or lower oesophageal sphincter tone in patients with ineffective oesophageal motility[16]. The second important observation having a potential therapeutic implication concerned the influence of sumatriptan on gastric fundic tone and sensitivity to distension[9]. Tack and co-workers[9] have shown that in some dyspeptic patients sumatriptan is able to restore impaired gastric accommodation to a meal and improve the early satiety symptom. A therapeutic potential for 5-HT$_1$ receptor agonist is suggested not only in functional dyspepsia, but in other functional gastrointestinal disorders, in particular IBS. The preliminary observation has revealed that sumatriptan causes a significant relaxation of the descending colon influencing the perception of colonic distension.

The present study has confirmed that sumatriptan affects not only the upper but also the lower part of the gastrointestinal tract, including anorectal function and rectal sensation thresholds. The sumatriptan-induced decrease in rectal sensitivity may have a therapeutic implication in IBS patients, particularly in patients with visceral hypersensitivity.

The effect of sumatriptan on the anal sphincter function should be also considered in patients using this drug in the form of suppository. In two patients after sumatriptan injection a short-lasting, transient proctalgia occurred. Hypothetically, it could result from the increase in the maximal resting pressure, or even spasm of the anal sphincter. However, no clinical observations have confirmed this hypothesis so far. On the other hand, various forms of the drug may differently affect gastrointestinal function. For example, it has been shown that, unlike the subcutaneous formulation, the intranasal administration of sumatriptan has no significant effect on gastric sensory and motor function, probably due to a low bioavailability of intranasally administered sumatriptan[17].

Since the resting anal canal pressure depends essentially on the internal anal sphincter (IAS) function the increase in its value after sumatriptan administration seems to result mainly from the IAS contraction. Up to now, research on neurological and pharmacological control of the IAS

function has evaluated quite precisely the role of the adrenergic and cholinergic systems. Recently, the role of non-adrenergic non-cholinergic (NANC) system involving nitric oxide (NO) as a neurotransmitter has been also investigated. Anorectal manometry and rectal sensitivity testing proved to be a useful method for evaluating the influence of pharmacological agents including serotonergic drugs acting on anorectal function in IBS patients[11]. It has been shown that serotonin induces contraction of the IAS, while ketanserin (a 5-HT_2 receptor antagonist) and cisapride (a 5-HT_4 receptor agonist and partial 5-HT_3 receptor antagonist) evokes the IAS relaxation in healthy subjects[18,19]. Prucalopride, a novel selective 5-HT_4 receptor agonist, seems not to influence the anorectal function either in IBS patients or in healthy controls[20], whereas tegaserod, the next 5-HT_4 receptor agonist, decreases sensitivity to rectal distension in healthy subjects[21]. In the study of Thumshirn *et al*[22] alosetron, a 5-HT_3 receptor antagonist, has no significant effect on gastrointestinal transit or rectal sensory and motor mechanisms in patients with non-constipated IBS. Interesting results have been obtained by Siproudhis *et al*[23] who investigated effects of two types of serotonergic antidepressants, amitriptyline and fluoxetine, on anorectal motility and visceral perception. Both antidepressants similarly relaxed the IAS, probably through a non-specific mechanism, without modifying visceral perception. Only amitriptyline relaxed the external anal sphincter[23].

The mechanisms underlying the sumatriptan-induced anorectal function changes remain unclear. The distribution of various types of 5-HT_1 receptors and particularly their role in the gastrointestinal function are also poorly identified. Anorectal function, and in particular visceral perception, are modulated at different levels of the brain-gut axis and theoretically, sumatriptan could be acting at each of these levels[24]. Potential mechanism for the effect of sumatriptan on anorectal function could occur via activation of the 5-$HT_{1B/D}$ receptors acting on enteric neurons. Alternatively, sumatriptan could be acting on sensory nerve terminals to modulate neurotransmitter release. A further possible mechanism could involve a central action of sumatriptan. However, pre-clinical data indicate that sumatriptan only poorly penetrates the blood-brain barrier making a central mechanism less probable.

A nitrergic pathway as a possible mechanism of the drug action on the gastrointestinal function has also been discussed. It has been already shown that the sumatriptan-induced relaxation of gastric fundus is partially mediated through the activation of an NANC mechanism, involving NO as a neurotransmitter, and the sumatriptan-induced relaxation of the gastric fundus is reversibly blocked by inhibition of NO synthase. However, regarding the fact that NO is the main neurotransmitter involved in the occurrence of RAIR, the lack of sumatriptan effect on the volume evoking RAIR might argue against the nitrergic mechanism. More likely a direct smooth muscle response may be modified. Sumatriptan induces not only the anal sphincter contraction, but as it has been shown in another study it causes also the increase in the lower oesophageal sphincter pressure.

Furthermore, it has been already shown that sumatriptan is able to induce endocrine secretion in men, and at the same time, for example somatostatin or glucacon can affect anorectal function. However, measurement of plasma somatostatin and glucagon concentration before and after administration of sumatriptan rules out their release as a mechanism by which sumatriptan may influence gastrointestinal function.

Sumatriptan may alter the perception of rectal distension due to its direct impact on the rectal tone. It has been already shown that the sumatriptan-induced gastric or colonic relaxations induce changes in visceral perception[9,10]. Likewise, higher volume thresholds during rectal sensitivity testing after sumatriptan administration may occur secondary to the drug-induced relaxation of the rectum. Therefore, further studies using a barostat or impedance planimetry are needed to explain this issue. The results obtained hitherto warrant further studies to clarify and verify the regulatory role of 5-HT_1 receptors in the gastrointestinal function and the mechanisms responsible for the effect of sumatriptan on the gastrointestinal sensorimotor function including receptor subtypes involved, and central *vs* peripheral mechanism. Selective and safe 5-HT_1 receptor ligands which are now lacking will be crucial for the future research.

In conclusion, the effect of sumatriptan on the anal sphincter function and rectal sensitivity thresholds indicates that 5-HT_1 receptors participate in the regulation of anorectal function. Better understanding of the role of these receptors in the pathogenesis of IBS may have some therapeutic implications, particularly in patients with visceral hypersensitivity. The possible application of anti-migraine drugs in the management of functional gastrointestinal disorders remains an open issue.

Preliminary results of this study have been presented in part at the 12th United European Gastroenterology Week held in Prague, Czech Republic, September 2004, and published as an abstract in *Gut* 2004; 53 (Suppl VI): A204.

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CLINICAL RESEARCH

Effect of gemcitabine on the expression of apoptosis-related genes in human pancreatic cancer cells

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Abstract

AIM: To investigate the expression of genes involved in the gemcitabine-induced cytotoxicity in human pancreatic cancer cells.

METHODS: A human pancreatic cancer cell line, PANC-1, was cultured. 1 x 10^4 PANC-1 cells were plated in 96-well microtiter plates. After being incubated for 24 h, gemcitabine was added to the medium at concentrations ranging 2.5 -1 000 mg/L. The AlamarBlue dye method was used for cell growth analysis. DNA fragmentation was quantitatively assayed using a DNA fragmentation enzyme-linked immunosorbent assay (ELISA) kit. *PAP* and *TP53INP1* mRNA expression was determined using the reverse transcription-polymerase chain reaction with semi-quantitative analysis. The expression of GSK-3β and phospho-GSK-3β proteins was examined with Western blot analysis.

RESULTS: The IC_{50} for the drug after a 48-h exposure to gemcitabine was 16 mg/L. The growth of PANC-1 cells was inhibited by gemcitabine in a concentration-dependent manner ($P < 0.0001$) and the cell growth was also inhibited throughout the time course ($P < 0.0001$). The DNA fragmentation rate in the gemcitabine-treated group at 48 h was 44.7 %, whereas it was 25.3 % in the untreated group. The *PAP* mRNA expression was decreased after being treated with gemcitabine, whereas the *TP53INP1* mRNA was increased by the gemcitabine treatment. Western blot analysis showed that phospho-GSK-3β^{ser9} was induced by the gemcitabine treatment.

CONCLUSION: Gemcitabine suppresses PANC-1 cell proliferation and induces apoptosis. Apoptosis is considered to be associated with the inhibition of PAP and GSK-3β, and the activation of *TP53INP1* and pospho-GSK-3β^{ser9}.

Key words: Gemcitabine; Pancreatitis-associated protein; TP53INP1; GSK-3β; PANC-1

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INTRODUCTION

Pancreatic cancer is the fifth most common cause of cancer death in Japan[1]. It is estimated that there are 20000 cases every year, which is similar to the number of deaths from this disease. The reasons for its very high mortality rate include the lack of early diagnosis, a low resectability at the time of initial diagnosis, and the rapid recurrence after resection. Surgery is rarely a curative option in pancreatic cancers because of local extension and metastasis. Chemotherapy for advanced pancreatic cancer is palliative. The use of 5-FU in combination with radiation in the locally advanced setting has been shown to enhance survival. In a randomized trial between gemcitabine and 5-FU, gemcitabine showed significantly better results in terms of the clinical benefit effect and survival[2].

Gemcitabine (2', 2'-difluorodeoxycitidine) is a nucleoside analogue well known for its antitumor activity in several solid tumors[3]. It is one of the drugs effective for pancreatic cancers in the clinical treatment[4]. Various general mechanisms of gemcitabine have been described[5-7]. A primary mechanism of gemcitabine is the blocking of DNA synthesis by inhibiting DNA polymerase activity[8]. Gemcitabine itself is not active until it enters the cells. Its intracellular transport is mediated by facilitated diffusion[9]. Intracellularly, gemcitabine is phosphorylated to its active metabolites by deoxycitidine kinase to difluorodeoxycitidine monophosphate (dfdCMP), difluorodeoxycitidine diphosphate (dfdCDP) and difluorodeoxycitidine triphosphate (dfdCTP). All three of the metabolites interfere at different steps in the processing of DNA. The dfdCTP is incorporated into

DNA and as such can obstruct DNA replication and repair. The dfdCTP can also be incorporated into RNA and can inhibit CTP-synthetase. The dfdCMP can also inhibit dCMP-deaminase. The dfdCDP is an inhibitor of ribonucleotide reductase, and its action was shown to lead to the depletion of the DNA precursor pool, dNTP. Inhibition of DNA synthesis leads to growth inhibition or cell death. In previous studies, gemcitabine has been shown to play a major role in the apoptosis of certain tumor cell lines[10-12]. There are several pathways that relate to gemcitabine-induced apoptosis. Nicole *et al*[13] reported that gemcitabine-mediated apoptosis is caspase-dependent in pancreatic cancers; Jones *et al*[14] showed that gemcitabine-induced apoptosis is achieved through the blocking of NF-kB in non-small cell lung cancer cells (NSCLC). In addition, gemcitabine-induced apoptosis is also related to signal-regulated kinase (ERK), Akt, Bcl-2 and p38 mitogen-activated protein kinase (MAPK) pathways[15-17]. Achiwa *et al*[18] indicated that the increase in human equilibrate nucleoside transport (hENT) expression is a determinant of gemcitabine sensitivity in NSCLC cells. However, the molecular mechanism of gemcitabine-induced apoptosis has not been fully elucidated.

Pancreatitis-associated protein (PAP) is a secretory protein of pancreatic acinar cells. It is almost absent in the normal pancreas, but is induced in acute and chronic pancreatitis. We have reported the expression of *PAP* mRNA in cancer tissues and have measured *PAP* levels in the sera and pancreatic juice of patients with gastrointestinal cancers[19-21]. We found that serum *PAP* levels were increased in 40% of patients with pancreatic cancer. We also reported that *PAP* levels in endoscopically aspirated pancreatic juice were positive in 55% of pancreatic cancers. *PAP* levels were significantly higher in both the serum and pancreatic juice in cases of pancreatic cancer, compared to chronic pancreatitis. Cytokines such as tumor necrosis factor-α, interferon-γ, and interleukin-6 induce *PAP* mRNA expression in the pancreatic acinar AR4-2J cell line. We found that the enhanced expression of *PAP* in pancreatic adenocarcinoma is caused by both ectopic expression in cancer cells and induction in acinar cells[22].

TP53INP1[23] was previously called stress-induced protein (SIP)[24] or p53-dependent inducible nuclear protein (p53DINP1)[25]. *TP53INP1* is strongly induced in acinar cells during acute pancreatitis in mice, and is also overexpressed in response to various stresses in vitro. *TP53INP1* gene expression is wild-type p53-dependent[26]. There is a functional p53-response element within the promoter region of the *TP53INP1* gene, and *TP53INP1* mRNA expression is activated in cells expressing wild-type p53 in response to various stresses. One of the major functions of TP53INP1 is promoting cellular apoptosis. Glycogen synthase kinase 3β (GSK-3β) is a multifunctional serine/threonine kinase mediating various cellular signaling pathways. The particular pathway depends on its substrates for phosphorylation[27]. Since GSK-3β is also an important mediator of an apoptotic signal, it is plausible that the GSK-3β deregulation observed in cancer cells confers resistance to chemotherapy, which is a major cause of treatment failure in human cancers[28].

In this study we investigated the effect of gemcitabine on the PANC-1 cells in terms of apoptosis-related factors.

MATERIALS AND METHODS

Cell culture and gemcitabine treatment

A human pancreatic cancer cell line, PANC-1, obtained from the American Type Culture Collection (ATCC, MD, USA), was maintained in Dulbecco's modified Eagle's medium supplemented with 100 mL/L fetal calf serum, penicillin, and kanamycin at 37℃ in a 50 mL/L CO_2, 950 mL/L air atmosphere. Gemcitabine (Eli-Lilly Japan, Kobe, Japan) at a concentration of 50 mg/mL was dissolved in the serum free culture medium and stored at -20℃ in the freezer. The concentration range of the treatment was from 2.5 mg /L to 1 000 mg/L.

Cell growth evaluation

The Alamarblue dye method was used for cell growth analysis. The 1×10^4 cells were plated in 96-well microtiter plates. After being incubated for 24 h, gemcitabine was added to the medium. Twenty μL of AlamarBlue dye solution (Iwaki Glassware, Inc., Tokyo, Japan) was added to wells containing 200 μL of medium at the time 12, 24, 48, and 72 h. After being incubated for 3 h, the cell growth was evaluated as the absorbance (A) using a spectrophotometer (Dai-Nippon Pharmaceutical Co., Osaka, Japan). An excitation wavelength of 540 nm was used, and the emission was read at 620 nm. The color of AlamarBlue stock is violet, and changes to red when oxidized. Each treatment was applied to 6 wells, and the experiments were repeated 3 times.

DNA fragmentation assay

DNA fragmentation was quantitatively assayed using a DNA fragmentation enzyme-linked immunosorbent assay (ELISA) kit (Boehringer Mannheim GmbH, Mannheim, Germany) according to the protocol. Cells were cultured in flat-bottom, 96-well microplates. After incubation in gemcitabine-supplemented media for 24 h, the cells were detached from the wells. The cells were lysed with lysis buffer, and the lysate was processed for streptavidin-coated microtiter plates. After incubation with biotin-labeled antihistone antibody and peroxidase-labeled anti-DNA antibody, the amount of fragmented DNA was determined using 2, 2'-azino-bis-3-ethylbenzthiazoline-sulfonate (ABTS) as a substrate. The plates were read on a Labsystems integrated EIA (Dai-Nippon Pharmaceutical Co., Osaka, Japan) at wavelengths of 540 nm and 620 nm. Each treatment was added to duplicate wells, and the experiment repeated 3 times.

Total RNA was extracted from pancreatic cancer cells using a SV Total RNA Isolation system kit (Promega, Madison, WI, USA). RNA concentrations were determined with spectrophotometry. RT was performed using a PowerScript™ Reverse Transcriptase kit (Clontech Laboratories, Inc., Palo Alto, CA, USA). First strand cDNA was synthesized from 8 μg of total RNA at 65 ℃ for 5 min after RT; the reverse transcriptase was inactivated by incubating at 42℃ for 60 min and the reaction was terminated by heating at 70℃ for 25 min. Then, cycles

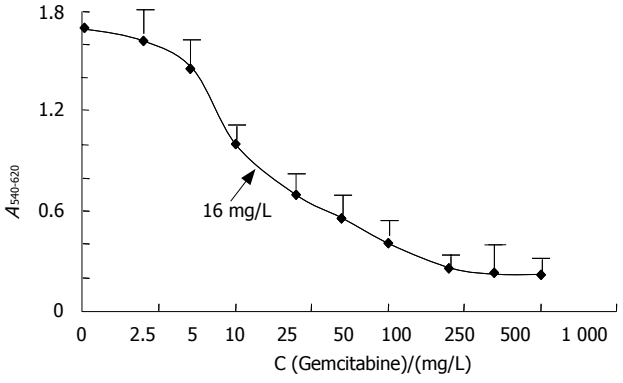

Figure 1 Effect of gemcitabine on the growth of PANC-1 cells.

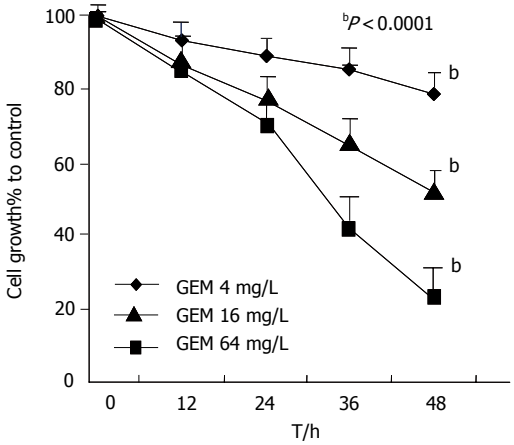

Figure 2 Inhibitory effect of gemcitabine on the growth of PANC-1 cells.

of amplification were performed on a DNA thermal cycler (Perkin Elmer Cetus, Inc., Norwalk, CT, USA) as follows: *PAP* primers were denatured at 91℃ for 60 sec, annealed at 54℃ for 60 sec, and polymerized at 72℃ for 45 sec. Then, extension was performed at 72℃ for 10 min. *TP53INP1* primers were denatured at 94℃ for 50 sec, annealed at 62℃ for 40 sec, and polymerized at 72℃ for 50 sec. The extension was performed at 72℃ for 10 min. The *PAP* primer pairs were sense: 5'-CTCCTGATTGCCTCCTCAAG -3' and antisense: 5'-AAACGTACCCTCTCTTTAGG -3', producing a fragment of 441 bp. *TP53INP1* mRNA was specifically amplified with the following primers: sense 5' -CATCCAGCCAAACTCTCAGTC -3' and antisense 5' -GCGACGAAGGCTATTTCTGT -3'. The size of the fragment was 703 base pairs (bp). The *GAPDH* of 452 bp was used as an internal control. Eight-microgram aliquots of the RT-PCR products were subjected to electrophoresis on a 20 g/L agarose gel and visualized with SYBR Gold (Molecular Probes, Inc., Eugene, OR, USA) at a 1:10000 dilution in dimethylsulfoxide, and exposed to ultraviolet 312-nm light.

Semiquantitative RT-PCR analysis

The gene expression of *PAP* and *TP53INP1* was semiquantitatively analyzed with an image analyzer (ATTO Densitograph ver. 3.02, ATTO, Inc., Tokyo, Japan). The relative expression intensity was calculated according to the following formula: PAP and TP53INP1 mRNA in a sample/GAPDH mRNA in a sample.

Western blot analysis

For Western blot analysis, 1×10^6 PANC-1 cells were lysed in a CelLytic™ mammalia tissue lysis/ extraction reagent buffer (Sigma-Aldrich, St. Louis, MO, USA). Aliquots were boiled for 5 minutes with Laemli buffer, resolved on a 125 g/L SDS-PAGE gel and eletrophoretically transferred to nitrocellulose membrane (Amersham, Buckingham, UK). Membranes were blocked for 1 h in TBS containing 50 g/L dry milk and 1 g/L Tween 20. Membranes were then incubated with either anti-rabbit phospho-GSK-3β (ser9) polyclonal antibody (Cell Signaling Technology, Inc. Beverly, MA, USA) or anti-mouse GSK-3β monoclonal antibody (BD Transduction Laboratories, Lexington, KY, USA) for one night at 4℃. After incubation with anti-rabbit or anti-mouse IgG second antibody labeled with peroxidase, the membranes were visualized with an ATTO image analyzer.

Statistical analysis

Experimental results were expressed as the mean ± SE. The difference between the means was evaluated with the Mann-Whitney *U* test. $P < 0.05$ was considered statistically significant. The statistical analysis was performed using StatView-5.0 (SAS Institute Inc. Tokyo, Japan).

RESULTS

Gemcitabine inhibited cell growth

The median 50% inhibitory concentration (IC_{50}) of gemcitabine after a 48-h exposure was 16 mg/L (Figure 1). We also measured the effect of 4, 16 and 64 mg/L gemcitabine at 12, 24, 36, and 48 h. The growth of PANC-1 cells was inhibited by gemcitabine in a concentration-dependent manner ($P < 0.0001$, Figure 2) and cell growth was also inhibited throughout the time course ($P < 0.0001$).

Gemcitabine induced apoptosis

1×10^4 PANC-1 cells were cultured in flat-bottom, 96-well microplates. After incubation in gemcitabine-supplemented media for 24 h, a DNA fragmentation assay was performed. The concentration of gemcitabine used in the DNA fragmentation assay was 16 mg/L. The DNA fragmentation rate in the gemcitabine-treated group was 44.7%, whereas the fragmentation rate in the untreated group was 25.3%. The fragmentation in the gemcitabine treated group was significantly higher than that in the untreated group ($P = 0.0157$).

Expression of PAP, TP53INP1 mRNA

We exposed the PANC-1 cells to 4, 16 and 64 mg/L gemcitabine for 48 h. The expression of *PAP* and *TP53INP1* mRNA was assessed using RT-PCR. The *PAP* mRNA expression was decreased after being treated with gemcitabine, and disappeared after being treated with 16 mg/L gemcitabine. The semiquantitative analysis showed the intensity of 16 mg/L gemcitabine-treated

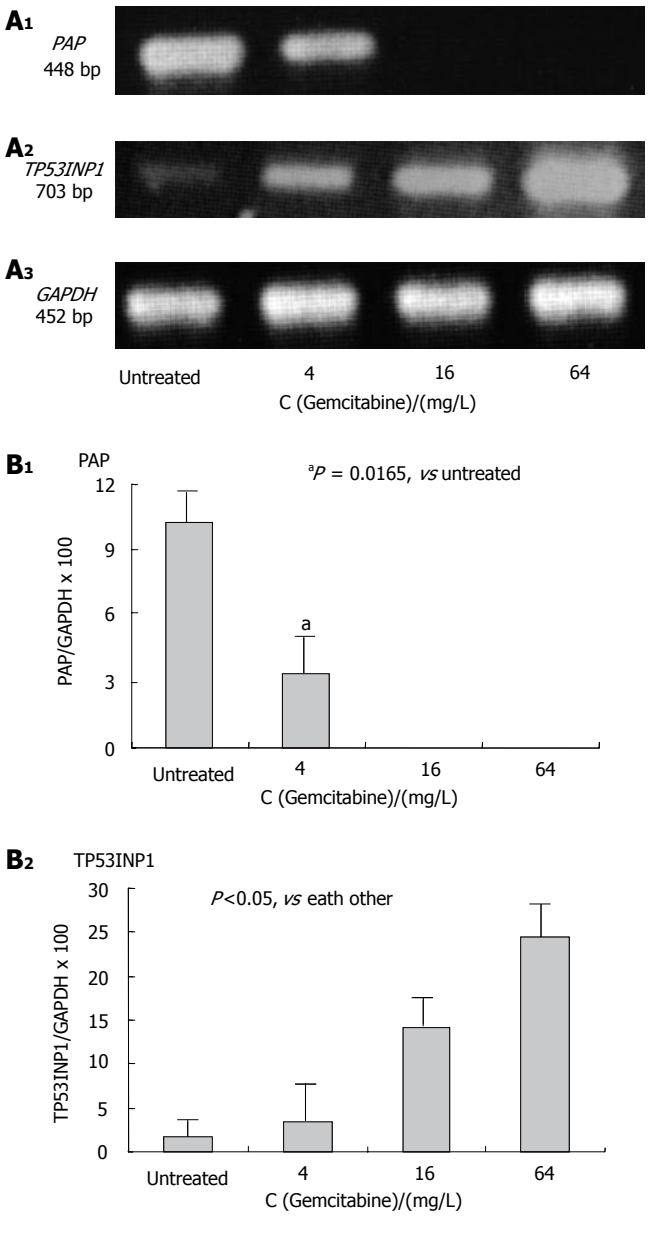

Figure 3 *PAP* mRNA expression after gemcitabine treatment.

cells to be significantly lower than that of the untreated cells ($P = 0.0165$, Figure 3). The *TP53INP1* mRNA was increased after exposure to gemcitabine. The intensity of the cells treated with 4, 16 and 64 mg/L gemcitabine was 2 times, 8.5 times and 14.6 times higher than in the untreated cells, respectively. The increasing density of *TP53INP1* mRNA was concentration-dependent. The *P* value of the interrelation of the untreated group to the 4 mg/L gemcitabine-treated group, 16 mg/L gemcitabine-treated group and 64mg/L gemcitabine-treated group were 0.0015, 0.0009 and 0.0352, respectively.

Expression of phospho-GSK-3β and GSK-3β proteins

The PANC-1 cell line showed no detectable phospho-GSK-3β^{ser9} in Western blotting analysis with a phospho-specific antibody, indicating GSK-3β activity in this cell line (Figure 4). On the other hand, phospho- GSK-3β^{ser9} was induced after 4 mg/L gemcitabine treatment, and gradually increased with the 16 and 64 mg/L gemcitabine

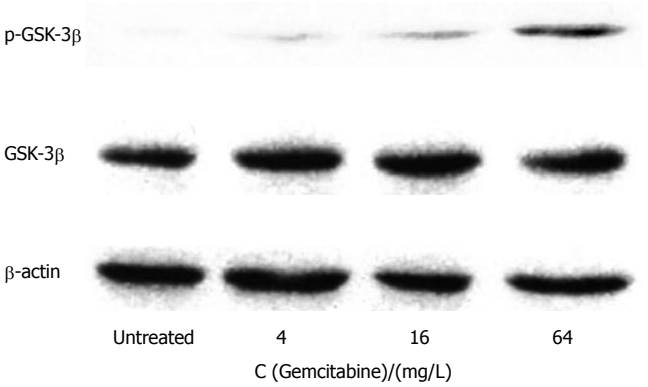

Figure 4 Effect of gemcitabine treatment on GSK-3β expression.

treatment. There was no significant difference in GSK-3β expression between the gemcitabine-treated and untreated groups.

DISCUSSION

Gemcitabine is a nucleotide analog that is converted to its triphosphate active form in cells and is subsequently incorporated into DNA to terminate strand elongation[13-17]. It shows a favorable clinical outcome in the treatment of pancreatic cancer and non-small cell lung cancer[14,18]. However, there have been few published *in vitro* analyses about gemcitabine. We therefore attempted to investigate the genes involved in the gemcitabine-induced cytotoxicity of pancreatic cancer cells. In the present study we showed that gemcitabine induced changes in expression of apoptosis-related genes in PANC-1 cells. The expression of anti-apoptotic pancreatitis-associated protein (PAP) was down-regulated, whereas the pro-apoptotic *TP53INP1* and p-GSK-3β were up-regulated after being treated with gemcitabine.

Apoptosis is believed to play an important role in tumors, and both pro- and anti-apoptotic factors are simultaneously activated in tumor development and progression. In this study, we showed that *PAP* expression was significantly decreased in the gemcitabine-induced anti-tumor process. The growth of human pancreatic cancer may require the up-regulation of *PAP* expression, which would suppress the apoptosis of cancer cells[19]. *PAP* is one of the effectors of antiapoptosis induced by tumor necrosis factor-α through NF-κB and MAP kinases in pancreatic acinar cells[29]. It was demonstrated that the activation status of the NF-κB, Akt and MAP kinase signaling pathways is often associated with anti-apoptotic signal transduction that is linked to chemotherapeutic agents[16]. NF-κB plays an important role in oncogenesis and promotes cellular resistance to anticancer therapy. Bandala *et al*[30] showed that gemcitabine treatment increased the activity of NF-κB, and Alexader *et al*[31] showed that the induction of NF-κB by gemcitabine was dose-dependent in five pancreatic cancer cell lines. In addition, gemcitabine specifically activates p38 MAP kinases in the human pancreatic cancer cell lines, PK-1 and PCI43[16]. Taken together, these findings confirmed that *PAP* is involved in the gemcitabine induced-apoptosis in a

dose-dependent manner.

Apoptosis is one of the major consequences of chemotherapy against malignancies. It is now established that the tumor suppressor p53 inhibits cell growth through the activation of cell cycle arrest and apoptosis. *TP53INP1* is a gene that is activated by wild type p53. It cooperates with HIPK2 to promote p53 phosphorylation at ser 46, and then induces apoptosis-inducing protein activation[25, 26]. In our conditions, we showed *TP53INP1* was up-regulated by gemcitabine treatment from 4 mg/L to 64 mg/L, in a dose-dependent manner. *TP53INP1* may be involved in gemcitabine-mediated apoptosis. The precise mechanism of the *TP53INP1* increase after gemcitabine treatment is unclear. It has been shown that phosphorylation of the NH_2-terminal residues of p53 mediates its stabilization and nuclear accumulation after anticancer drug treatment[28].

Beurel *et al*[28] has reported that GSK-3β is hyperphosphorylated on serine 9 in human hepatoma cell lines as well as in human and murine tumoral livers. We examined the impact of gemcitabine on GSK-3β activity in PANC-1 cells by measuring the phosphorylation level of GSK-3β at serine 9 as an indicator of GSK-3β inactivation. GSK-3β can act as a positive or negative physiological regulator of the p53 protein. During endoplasmic reticulum stress, p53 is inhibited through a mechanism involving its phosphorylation at ser 315 and ser 376 by GSK-3β. On the other hand, GSK-3β plays a crucial role in cell survival mediated by nuclear factor-kappaB (NF-κB) signaling. Phospho- GSK-$3\beta^{ser9}$ is an inactive form in normal tissues that suggests the regulation of the balanced expression of the active and inactive forms of this kinase. In our data, phospho- GSK-$3\beta^{ser9}$ was induced by gemcitabine treatment, indicating that GSK-3βgradually became inactive in the gemcitabine-treated cells. Our results are similar to those of Beurel *et al*[28], who showed that GSK-$3\beta^{ser9}$ phosphorylation by lithium treatment on tumor cells conferred resistance to anticancer therapy.

In conclusion, gemcitabine decreases cell proliferation of a human pancreatic cancer cell line, PANC-1, and promotes cellular apoptosis. The antiapoptotic gene, *PAP*, is down-regulated by gemcitabine treatment, whereas the pro-apoptotic *TP53INP1* gene and GSK-$3\beta^{ser9}$ protein are up-regulated. Gemcitabine can induce apoptosis in cancer cells through GSK-3β and *PAP* inhibition, and *TP53INP1* and GSK-$3\beta^{ser9}$ activation.

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CLINICAL RESEARCH

Mangafodipir trisodium-enhanced magnetic resonance imaging for evaluation of pancreatic mass and mass-like lesions

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Abstract

AIM: To investigate the role of mangafodipir trisodium (MnDPDP) in focal pancreatic masses and mass-like lesions by evaluating contrast uptake features of the lesions and pancreatic parenchyma after contrast medium injection.

METHODS: A total of 37 patients with pancreatic mass or mass-like lesions were examined by unenhanced and MnDPDP-enhanced magnetic resonance imaging (MRI).

RESULTS: MRI was obtained 20-40 min after infusion of MnDPDP and homogeneous contrast enhancement was observed in normal pancreas parenchyma. In patients with atrophic pancreas there was no enhancement in pancreatic parenchyma on MnDPDP-enhanced MRI. In 37 patients with 41 pancreatic masses and mass-like lesions, contrast enhancement was observed at 5 lesions on MnDPDP enhanced MRI. Three of these 5 lesions were focal pancreatitis and the other 2 were adenocarcinoma. No contrast enhancement was determined in 36 pancreatic masses and mass-like lesions in 32 patients.

CONCLUSION: MnDPDP contrast-enhanced MRI, especially in cases with no parenchyma atrophy, can distinguish focal pancreatic lesion margins. Information about the function of pancreatic parenchyma can be obtained out of tumor. MnDPDP facilitates staging of pancreatic tumors by detection of metastatic lesions in the liver. In addition, diminished hetergenous uptake of MnDPDP in patients with pancreatitis may be helpful in differential diagnosis.

Key words: MnDPDP; Pancreas; MRI

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INTRODUCTION

Diagnosis of focal pancreatic lesions is a significant challenge for radiologists and surgeons. Focal pancreatic lesions originate from the exocrine (mainly ductal adenocarcinomas) or endocrine part of gland (apudomas), cysts and/or inflammatory lesions. The diagnosis of apudoma is based on the elevated levels of circulating hormones, while the diagnosis of all other focal pancreatic lesions is mainly based on diagnostic imaging[1].

The relation of tumor with its surrounding structures can be described by cross-sectional techniques such as ultrasonography (US), CT and MRI. Ultrasonography has been proved as a first screening mode to depict the panceatic head and duct, the common bile duct and major vessels, but its value is limited by air in the digestive tract and its operator. Contrast enhanced triphasic spiral CT has an accuracy of only 70% for local staging[2]. Surgical procedures are therefore sometimes needed for diagnostic purposes[3,4]. Consequently there is a need for imaging methods with a higher sensitivity and specifity for identifying and staging pancreatic lesions to diminish the number of unnecessary laparotomies.

MnDPDP (Teslascan, Amersham, GE Heralth Care) is an organ-specific paramagnetic contrast agent that has been developed for imaging of the hepatobiliary system. Significant tissue uptake is seen mainly in the liver but much less in the renal cortex, adrenal glands, pancreas and other abdominal organs[5-7]. MnDPDP enables to get images with a higher signal-to-noise (S/N) ratio[8].

In this study, pancreatic mass and mass-like lesions were examined by MRI performed using MnDPDP. The aim of this study was to investigate the role of MnDPDP in focal pancreatic masses and mass-like lesions by evaluating features of their contrast uptake after contrast medium injection.

Table 1 Localization of pancreatic mass and mass-like lesions

	Patients (*n*)	Localization			Lesions (*n*)
		Head	Corpus	Tail	
Pancreas Ca	24	16	5	4	25[1]
Gastrinoma	1			1	1
Lymphoma	1			1	1
Lipoma	1			1	1
Focal pancreatitis	3	2		1	3
Pseudocyst	4	1	4	2	7[2]
Hydatid cyst	1	1			1
Choledochal cyst	1	1			1
Metastase	1		1		1
TOTAL (%)	37	21 (51.2)	10 (24.3)	10 (24.3)	41 (100)

[1]Two masses in a patient.
[2]Two masses in a patient and three masses in the other.

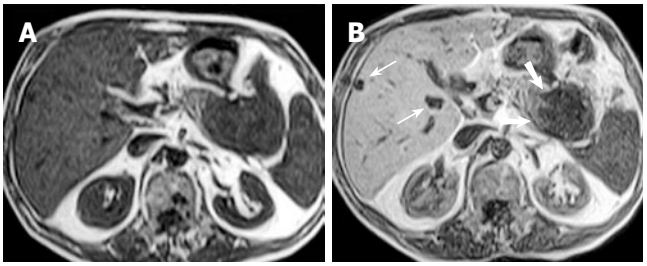

Figure 1 T1-weighted axial SE contrast-unenhanced (A) and MnDPDP-enhanced (B) images of pancreatic adenocarcinoma with liver metastases. MnDPDP enhancement was not determined in the lesion while the normal parenchyma of the pancreas showed homogeneous contrast enhancement. MnDPDP- enhanced image showed the better delination of the tumor (thick arrows). Tiny liver metastases (thin arrows) were not determined on unenhanced MR image but on MnDPDP-enhanced MRI.

MATERIALS AND METHODS

Pancreatic mass lesions or suspicious mass were determined in 37 patients (24 men, 13 women) with a mean age of 58.1 years (range 23-83 years) by US and CT. All patients underwent MnDPDP-enhanced and unenhanced MRI. The patients who received any contrast agent within 1 h before MnDPDP-enhanced MRI or had obstructive hepatobiliary disease or biliary stasis or severe renal impairment, or were contraindicative for CT or MRI or previously enrolled into this study, or defined as pregnancy or lactation, were excluded. The study was approved by the local ethical committee.

MR imaging was performed at 1.5 T (Picker Edge, Picker Internationals Highlands Height, OH) using a body coil. Sequences of axial T2-weighted fast-spin-echo (TR/TE: 8600-9700/96 msec matrix 192×256, slice thickness 3,5 mm, flip angle 90, FOV 40-55 cm), axial and coronal T1-weighted gradient echo (TR/TE: 155/8.1 msec, matrix 160x256, slice thickness 3 mm, gap 0,5 mm, flip angle 60, FOV 40-55 cm, breath-hold 15-20 sec) were applied in all patients before administration of contrast agent. Images of the coronal plane were obtained using only one of the three sequences (SE, gradient echo or fat sat gradient echo).

MnDPDP was injected intravenously at a dosage of 10 μmol/kg (2-3 mL/min) for 15 minutes. Postcontrast imaging was obtained using the same protocol and sequences (except for T2 weighted fast SE sequence) approximately 30 minutes after contrast administration. After contrast medium injection, the size, number, appearance, characteristics of the lesions were compared with normal pancreatic parenchyma. Also contrast uptake level in the lesions and pancreatic parenchyma were evaluated.

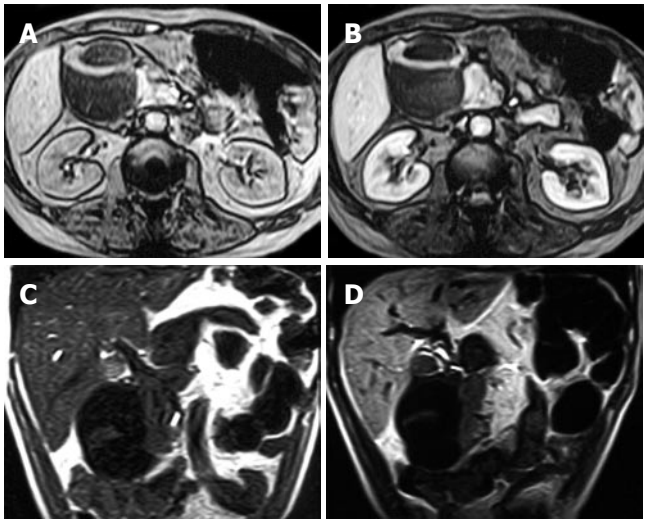

Figure 2 Unenhanced (A) and MnDPDP-enhanced (B) axial T1-weighted GRE images, unenhanced (C) and MnDPDP-enhanced (D) coronal T1-weighted SE images in pancreatic parenchyma out of lesion and liver parenchyma after MnDPDP infusion. Minimal contrast enhancement in the septa of mass was found on MnDPDP-enhanced images (B and D).

RESULTS

Diagnosis of all patients was confirmed by radiological, clinical and histopathological examination (Table 1). Twenty-three of the 37 patients underwent surgical intervention and percutaneous biopsy was performed in 5 patients with primary or metastatic lesions. In the remaining 9 patients, diagnosis was confirmed by clinical and radiological follow-up. Twenty-four patients with pancreatic carcinoma underwent MnDPDP-enhanced abdominal MRI. In 22 of 24 patients, contrast medium uptake in the mass lesions was not determined (Figure 1). Minimal heterogeneous contrast enhancement was seen in one of the 2 patients with contrast-enhanced masses and contrast enhancement of the septa was observed in the other patient with mucinous cystadenocarcinoma (Figure 2).

In patients with pancrea carcinoma contrast enhancement of pancreatic parencyhma out of the mass was evaluatedtic. Contrast enhancement was determined in 13 cases and no contrast enhancement was observed in 11 cases. Homogeneous contrast enhancement of pancreatic parencyhma was seen in 11 cases. MnDPDP-enhanced images revealed a significant improvement in the demarcation and depiction of pancreatic masses as compared to non-enhanced images. Contrast uptake of parenchyma out of mass was minimal in the other 2 cases. In these cases of pancreatic head carcinoma, pancreatitis was found with enlarged and heterogeneous contrast enhancement at the pancreatic parencyhma. In 11 patients with pancreas car-

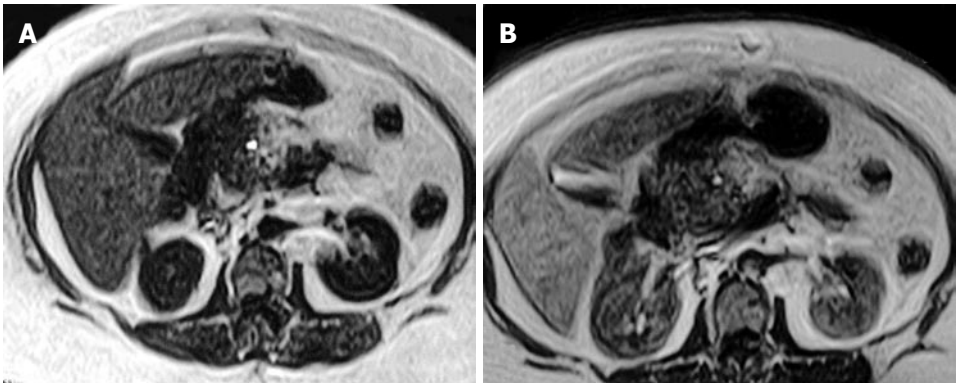

Figure 3 Contrast-unenhanced T1-weighted axial SE image demonstrating focal enlargement of the pancreatic head with a low signal intensity, simulating the appearence of pancreatic cancer (A) and MnDPDP-enhanced T1-weighted axial SE image showing heterogeneous and reduced contrast enhancement in this focal lesion (B).

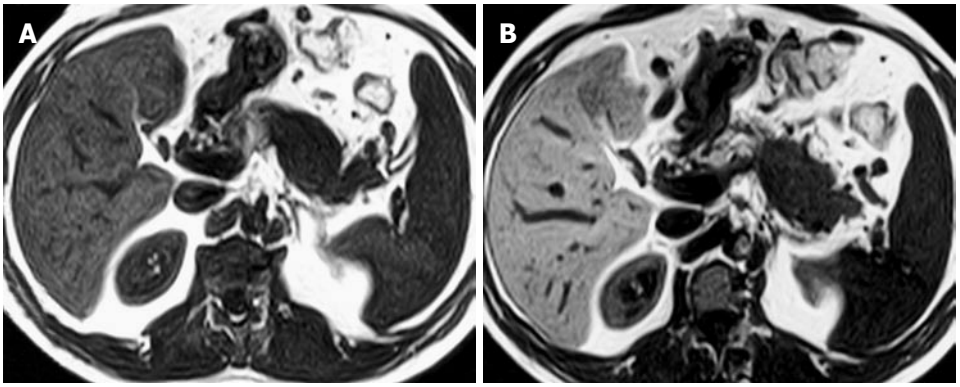

Figure 4 Unenhanced (A) and MnDPDP-enhanced (B) axial T1-weighted SE images showing better delineation of the tumor after MnDPDP infusion.

cinoma, pancreatic parencyhma was atrophic, indicating that these patients after MnDPDP infusion had no contrast enhancement in parencyhma out of the mass. In 3 patients with focal pancreatitis, the lesions were located at the tail in 1 case and at the head in 2 cases. Heterogeneous and reduced contrast enhancement in the focal lesions and homogeneous enhancement of parencyhma out of the lesion were observed in all the 3 cases after MnDPDP infusion (Figure 3). In patients with pancreas pseudocyst, metastatic lesion, hydatid cyst, aggressive type non-Hodgkin's lymphoma, lipoma, choledochal cyst and gastrinoma (Figure 4), no contrast enhancement was seen in all masses after MnDPDP infusion. In 37 patients with pancreatic masses or mass-like lesions, minimal contrast enhancement was established in 5 of 41 lesions (12.2%) by MnDPDP-enhanced MRI. No contrast enhancement was determined in 36 (87.8%) pancreatic masses or mass-like lesions in 32 patients.

DISCUSSION

Ductal adenocarcinoma of the pancreas comprises 75-90 % of all tumors of the pancreas[9]. Abdominal discomfort, back pain, obstructive jaundice and weight loss are the most common symptoms of pancreatic cancer[10]. The only potential cure for pancreatic neoplasms is surgery. Therefore early diagnognosis and assessment of tumor resectability are fundamental to achieve a successful treatment[11]. Bluemke *et al*[3] reported that ductal adenocarcinoma can be detected with helical CT and that further efforts to improve the preoperative staging of pancreatic ductal adenocarcinoma with helical CT should be directed toward improving the detection of small pancreatic ductal adenocarcinomas. Vellet *et al*[12] also reported that non-contour deforming small adenocarcinomas may not be detected by dynamic CT. Small pancreatic masses can be readily detected by MnDPDP-enhanced MRI and the sensitivity of MRI in staging of tumor is increased[13]. Gehl *et al*[14] reported that pancreatic enhancement is increased by 98% after MnDPDP injection. It was also found that after MnDPDP infusion, signal intensity of normal pancreatic parencyhma rises while tumor remains with less signal intensity[15].

Tumor-forming pancreatitis, also known as pseudotumorous pancreatitis or inflammatory pancreatic mass, is presented as a mass-like glandular enlargement. Focal enlargement of the pancreas typically involving head of the pancreas and common bile duct may occur, simulating the development of malignant neoplasms. Since the signal intensity and nonspecific contrast enhancement patterns of this tumor are similar to those of malignant tumor, their differential diagnosis is usually diffucult[16]. In our study, minimal heterogeneous contrast enhancement was observed in 3 patients with local pancreatitis by MnDPDP-enhanced MRI.

In patients with pancreatic adenocarcinomas, the contrast enhancement was compared to the proximal and distal part of tumor after MnDPDP infusion and less enhancement was found in the distal part of tumor. This may be caused by the obstruction of the pancreatic duct which is often found adjacent to the distal part of tumor[1]. In our study, pancreatic contrast enhancement at the distal part of tumor was poor in 2 cases because of pancreatitis adjacent to the distal part of tumor. Duct dilatation and atrophy of the pancreatic tail and body can be seen due to obstruction of the pancreatic duct in malignant tumor at the head of the pancreas. The parenchyma may have

a poor function even though atrophy does not occur, contrast enhancement of parenchyma becomes markedly less[8].

In our study, no uptake of contrast medium was observed in 11 patients with pancreatic malignant tumor by MnDPDP-enhanced MRI. Contrast enhancement of tumor was not observed in patients with tumor (out of 2 cases). On MnDPDP-enhanced T1-weighted images, minimal contrast-enhanced mass was detected in 1 patient with adenocarcinoma at the head of pancreas and contrast enhancement was observed in septa of mass of the other patient with mucinous cystadenocarcinoma, demonstrating that MnDPDP-enhanced MRI gives a better delineation of the tumor, especially in patients with no parenchyma atrophy. It may be difficult to distinguish the lesions simulating the occurrence of malignant neoplasms in patients with focal pancreatitis from malignant tumors. Diminished heteregenous uptake of MnDPDP of the lesions in patients with pancreatitis may be helpful in differential diagnosis.

MnDPDP is used as an organ specific contrast agent orginally designed for liver imaging. Since the agent is taken up by hepatocytes, increased contrast between normal liver parenchyma and metastatic liver lesions can be achieved. MnDPDP facilitates staging of pancreatic tumors by providing easily detection of metastatic lesions to the liver. MnDPDP may improve the staging of pancreatic cancer by increasing the sensitivity of MRI in the detection of liver metastases. MnDPDP-enhanced MRI can improve the accuracy of detection ratio and staging of focal pancreatic lesions, and is a safe and well tolerated noninvasive diagnostic method.

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CLINICAL RESEARCH

Hyperhomocysteinemia and hypercoagulability in primary biliary cirrhosis

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Abstract

AIM: To assess the hypercoagulability in PBC and its relationship with homocysteine (HCY) and various components of the haemostatic system.

METHODS: We investigated 51 PBC patients (43F/8M; mean age: 63±13.9 yr) and 102 healthy subjects (86 women/16 men; 63±13 yr), and evaluated the haemostatic process in whole blood by the Sonoclot analysis and the platelet function by PFA-100 device. We then measured HCY (fasting and after methionine loading), tissue factor (TF), thrombin-antithrombin complexes (TAT), D-dimer (D-D), thrombomodulin (TM), folic acid, vitamin B6 and B12 plasma levels. C677T 5,10-methylenetetrahydrofolate reductase (MTHFR) polymorphism was analyzed.

RESULTS: Sonoclot RATE values of patients were significantly ($P < 0.001$) higher than those of controls. Sonoclot time to peak values and PFA-100 closure times were comparable in patients and controls. TAT, TF and HCY levels, both in the fasting and post-methionine loading, were significantly ($P < 0.001$) higher in patients than in controls. Vitamin deficiencies were detected in 45/51 patients (88.2%). The prevalence of the homozygous TT677 MTHFR genotype was significantly higher in patients (31.4%) than in controls (17.5%) ($P < 0.05$). Sonoclot RATE values correlated significantly with HCY levels and TF.

CONCLUSION: In PBC, hyper-HCY is related to hypovitaminosis and genetic predisposing factors. Increased TF and HCY levels and signs of endothelial activation are associated with hypercoagulability and may have an important role in blood clotting activation.

Key words: Homocysteinemia; Hypercoagulability; Primary biliary cirrhosis; Tissue factor; Folic acid

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INTRODUCTION

Primary biliary cirrhosis is deemed to be an autoimmune chronic cholestatic disorder of the liver that primarily affects middle aged women. Currently, the diagnosis of PBC is often made when the patient is still asymptomatic, with abnormal liver biochemistry and/or antimitochondrial antibodies (AMA). The symptomatic patients may have fatigue, generalized pruritus, osteoporosis, fat soluble vitamin deficiencies and portal hypertension[1,2]. The disease generally progresses slowly but survival is less than age- and gender-matched general population and natural history may vary greatly from patient to another[3].

Thrombosis of portal veins has been detected in 40% of PBC livers resected at orthotopic liver transplantation (OLTx) and is correlated with history of bleeding varices[4]. It has been hypothesized that portal veins thrombosis may be responsible for causing development of non-cirrhotic portal hypertension and progression of liver fibrosis[5,6]. A higher incidence of thrombosis of portal venous tree may be promoted by a hypercoagulable state[7] and recent evidence has demonstrated that PBC patients were hypercoagulable on thromboelastography[8,9]. Scarce data are available on the possible causes of this hypercoagulability. In particular, plasma coagulation factors have not been completely evaluated in these patients.

Hyperhomocysteinemia has been found to be associated with a hypercoagulable state and liver fibrosis[10,11]. It has been documented that mean basal and post-methionine load serum HCY levels are significantly higher in patients with PBC than healthy controls[12]. However these findings

need to be confirmed and no data are available on the genetic and acquired causes of such hyperhomocysteinemia and its relationship with hypercoagulability in patients with PBC.

The aim of this study was to investigate basal and post-methionine load serum homocysteine levels, various components of plasmatic coagulation, platelet function and their relationship with hypercoagulability in PBC patients.

MATERIALS AND METHODS

Subjects

Fifty-one consecutive patients with a diagnosis of PBC (43 women and 8 men; age: median 63±13.9 yr (range 20-76) referred to the Gastroenterology Unit of the University of Florence from December 2001 to July 2002, were enrolled. We divided the patients into four staging severity groups: 12 patients in stage I, 11 in stage II, 15 in stage III and 13 in stage IV. Exclusion criteria were renal insufficiency, consumption of alcohol, therapy with steroids, anticoagulants, NSAIDs or methotrexate, folic acid and vitamin B12 supplementation. All patients were stable with no history of bleeding or infectious complications at least 6 wk before evaluation. Routine laboratory investigations included aPTT, PT, fibrinogen, liver function tests (bilirubin, alkaline phosphatase, albumin, aspartate and alanine transaminases), creatinine, cholesterol, iron, calcium, and antimitochondrial antibodies (AMA). All cholestatic patients received vitamin K. All patients had a screening for thyroid-associated disease and intestinal bowel diseases.

One hundred and two healthy subjects with no previous history of liver disease, comparable for age and sex (86 women and 16 men; median 63±13 (range 18-75) yr), recruited from blood donors of our hospital and laboratory volunteers, were used as control group. Patients and controls gave their informed consent to use part of their blood samples for an experimental study.

Experimental procedures

Venous blood samples were collected from the basilical vein after discarding the first 2 mL of blood. We determined HCY plasma levels in the fasting state and 4 h after an oral load with methionine. One hundred mg/Kg of body weight of L-methionine was administered in approximately 200 mL of fruit juice immediately after the fasting phlebotomy.

Sonoclot™ analysis

The haemostatic process in whole blood has been measured by the Sonoclot™ (Sienco Company, Morrison, CO) analysis, a viscoelastic test on whole blood collected in tubes containing 0.129 M sodium citrate. (Saleem 83) Sonoclot analysis was performed within 30 min from the phlebotomy, with 360 μL citrated blood recalcified with 15 μL of calcium chloride (0.25 mol/L). The following variables were analyzed: (1) the clot RATE, i.e. the gradient of the primary slope measured as Clot Signal Units per minute which is an index of clot formation affected by both platelets and coagulation proteins; (2) the time to peak amplitude (TP) (minutes) which reflects the clot retraction away from the surface of the probe and is mainly influenced by the platelet function.

Platelet function analysis

Platelet function was evaluated by the PFA-100 ® device (Dade-Behring) on whole blood collected in tubes containing 0.129 M sodium citrate. The closure times (CT) were determined on duplicate samples (0.8 mL) within 2 hours of collection, using cartridges containing collagen-coated membranes with epinephrine (Col/Epi cartridge) or ADP (Col/ADP cartridge) as previously described[9].

Blood coagulation parameters

D-dimer (D-D), thrombin-antithrombin complex (TAT), tissue factor (TF) and thrombomodulin (TM) plasma levels were evaluated using ELISA method (D-D: Agen, Brisbane, Australia; TAT: Dade Behring, Marburg, Germany; TF: American Diagnostica, Greenwich,CT,USA; TM: Diagnostica Stago, Asnieres, France) on blood samples collected in tubes containing 0.129 M sodium citrate, immediately centrifugated at 4 ℃ and stored in plasma aliquots at -80 ℃ and assayed within two weeks.

Homocysteine assay

To determine HCY, whole blood was collected in tubes containing ethylenediaminotetracetate (EDTA) 0.17 mol/L, immediately put in ice and centrifuged within 30 minutes at 4 ℃ (15000 x g for 15 min). The supernatant was stored in aliquots at -80 ℃ until assay. Homocysteine plasma levels were detected by FPIA method (Abbott, Wiesbaden, Germany).

Vitamin pattern

Serum folic acid and vitamin B12 were measured by radioassay (ICN Pharmaceuticals, NY)). Serum vitamin B6 was measured with HPLC method and fluorescence detecting (Immundiagnostik, Benheim, Germany).

MTHFR polymorphism

Genomic DNA was extracted from peripheral blood leukocytes. C677T polymorphism in the MTHFR gene was carried out on genomic DNA by PCR amplification as already described[13].

Statistical analysis

All analyses were performed with SPSS 10.0 for Windows (SPSS, Chicago, IL, USA). The non-parametric Mann-Whitney test for unpaired data was used for comparisons between single groups. Spearman's rank correlation coefficient (for non-parametric data) was used for the correlation analysis. Fasting and post-methionine hyperhomocysteinemia were defined based on the 95th percentile cut-off of the control population (Fasting: M=15 μmol/L, F=13 μmol/L; Post-methionine: M=38 μmol/L, F=35 μmol/L).

For genetic analysis, Hardy-Weinberg equilibrium was assessed using χ^2 analysis. All probability values are two-tailed with values less than 0.05 considered to be

Table 1 Laboratory characteristics of patients investigated according to the stage of disease[1]

Stage	I	II	III	IV
Patients (*n*°)	12	11	15	13
Alkaline phosphatase (U/L)	273 (50-797)	447 (145-1315)	397 (190-1956)	289 (52-1960)
AST (U/L)	28 (13-6)	38 (25-400)	35 (14-63)	49 (11-144)
ALT (U/L)	37 (14-89)	54 (17-100)	39 (17-72)	47 (8-218)
Total Bilirubin (mg/dL)	0.7 (0.4-2.4)	0.9 (0.2-1.2)	0.9 (0.3-1.5)	1.0 (0.5-1.6)
Cholesterol (mg/dL)	190 (112-235)	220 (104-285)	219 (110-275)	208 (154-314)
aPTT (sec)	32 (27-63)	28 (26-43)	32 (25-37)	32 (26-36)
PT (%)	98 (90-105)	100 (91-120)	99 (81-100)	93 (58-100)
Fibrinogen (mg/dL)	379 (279-650)	380 (268-463)	327 (276-590)	380 (100-586)

[1]Data are expressed as median (range).

Table 2 Platelet and blood coagulation tests

	PBC patients *n* = 51	Controls *n* = 102
Sonoclot RATE (U/min) all pts	29 (14-49)[b]	21 (14-31)
• <150 x 10^3/μL plts (*n* = 22)	30 (14-49)[b]	
• ⩾150 x 10^3/μL plts (*n* = 29)	28 (19-41)[d]	
Sonoclot TP (min) all pts	12 (6-30)[d]	9 (6-15)
• <150 x 10^3/μL plts (*n* = 22)	15 (6-30)[d]	
• ⩾150 x 10^3/μL plts (*n* = 29)	10 (6-24)	
PFA/EPI - CT (secs) [⩾150 x 10^3/μL plts (*n* = 29)]	151.5 (60-222)	146 (111-179)
PFA/ADP- CT (secs) [⩾150 x 10^3/μL plts (*n* = 29)]	106 (61-180)	102 (61-148)
TAT (μg/L)	2.8 (1.5-87.3)[b]	2.5 (1.1-4.2)
D-Dimer (ng/mL)	16.0 (2-173)	23.0 (3-55)
TF (pg/mL)	255.6 (24.8-466.1)[d]	121.9 (24.8-466.1)
TM (μg/mL)	23.7 (2.6-153.5)[d]	13.8 (7.6-23.1)

[b]$P < 0.01$, [d]$P < 0.001$ *vs* Controls; TP = time to peak; PFA/EPI - CT = closure time with epinephrine; PFA/ADP-CT = closure time with ADP. Data are expressed as median (range).

statistically significant.

RESULTS

Laboratory characteristics of patients investigated according to the stage of disease are shown in Table 1.

Blood Coagulation and Platelet Analysis

Sonoclot RATE values of PBC patients were significantly higher than those of controls ($P < 0.001$). In both the 29/51 (57%) PBC patients with a platelet count in normal range and the 22/51 (43%) PBC patients with thrombocytopenia as well as 26/51 (51%) PBC patients, the Sonoclot RATE value was higher than 95% of controls ($P < 0.001$) (Table 2).

Sonoclot TP values were significantly higher only in PBC patients with thrombocytopenia. Indeed, a significant difference of Sonoclot TP ($P < 0.001$) values was observed between PBC patients with a normal platelet count and with thrombocytopenia (Table 2). The hypercoagulable state and the increased TF circulating levels were independent of the disease's stage, and were presented even in the first stage of the disease (data not shown).

PFA-100 closure times after stimulation with epinephrine or ADP were comparable in patients with PBC and controls (Table 2). In 9/51 (17.6%) PBC patients, D-dimer plasma levels were higher than 95% of controls but the difference did not reach the statistical significance ($P = 0.16$).

TAT, thrombomodulin and tissue factor levels were significantly higher in patients than in controls (TAT 2.8±13.9 (1.5-87.3) μg/L *vs* 2.5±0.52 (1.1-4.2) μg/L; TM 23.7±3.5, (2.6-153.5) ng/mL *vs* 13.8±4.4 (7.6-23.1) ng/mL; TF 255.6±223.0 (81.4-1259.6) pg/mL *vs* 121.9±81.9 (24.8-466.1) pg/mL; $P < 0.001$).

Homocysteine

HCY plasma levels, both in the fasting state and post-methionine loading, were significantly higher in patients than in controls (Fasting: 12.1±8.76 (1.5-58.8) μmol/L *vs* 9.9±1.7 (6.4-18.0) μmol/L; Post-methionine 30.1±14.4 (9.2-99.6) μmol/L *vs* 28.0±5.23 (16.4-38.9) μmol/L, $P < 0.001$) (Table 3). Totally, hyperhomocysteinemia (defined as a concentration of fasting and/or post-methionine HCY above 95% of controls) was diagnosed in 23/51 patients (45.1%) (8 only fasting; 4 only PM; 11 both). No significant difference in plasma HCY levels was detected between four staging severity groups.

Folic acid, vitamins B12 and B6

Vitamin deficiencies (defined as a vitamin concentration below the 10th percentile of controls) were detected in 45/51 patients (88.2%).

Deficiency of folate (defined as a concentration less than 6.4 ng/mL) was documented in 39/51 (76.5%); deficiency of vitamin B12 (defined as a concentration less than 243.7 pg/mL) was found in 6/51 patients (11.8%). Deficiency of vitamin B6 (defined as a concentration less than 3.4 pg/mL) was found in 3/51 patients (5.8%). Three patients had low levels of both folic acid and vitamin B12, 1 patient of both vitamin B12 and B6 and 2 patients of both folic acid and vitamin B6.

Vitamins median values are shown in Table 3.

MTHFR C677T polymorphism

The allele frequency of the C677T polymorphism was 0.52 in patients and 0.45 in controls. The distribution of the three genotypes in controls was as follows: TT 17.5 %; CT 55.3%; CC 27.2%. The genotype distribution in patients was as following: TT 31.4%; CT 41.2%; CC 27.4%. The prevalence of the homozygous TT677 genotype was significantly higher in patients (31.4%) than in controls (17.5%) ($P < 0.05$).

Table 3 Homocysteine and vitamin levels in PBC patients and controls

	PBC Patients ($n = 51$)	Controls ($n = 102$)
Fasting HCY (μmol/L)	12.1 (1.8-58.8)[b]	9.9 (6.4-18.0)
Post-methionine HCY (μmol/L)	30.1 (9.2-99.6)[b]	28.0(16.4-38.9)
Folic Acid (ng/mL)	5.3(1.2-13.4)[b]	10.7(5.4-18.5)
Vitamin B12 (pg/mL)	335 (201-977)[a]	304.9(176-427.1)
Vitamin B6 (pg/mL)	6.6 (1-20)[b]	10.0 (3-17)

[a]$P < 0.05$ *vs* controls; [b]$P < 0.001$ *vs* control.

Table 4 TTMTHFR polymorphism and vitamin state in 51 PBC patients *n* (%)

MMTHFR	Low folate, normal HCY	Normal folate, high HCY	Low folate, high HCY	Normal folate, normal HCY	Total *No* patients
CT	8 (38)	0	10 (47.6)	3 (14.45)	21
CC	8 (57)	0	2 (14.5)	4 (28.5)	14
TT	3 (18.7)	2 (12.5)	9 (56)	2 (12.5)	16
Total	19	2	21	9	

Patients with the homozygous TT677 genotype had higher, but not statistically significant HCY levels than those with C677T and CC677 genotypes (TT = 13.3 (1.8-58.8) μmol/L; CT = 12.3 (7.9-42.6) μmol/L; CC = 10.2 (6.0-21.7) μmol/L).

TTMTHFR polymorphism and/or vitamin deficiencies were present in all hyperhomocysteinemic patients (Table 4). In other words, in patients with normal folic acid plasma levels homocysteinemia was normal except for two patients with TTMTHFR polymorphism.

Correlation between the parameters investigated

A significant correlation between Sonoclot rate values, TAT ($r = 0.44$, $P < 0.001$), TF plasma levels ($r = 0.30$, $P < 0.05$) and basal HCY ($r = 0.45$, $P < 0.001$) was observed. Sonoclot rate was significantly higher in patients with high fasting state and/or post-methionine than in the other patients [34 ± 7.4 (22-45) U/min *vs* 26 ± 5.4 (14-49) U/min (Figure 1)]. Moreover, a significant correlation was detected between HCY, TM ($r = 0.54$, $P < 0.001$) and TF ($r = 0.55$, $P < 0.05$).

TAT levels correlated significantly with TF ($r = 0.43$, $P < 0.05$) while HCY plasma levels correlated significantly with cholesterol plasma levels ($r = 0.55$, $P < 0.001$).

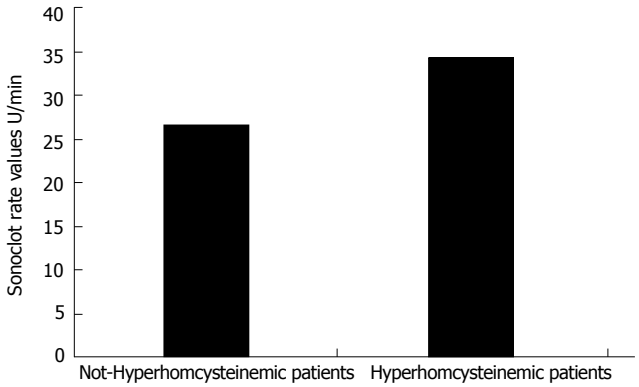

Figure 1 Sonoclot rate values in PBC patients with or without hyperhomocysteinemia.

DISCUSSION

Hypercoagulability in PBC has been previously documented with thromboelastography[8,9], and here we have confirmed this finding with Sonoclot, another technique of analysis of haemostatic process, and demonstrated high TAT circulating levels in 51 PBC patients.

Hypercoagulability may have two different roles in natural history of PBC: it could promote portal veins thrombosis and liver damage and on the other hand it could be responsible for a more favourable prognosis of variceal bleeding[14,15] and a lower blood loss at liver transplantation[11,16-19]. It has been hypothesized that portal veins thrombosis may be responsible for causing development of regenerative nodules and non-cirrhotic portal hypertension and progression of liver fibrosis[5,6]. In PBC patients hypercoagulability may contribute to the high incidence of oesophageal varices in early histological stages associated with the presence of regenerating nodules[20]. A recent study demonstrated that anticoagulant therapy with a thrombin antagonism can reduce fibrogenesis in rat liver[21], it could be interesting to evaluate if a correction of hypercoagulability could reduce the intrahepatic thrombosis formation and development of liver fibrosis and portal hypertension.

Moreover we found that TF might be involved in determination of thrombophilic status, in fact TF levels are higher in PBC patients than in healthy controls and are related to Sonoclot Rate values. TF is a glycoprotein present on the surface of the plasma membranes of monocytes, endothelial cells and smooth muscle cells[22,23], and it is the primary cellular trigger of the coagulation cascade. In our patients clinical conditions that may affect TF concentrations such as infections, neoplasia, or heparin administration have been ruled out. Lymphocyte activation is able to induce tissue factor expression by monocytes[23]. PBC is characterized by an intense biliary and systemic inflammatory CD4+ and CD8+ T cell response[23,25] and may be responsible for tissue factor expression and subsequent elevation of circulating levels.

Our data confirm the finding of elevated homocysteine levels in patients with PBC. Hyperhomocysteinemia has been found to be associated with a hypercoagulable state and liver fibrosis[10]. Here we demonstrated that HCY levels are associated with hypercoagulability and, as reported by others in the setting of thrombotic diseases[26,27], in this study we offer the "*in vivo*" demonstration that in PBC patients high levels of HCY are related to increased TF plasma concentration. TF expression by endothelial cells and monocyte-macrophages may be induced by HCY. This effect has been documented *in vitro* studies, in experimental animals and in hyperhomocysteinemic patients in a specific and dose-dependent manner[28]. HCY may be one of the mechanisms involved in the endothelial stimulation, as documented by the significant correlation with TM levels, which are significantly higher in patients than in controls.

Platelet function has been investigated by two parameters: Sonoclot TP value and PFA-100 closure time. Both of them have been found to be comparable or even higher than healthy controls values. These data are similar to those presented by Pihush *et al*[9].

As regards fibrinolysis, our data demonstrate that the hypercoagulable state in PBC patients is not associated with a comparable activation of fibrinolysis, as no difference in D-D levels was documented between patients and controls. Further studies are necessary to thoroughly investigate fibrinolysis, and in particular its main inhibitor, plasminogen activator inhibitor-1 (PAI-1), which may be released by stimulated monocytes and endothelial cells[29]. This behaviour of fibrinolytic system may contribute to determine a prothrombotic state.

In this study the main possible genetic and acquired alterations of HCY metabolism in PBC were investigated. We demonstrated the presence of vitamin deficiencies related to methionine metabolism (folic acid, vitamin B6 and vitamin B12) in about 90% of patients. In particular, the majority of patients had a folate deficiency. This is a novel finding that arises from the question about the potential clinical utility of a low-cost vitamin supplementation in these patients. It should be investigated if folate supplementation could correct hyperhomocysteinemia and/or hypercoagulability. Folate levels may be low due to inadequate dietary intake, malabsorption, increased utilization or to effects of drugs[30]; in our patients we excluded the presence of drugs able to interfere with folate absorption, such as methotrexate. Disease activity may contribute to increased demand for folate due to inflammation[32]. However, we found no correlation between disease activity, as measured by histological stage, and folate or HCY levels. Therefore, these data may point to inadequate intake as a significant factor that affects folate levels. Another hypothesis is the presence of an impairment of the folate enterohepatic circulation. This cycle plays an important role in the homeostasis of folic acid. It has been demonstrated that the interruption of bile circulation by bile drainage leads, to a rapid fall in serum folate levels to 30% of base line within 4-6 h[33]. Cholestasis could make this system of vitamin reutilization ineffective, and could induce folate low serum levels.

No data are available on the prevalence of C677T 5,10-methylenetetrahydrofolate reductase (MTHFR) polymorphism in PBC patients. This polymorphism is one of the most frequent genetic factors responsible for the alteration of HCY levels, especially in the presence of a suboptimal folate status[34,35]. We found a significant higher prevalence of the homozygous TT677 genotype in PBC patients. In these patients MTHFR has a reduced activity caused by a C-T substitution at nucleotide 677[36]. However, in order to investigate the prevalence of this genetic polymorphism in PBC further larger studies are needed.

In PBC serum cholesterol levels markedly increase with worsening of cholestasis[37,38]. In this study, as in others,[39,40] a significant in vivo association between HCY and cholesterol circulating levels was found, suggesting another possible explanation, together with cholestasis, for hypercholesterolemia in this disease.

Recently, in cultured human hepatocytes it was demonstrated that hyperhomocysteinemia determines an oxidative stress of the endoplasmic reticulum which activates the sterol regulatory element-binding proteins (SREBPs)[41]. The activation of SREBPs is associated with increased expression of genes responsible for cholesterol/triglyceride biosynthesis, and may be an explanation to our findings.

In conclusion, hypercoagulability and hyperhomocysteinemia exist in patients with PBC, and there is an association between these two parameters. TF may have a role in determination of blood clotting activation and hyperhomocysteinemia is related to hypovitaminosis and genetic predisposing factors. Further studies are needed to clarify if hyperhomocysteinemia and hypercoagulability may have a role in progression of liver damage and if they may be influenced by vitamin supplementation.

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RAPID COMMUNICATION

Soluble CD40 ligand in prediction of acute severe pancreatitis

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Abstract

AIM: To assess the early predictability of the soluble CD40L (sCD40L) in pancreatitis severity.

METHODS: Between February 2000 and February 2003, 279 consecutive patients with acute pancreatitis were prospectively enrolled in our study. In this report, 40 patients with mild and 40 patients with severe pancreatitis were randomly studied. sCD40L concentrations were measured 48 hours after admission.

RESULTS: sCD40L levels were significantly higher 48 hours after admission in severe pancreatitis than in mild pancreatitis. Using a cutoff of 1000 pg/L, the sensitivity and specificity of sCD40L to detect a severe course of the disease were 78% and 62% respectively compared to 72% and 81% for CRP. Logistic regression analysis found that CRP was the only statistically significant marker able to detect a severe course of the disease.

CONCLUSION: These findings indicate that CRP remains a valuable marker to determine the severity and prognosis of acute pancreatitis whereas sCD40L levels should be assessed in further studies.

Key words: Acute pancreatitis; Prognostic factor; sCD40

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INTRODUCTION

Acute pancreatitis (AP) is a disease of variable severity. While approximately 80% of patients experience mild attacks, the remaining 20% still suffer from severe disease, with a mortality rate as high as 30%[1,2]. Since treatment of patients with severe disease is currently mostly supportive and directs at either prevention or control of the local and systemic complications, the early prediction of the severity of AP is the main goal of clinicians in charge of such patients[2]. Multifactorial scales including Ranson and APACHE II scoring systems have been used in acute pancreatitis since the 1980s. Ranson scale has been used in the 1980s in virtually all studies dealing with acute pancreatitis[3,4]. The list of 11 numerical parameters proposed by Ranson has no been fully discussed because only 5 criteria are obtained at admission and the remaining 6 during the subsequent 48 hour period, delay the performance of an early prediction. Moreover, the complexity of these scales and perhaps the fact that CT scanners are not available in every hospital account for the increasing interest in serum markers[5].

The pathophysiology of acute pancreatitis includes the activation and release of pancreatic enzymes in the interstitium, pancreatic autodigestion, and a multiple organ dysfunction following the release of these enzymes into the systemic circulation[6,7]. There is evidence that synthesis and release of pro-inflammatory cytokines and chemokines are also responsible for local and distant injuries[8,9]. If the concentration of these biological factors is correlated to the severity of the disease and if they are detected before the occurrence of multiple organ dysfunction, these factors might be important for the rapid scoring of the disease severity in the acute phase[5].

CD40, a proteic member of the tumour necrosis factor receptor family, is expressed on the membrane of a variety of cells, including B lymphocytes, monocytes, biliary and acinar cells as recently shown by our group[7,10]. CD40 binds to its ligand CD40L, a membrane glycoprotein, to mediate major immunoregulatory signals involved in auto-immune disease, graft rejection, inflammatory bowel disease, development of atherosclerosis and acute experimental pancreatitis[11]. We postulated that CD40-CD40L interactions might also play a role in the pathogenesis of human pancreatitis and that the soluble form of CD40L

Table 1 Characteristics of the population

	Control group	Mild pancreatitis	Severe pancreatitis	*P* value
n =	10	40	40	
Male/female	5/5	27/13	31/9	0.33
Age (year)	33.5 (31.2-35.3)	52.5 (51.5-62.7)	58.5 (56.1-66.6)	0.21
Serum amylase (U/L)	98 (75-128)	356 (352-892)	474 (451-1238)	0.34
Delay after onset (hours)	-	20.1 (18.2-22.4)	22.2 (19.5-23.2)	0.58
Cause of AP				
-biliary	-	17	21	0.5
-alcohol	-	14	16	0.6
-other	-	2	1	0.6
-idiopathic	-	7	2	0.15
Ranson > 3	-	8	19	0.017
Ranson value	-	2 (1.5-2.1)	2 (1.9-2.6)	
sCD40L (pg/L)	22 (16.3-28.1)	795 (692-1301)	1215 (1186-1753)	
CRP levels (mg/L)	12.2 (9.3-15.5)	42.5 (50.1-101.7)	225.5 (199.8-319.4)	0.0007
CT scan				
-normal	-	5	0	0.021
-edematous	-	28	19	0.04
-necrosis	-	19	9	0.02
-collection	-	8	27	0.0001
Death	-	0	3	0.07

Values are expressed as median values (CI 95%).

(sCD40L) levels might be correlated to the severity of the disease. The purpose of this study was to assess the early predictability of sCD40L in pancreatitis severity.

MATERIALS AND METHODS

The study protocol was approved by the local Ethics Committee. Upon admission, all patients with acute pancreatitis meeting the criteria of inclusion were identified and proposed to be included in the study. Before inclusion, each patient gave an informed consent. Between February 2000 and February 2003, 279 consecutive patients with acute pancreatitis were prospectively enrolled in our study. We assessed the role of sCD40L in the prediction of the severity of pancreatitis in this preliminary study. Consequently, 40 patients with mild and 40 patients with severe pancreatitis were randomly evaluated in the current study (Table 1). Ten healthy controls from our own divisions were also used for comparison. Inclusion criteria were typical abdominal pain associated with serum amylase twice the upper limit value (range: 26-128 U/L). The diagnosis was subsequently confirmed by CT scan with a Picker 1200 or a PQ-2000 instrument. Before scanning 150 ml of diluted oral contrast material (Telebrix Gastro, France) was administered to visualize the gastrointestinal tract. Intravenous contrast material (Omnipaque 350, Shering, Germany) was injected and multiple scans were obtained from the pancreas to the pelvis as previously described[12]. Pancreatic necrosis was considered significant if there was > 30% necrosis of the gland. Pancreatitis severity was defined according to the Atlanta classification and included organ failure (e.g.,shock, pulmonary insufficiency, renal failure), the presence of local complications (pancreatic necrosis, pseudocysts, abscess), or both[13]. Blood samples drawn 48 hours after admission were used to measure CRP and sCD40L using commercially available kits (Behring, Marburg, Germany and Bender MedSystems Vienna, Austria).

Statistical analysis

Serum markers were compared during severe and mild attacks by univariate analysis (Student' *t*-test, Mann-Whitney). SCD40L, CRP and Ranson scores were evaluated by receiver operating characteristics (ROC) curves for selected sCD40L and historically determined (CRP and Ranson) cutoff levels[3,4,5]. The cutoff for CRP (150 mg/L) and Ranson (severe > 3) was chosen according to the literature[4,5]. The cutoff for sCD40L was chosen according to its ROC curve. A logistic regression analysis for the binary outcome of mild or severe pancreatitis was performed. $P < 0.05$ was considered statistically significant. Statistics calculations were done using SPSS Software package (Chicago, USA).

RESULTS

The delay between the onset of abdominal pain and admission ranged from 7 hours to 2 d (Table 1). The aetiology of acute pancreatitis was biliary in 38 patients (48%), alcoholism in 30 (38%), drug-induced pancreatitis in 2 (3 %), hypertriglyceridemia in 1 (2 %) and idiopathic in the remaining 9 patients (11%) (Table1).

CT scan was obtained within 36 hours after admission in each patient and showed a normal pancreas in 5 patients, edema in 47 patients and necrosis in 28 patients (Table 1). Acute fluid collections were found in 35 patients (43 %) (Table1). The Ranson score established at 48 hours was $\geqslant 3$ in 27 patients (33 %), 19 of whom had severe pancreatitis and 8 mild pancreatitis. Sensitivity, specificity, positive predictive value, and negative predictive value of Ranson

Table 2 Performances of sCD40L, CRP and RANSON score

	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
sCD40L (cutoff 1000 pg/L)	77	62	63	74
CRP (150 mg/L)	72	81	80	74
RANSON Score (>3)	48	80	70	60

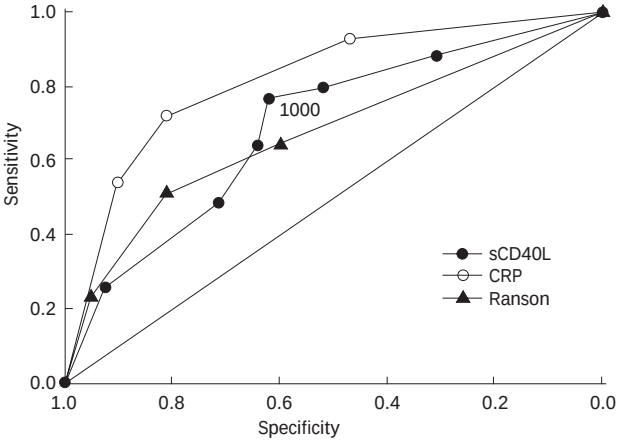

Figure 2 sCD40L (400, 800, 1000, 1200 1400 and 2000 pg/L), CRP (50, 150 and 200 mg/L) and Ranson (Ranson score of 2, 3 and 4) ROC curves with selected or historically determined cutoffs in patients with mild and severe pancreatitis.

score to predict a severe attack were 48% and 80%, and 70% and 60% respectively (Table 2). Three patients group with severe pancreatitis (7.5 %) died, two of whom were of biliary origin (Table 1).

Healthy controls had very low levels of sCD40L as well as CRP (Table 1). By comparison, levels of sCD40L and CRP were 795 pg/L (CI 95 %: 692-1301) and 42.5 mg/L (CI95%: 50-101) in patients with mild pancreatitis and 1215 pg/L (C I95% 1186-1753) and 225 mg/L (CI 95 % 199-319) ($P<0.0007$ and $P<0.0001$) in patients with severe pancreatitis respectively (Table 1 and Figure 1). No significant correlation was found between serum levels of sCD40L and the nature of the complications that occurred in patients with severe pancreatitis. There was only a trend towards correlation between sCD40L and the occurrence of multiple organ failure. The three patients who died from severe pancreatitis had intermediate levels of sCD40L (range from 184 to 236 pg/L). The sensitivity, specificity, positive predictive value, and negative predictive value of CRP to predict a severe attack were 72% and 81% respectively and 80% and 74% respectively (Table 2). Ranson score, CRP and sCD40L results were evaluated by their ROC curves for selected cutoff levels (Figure 1). Using a cutoff of 1000 pg/L for sCD40L as defined by the ROC curve, the sensitivity, specificity, positive predictive value and negative predictive value of sCD40L to predict a severe attack were 77% and 62% respectively, whereas the positive and negative predictive values were 63 % and 74% (Table 2). Logistic regression analysis for the binary outcome of mild or severe pancreatitis was performed to better delineate the performance of Ranson score, CRP and sCD40L (Figure 2). The forward stepwise analysis

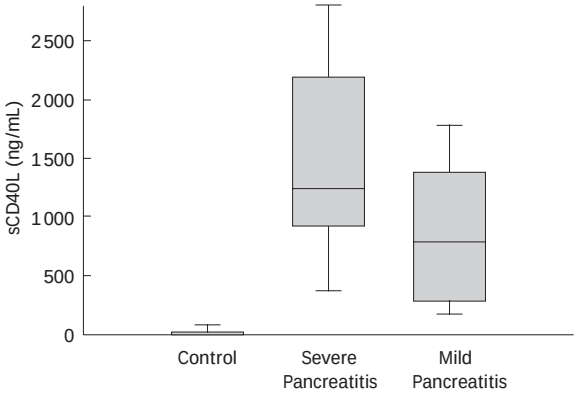

Figure 1 Soluble CD40L concentrations in healthy controls, severe and mild pancreatitis patients. Horizontal bars delineate the mean value.

found that CRP at d 2 was the only statistically significant parameter to predict a severe disease ($P<0.001$).

DISCUSSION

Predicting the course of acute pancreatitis remains a challenge for the physician[2]. Moreover, the 48-hour delay necessary to collect the standard scores and the complexity of multifactorial scales have prompted many physicians to investigate new markers[5]. CRP is an acute phase protein produced by the liver during inflammatory conditions that was first described in 1930[3]. In the mid-1980s, several studies showed that CRP could be considered as a valuable prognostic factor for severe pancreatitis[3,5,14]. Indeed, a better understanding of the pathophysiology of acute pancreatitis may contribute to a better selection of a specific prognostic marker. To this end, CD40-CD40L interaction, a major pro-inflammatory system widely distributed on a variety of leukocytes, has been recently studied in experimental rodent pancreatitis[7]. The expression of CD40 and its ligand were detected on pancreatic acinar cell surface, and peripheral T lymphocytes showed a progressive increase in the expression of CD40L during the course of experimental pancreatitis. Taken together, we thought that these findings might be applied to human pancreatitis. In the current report where 40 patients with mild and 40 patients with severe pancreatitis were randomly evaluated, we showed for the first time that the levels of the soluble form of CD40L (sCD40L) were higher in severe than in mild pancreatitis 48 hours after admission. The usefulness of this potential new marker has been compared to CRP, the most widely accepted serum marker that is used on a daily routine in almost all hospitals[3,7] and to Ranson score. We found that the sensitivity of sCD40L to predict a severe course was better than that by CRP. The negative predictive value of sCD40L that allows the physician to really exclude a severe disease was superior than that by CRP. A marker characterized by a better negative predictive allows cost savings since it can avoid useless observation time in emergency room or intensive care unit[15,16]. However, logistic regression analysis found that CRP was the only statistically significant marker able to identify a patient who developed a severe course of the disease. One explanation for this result is that our study was limited by the

small number of patients evaluated. Consequently, correlation between the levels of sCD40L and the occurrence of complications or death for example could not be studied.

Of note, Ranson score appeared insufficiently predictive of severe attacks, a feature already reported in several previous prospective studies[14,17,18]. To predict the severity of pancreatic disease itself, before the occurrence of multiple organ dysfunction, numerous other factors besides CRP have been evaluated so far with various performances. These factors are either enzymes released from the pancreas such as trypsinogen activation peptide[16] or mediators induced by inflammation in the pancreas itself or in remote organs such as procalcitonin[19], adhesion molecules[20] or cytokines[21]. For most of them, the higher the blood concentrations of these enzymes or mediators are, the more severe the disease is. Soluble CD40L could be one of the next markers of pancreatitis severity but further larger studies are needed to validate its clinical use in the early assessment of pancreatitis severity.

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RAPID COMMUNICATION

Association of the HLA-DRB1*0701 allele with perinuclear anti-neutrophil cytoplasmatic antibodies in Mexican patients with severe ulcerative colitis

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Abstract

AIM: To determine the association between the HLA-DRB1 alleles and perinuclear anti-neutrophil cytoplasmatic antibodies (p-ANCA) positive in Mexican patients with ulcerative colitis (UC).

METHODS: Ninety Mexican mestizo patients (45 females) with UC, confirmed by biopsy, were studied. High resolution HLA typing was performed by PCR-SSO reverse dot blot and PCR-SSP. Molecular typing techniques were applied to define HLA-DRB1 alleles. Enzyme-linked immunosorbent assay and immunofluorescence techniques were used to detect p-ANCA.

RESULTS: Forty-eight (53%) UC patients were positive for p-ANCA by ELISA and IF. We found that p-ANCA-positive UC patients had a significantly increased frequency of HLA-DR7 compared with p-ANCA-negative controls (22% *vs* 5.1%; pC = 0.02, OR = 5.2, CI 95%: 1.06-37.82). Disease activity was scored as severe in 20 patients, moderate in 8, mild in 14 and no activity in the remaining 38 patients according to the Truelove and Witts criteria. Subgroup analysis showed a significantly increased frequency of the HLA-DRB1*07 allele in 15 of 20 UC patients with severe activity of UC and p-ANCA positivity [100% *vs* 0%; pC = 0.0000001; OR = 35]. No significant differences were found between p-ANCA positive patients, HLA-DR alleles and other clinical features such as extraintestinal manifestations, proctocolectomy and extension.

CONCLUSION: The HLA-DRB1*07 is associated with p-ANCA positive UC Mexican patients.

Key words: HLA-DR; p-ANCA; Ulcerative colitis; Mexicans

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INTRODUCTION

Perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCA) have been found consistently in patients with ulcerative colitis (UC)[1,2] however, their pathogenic role is still uncertain. The prevalence of p-ANCA in UC varies greatly from 39 to 80 percent in the ethnic group studied[2].

A genetic involvement in UC is supported by observations on familial aggregation of the disorder and concordance in monozygotic twins[3]. Considering the central role of the immune system in mediating the tissue damage in IBD, genes that participate in the development and regulation of the immune response, such as the HLA class II genes, are candidates for conferring the genetic susceptibility. Putative associations of UC with the polymorphic genes of the major histocompatibility complex (MHC) located on the short arm of the chromosome 6 suggest a role of these in the genetic susceptibility to develop UC in several populations of different ethnic and geographic background[4].

UC is a clinical and genetic heterogeneous disorder described in several ethnic groups[5-7]. An association between HLA-DR alleles and UC, in particular p-ANCA positive has been reported in patients from Italy[6], China[8] and United States[9].

Mexican mestizo individuals have a proportion of 56% native American Indian genes, 40% Caucasian genes, and 4% African genes[10], thus they are suitable to study the role of ethnicity in the susceptibility to develop UC and

p-ANCA positive in UC. Mestizo represents a complex mixture of European (Caucasian) and American native inhabitants (mongoloid) genetics, and constitutes the core of the Mexican and the Latin American populations.

The purpose of this study was to determine the association between the HLA-DRB1 alleles and p-ANCA positive in Mexican mestizo patients with UC.

MATERIALS AND METHODS

Patients

Ninety Mexican Mestizo patients with ulcerative colitis confirmed by histology were studied. Details of demographic and clinical characteristics of UC were obtained by a questionnaire, review of records and personal interview. No patient had a family history for UC. Disease extension was defined by colonoscopy. Thus, disease was classified either as extensive colitis (inflammation proximal to the splenic flexure) or distal colitis. Disease activity was scored by Truelove and Witts criteria[11].

Blood samples were obtained from 99 Mexican mestizo healthy, unrelated individuals with no family history of UC, ethnically matched to patients with UC as controls. Mexican mestizo individuals included in the present study accounted for 56% native American Indian genes, 40% white genes and 4% Black genes[11].

Methods

HLA typing Genomic DNA was isolated from peripheral venous blood by a modified "salting out" technique[12], precipitated with ethanol and resuspended in sterile distilled water at a final concentration 0.1-1.0 μg/μL before use.

Generic HLA-DRB1 typing was performed by PCR-SSO reverse dot blot hybridization (Amplicor, Hoffmann La Roche, Basel, Switzerland). High resolution HLA typing was performed by dot blot hybridization of amplified DNA with sequence-specific oligonucleotide probes labeled with digoxigenin di-deoxy-uridine-triphosphate (Dig-11-ddUTP). Information about DRB1 sequence was obtained from the 12th International Histocompatibility Workshop[13].

Indirect Immunofluorescence (IFL) Detection of ANCA was done according to the recommendations of the international workshop[14]. Human neutrophils were isolated from peripheral blood from patients and normal healthy volunteers by gradient centrifugation in 2% methylcellulose solution. The slides were fixed in 98% ethanol at 4℃ for 5 min and dried quickly in air. After wetting with phosphate-buffered saline (PBS) in a dilution of 1:40 and diluted two fold until dilution reached 1:320. Following incubation for 1 h at room temperature, the slides were washed three times with PBS and bound antibodies were incubated and detected with fluorescein isothiocyanate (FITC)-conjugated F(ab') rabbit anti-human IgG, diluted 1:100 in PBS for 30 min at room temperature. Subsequently, the slides were washed three times with PBS and covered with glycerin-phosphate-buffered saline. A titer $\geqslant$1:80 was considered positive, based on the results obtained from healthy controls.

Antigen-specific enzyme-linked immunosorbent assays (ELISA). This method was performed to identify the putative antigens recognized by the p-ANCA positive sera. Human Lf, MPO or cathepsin G was diluted to 2 μg/mL in carbonate buffer (pH 9.6) and 100 μL of each solution was placed in the wells of a 96-well microplate and left for 24 h at 4℃. After the plate was washed three times with PBS, 100 μL of diluted sera (1:100 in PBS containing 0.1 % Tween 20 and 0.1 % skim milk) was added to each well, and the plate was incubated for 1 h at 37℃. After washing, the enzyme reaction was performed, and color development was measured with a microplate colorimeter. Optical density values >2 SD were considered positive[15].

Statistical analysis Gene frequencies were compared using a 2×2 contingency table and Chi square test. Odd ratios have been calculated for the disease in carriers of specific alleles. Comparisons of allele frequencies between subgroups were performed using the EPI-INFO statistical package (Version 5.0; USD incorporated 1990, Stone Mountain, Georgia). All p values quoted were corrected by the Bonferroni test for multiple comparisons, while the number of alleles studied was taken into account. Statistical significance was considered when $P<0.05$.

RESULTS

Clinical features

The average disease duration was 7.2±6.6 years. Extensive colitis was present in 60% and distal colitis in 40%. Disease activity was scored as severe in 20 patients (25%), moderate in 8 (10%), mild in 14 (18%) and no activity in the remaining 38 patients (47%). Extraintestinal manifestations were present in 40%: arthritis (14.6%), primary sclerosing cholangitis (10.4%), sachroiliitis (6%), erythema nodosum (6%), ankylosing spondylitis (1.5%) and aphthous ulceration (1.5%). Fourteen patients (15.5%) required colectomy due to refractory medical therapy.

Positive p-ANCA

Positive p-ANCA was detected by IFL and ELISA methods in 48 of 90 patients with UC (53%) whereas c-ANCA was detected in 4 patients with UC. The frequencies of positive p-ANCA were increased significantly in the patients with UC as compared to controls. Titers of p-ANCA ranged 1:80-1:320.

HLA distribution

Patients with positive p-ANCA showed a significantly increased frequency of the HLA-DRB1*07 as compared to p-ANCA negative patients (pC = 0.02, OR = 5.2, CI 95 %: 1.06-37.82) and healthy controls (pC = 0.04, OR = 2.9, CI 95 %: 1.01-20.56). The remaining HLA-DR alleles did not show statistically differences between UC patients with positive p-ANCA and negative p-ANCA as shown in Table 1.

Subgroup analysis showed a significantly increased frequency of the HLA-DRB1*07 allele in patients with severe activity of UC and positive p-ANCA (15/15) as compared to 5 patients with severe disease and negative p-ANCA and no HLA-DRB1*07 [100 % *vs* 0%; pC = 0.0000001;

Table 1 Gene frequencies (g.f.) of HLA-DR alleles in patients with UC and healthy controls

HLA	p-ANCA + N = 96		p-ANCA – N = 84		Healthy controls N = 198	
	n	g.f.	*n*	g.f.	*n*	g.f.
DR7	21	0.218[1,2]	4	0.047	22	0.111
DR2	16	0.166	13	0.154	14	0.090
DR4	14	0.145	17	0.202	47	0.237
DR1	13	0.135	12	0.142	10	0.050
DR8	10	0.104	11	0.130	33	0.165
DR5	8	0.083	11	0.130	20	0.100
DR6	6	0.062	9	0.107	21	0.109
DR3	5	0.052	5	0.059	11	0.055
DR10	3	0.031	2	0.023	1	0.005

N = Number of chromosomes *n*= number of alleles.
[1]p-ANCA positive *vs* negative: pC = 0.02, OR = 5.2, CI 95%: 1.06-37.82.
[2]p-ANCA positive *vs* controls: pC = 0.04, OR = 2.9, CI 95%: 1.01-20.56.

OR=35]. No significant differences were found between p-ANCA positive patients, HLA-DR alleles and other clinical features such as extraintestinal manifestations, proctocolectomy and extension.

Disease activity

Data about disease activity were collected without knowledge of the results of ANCA testing. Interestingly, a significant association was found between severe activity of the disease and positive p-ANCA [75 % *vs* 25 %; pC = 0.00008; OR = 11; CI 95 %: 4.0-34.0]. No association was found between moderate, mild or inactive disease and the presence of p-ANCA (Table 2).

Extension We found that 22 UC patients with extensive colitis (47.8 %) were positive for p-ANCA while 19 patients with distal colitis were positive for p-ANCA (55.8 %). No statistical association was found between p-ANCA positivity and the extension of the disease [*P* = 0.47; OR = 0.72; CI 95 %: 0.27-1.94].

Proctocolectomy Seven patients with proctocolectomy were positive for p-ANCA as compared to 34 patients (51.5 %) who did not undergo the surgical procedure and were p-ANCA positive. No association was found between both groups [*P* = 0.91; OR = 0.94; CI 95 %: 0.26-3.43].

EIMs Futhermore, association was no found between each of the extraintestinal manifestations such as arthritis, primary sclerosing cholangitis, sachroiliitis, erythema nodosum, ankylosing spondilytis or aphthous ulceration and the presence of p-ANCA.

Type of treatment We followed up 80 patients treated with corticosteroids (local and systemic), 5-ASA (local and systemic) and azatioprin, and 10 untreated patients who served as controls. No association was found between p-ANCA positivity and any kind of medical treatment.

The prevalence of p-ANCA was higher in treated patients (45 %) than in untreated patients (6 %), probably reflecting the greater severity of the cases that required surgical treatment. After stratifying patients according to p-ANCA status, prevalence rates were not different as far as age, gender, age at first diagnosis, disease extension, surgery, extraintestinal manifestations were concerned.

No association was found between the number of clinical relapses and p-ANCA positive determination (data not shown).

Table 2 Clinical features of UC patients with positive and negative p-ANCA

Clinical feature	p-ANCA + *n* = 48	p-ANCA - *n* = 42	*P* value
Age at diagnosis (yr)	31 ± 8	33 ± 7	0.34
Gender (F/M)	23/25	22/20	0.62
Disease duration (yr)	7.4 ± 6.3	6.8 ± 6.2	0.45
Clinical relapses	2 ± 1	3 ± 2	0.25
Medical Treatment			
5-ASA	90%	85%	0.75
Corticosteroids	44%	45%	0.90
Azatioprine or 6-MP	24%	22%	0.82
Extensive colitis	29	27	0.47
Distal colitis	19	15	
Colectomy	7	7	0.94
No Colectomy	41	35	
EIMs present	21	25	0.67
EIMs absent	27	17	
Disease activity			
Absent	19	21	0.82
Mild	9	10	0.71
Moderate	5	6	0.65
Severe	15	5	0.000008

[a]OR = 11; CI 95%:4.0-34.0.
n = Number of patients.

DISCUSSION

This study provides evidence of the important role of MHC genes class II in the development of autoantibodies in patients with ulcerative colitis. The relevant finding was the increased frequency of the HLA-DRB1*07 allele in UC patients with positive p-ANCA compared with UC patients with negative p-ANCA. This association has not been reported in other ethnic groups. Several studies on Caucasians have demonstrated the association between the HLA-DR2 and the presence of p-ANCA positive UC patients from United States[16], Germany[17] and Italy[18]. In Chinese patients, a strong association is found between the HLA-DQ alpha 1c allele and the presence of ANCA positive[8].

On the other hand, other studies have not found association of the presence of positive p-ANCA and HLA-DR alleles in Jewish[2], French[18] and Korean[19] UC patients. Previous Mexican studies have reported association between HLA-DR1 and polymorphisms in the promoter region of tumor necrosis factor alpha and the genetic susceptibility to develop UC in this population[7,20].

The HLA-DRB1*07 has not been reported to have an association with positive p-ANCA in patients with UC from other ethnic groups. Interestingly, this allele is associated with chlorpromazine-induced lupus anticoagulant in patients with chronic psychiatric disorders[21]. We also found that the HLA-DR7 was associated with the production of anti-phospholipid antibodies in a group of Mexican mestizo patients with systemic lupus erythematosus[22]. The differences in the associated alleles could also be explained as a result in the genetic variation due to the role

of ethnicity in certain groups.

Finally, the association of the HLA-DRB1*07 allele with p-ANCA may be a result of immunoregulatory mechanisms related to the antigen presentation of protein fragments (autoantigens) to T cells, and may also play an important role in the development of autoimmunity, including the production of several autoantibodies such as lupus anticoagulant and anti-phospholipid antibodies as mentioned above.

This novel association between HLA-DRB1*07 allele and positive p-ANCA in Mexican UC patients suggests that this allele could be a marker of severe activity in patients with UC and plays a role as genetic marker for the production of different kind of autoantibodies in our population.

In conclusion, the HLA-DRB1*07 allele plays a genetic role in the production of p-ANCA in Mexican mestizo patients with ulcerative colitis.

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RAPID COMMUNICATION

Elevated plasma cryofibrinogen in patients with active inflammatory bowel disease is morbigenous

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Abstract

AIM: To investigate the role of cryofibrinogen (CF) in active inflammatory bowel disease (IBD).

METHODS: CF was assayed in 284 subjects: 61 with active and 63 with inactive ulcerative colitis (UC), 45 who had proctocolectomy, 35 with active and 20 with inactive Crohn's disease (CD), 40 with other diseases and 20 healthy controls. Trypsin inhibitor (TI) and TI antibody (TI-Ab) were measured in plasma and CF complex by ELISA.

RESULTS: CF in active UC was strikingly high compared with all other groups (χ^2<0.001). Similarly, CF was significantly higher in active CD than in inactive CD or in controls (χ^2<0.01). In UC, high CF and TI-Ab were associated with the need for operations. Further, high CF, CF/fibrinogen ratio, low TI and high TI-Ab in plasma were associated with disease activity or refractoriness to medication. Elevated CF was not associated with acute reactants like C-reactive protein and white blood cell counts except for erythrocyte sedimentation rate, suggesting that elevated CF was not a consequence of acute inflammation.

CONCLUSION: Elevated CF in active IBD appears to be morbigenous. CF promotes IBD via two main mechanisms, quenching of TI (an anti-inflammatory substance) and impairing microvascular perfusion by forming protein aggregates. CF may also serve as a biomarker of chronic IBD. Additional studies are warranted to fully evaluate the role of CF in IBD and the outcome should contribute to a better understanding of the pathogenesis of IBD.

Key words: Inflammatory bowel disease; Cryofibrinogen; Trypsin inhibitor; Trypsin inhibitor antibody; Acute colitis

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INTRODUCTION

In 1955, Korst and Kratochivil[1] for the first time described "cryofibrinogen (CF)" as an abnormal protein, which is reversibly cold-precipitable in anticoagulated blood and could form fibrin and clot with thrombin. Now, CF is known as a cold-precipitable protein complex composed of fibrin, fibrinogen and fibrin split products found in plasma but not in serum of some individuals[2-4]. Further, cryofibrinogenaemia is now suspected to be associated with acute and chronic inflammatory diseases, lymphoproliferative disorders, and vascular complications[2-8]. However, it is true to say that up to now, CF has been best known as a risk factor for thrombo-embolic complications[2-9].

Regarding inflammatory bowel disease (IBD) and CF, several independent risk factors for thrombotic vascular diseases have recently been reported in IBD[9-13]. For example, it is known[11] that granulomatous vasculitis and fibrin deposition are components of the inflammatory process in Crohn's disease (CD). Similarly, presence of micro-thrombi in rectal biopsy specimens from patients with either ulcerative colitis (UC) or CD has been reported[12]; the incidence of systemic thrombo-embolic events in patients with IBD is known to be higher than that in the general population[13], reflecting a primary coagulation abnormality in patients with IBD[9-13].

Another biochemical substance, which was investigated in this study in relation to IBD, is trypsin inhibitor (TI). TI is best known for inhibiting trypsin to spare bystander tissues from unwanted damage[14]. Additionally, the literature provides convincing evidence for an anti-inflammatory action by TI as one of the body's naturally occurring immunoregulators[14-16] that include suppression of TNF generation[7]. In view of the above background, in

this study we hoped that finding the relationships between IBD, CF and TI might contribute to the understanding of the pathogenesis of IBD.

MATERIALS AND METHODS

Subjects

Between 1994 and 2001, a total of 284 subjects were investigated in this study. The demography of the study populations is presented in Table 1. There were 169 patients with UC (61 with active UC, 63 with remitted UC and 45 had proctocolectomy), 55 with CD (35 with active CD and 20 with remitted CD), 40 disease controls and 20 healthy controls.

Determination of disease severity and ongoing medications

Patients with active UC had bloody diarrhoea more than 6 times/d, a score of at least 14 on Lichtiger's clinical activity index (CAI)[17] and colonoscopy revealed Matts' s endoscopic grade 3[18]. Patients with active UC received total parenteral nutrition (all had severe UC) together with 5-aminosalicylic acid (5-ASA) or sulphasalazine orally and intravenous (iv) prednisolone (30-80mg/d) except for 5 patients who had their first UC episode before starting drug therapy. The remaining 45 patients with UC who had proctocolectomy with ileal pouch anal anastomosis (IPAA) at least 6 mo prior to this investigation represented the post-operative UC group. None of the 55 patients with CD received steroid therapy. Patients with active CD ($n=35$) had a mean Crohn's disease activity index (CDAI) of 266[19]. The subgroup with inactive CD ($n=20$) had a CDAI of less than 150 (clinical remission level). Forty age and sex matched hospital patients with other diseases were included as a disease control group (Table 1). Of these, 10 had non-IBD acute colitis caused by bacteria [enteropathogenic *Escherichia coli*, Vero toxin (-) in 6, *Salmonella enteritidis* in 3, and *Klebsiella peumoniae* in one], 4 with rheumatoid arthritis, 4 with primary biliary cirrhosis, 2 with autoimmune hepatitis, 2 with Bechet's disease, 2 with systemic lupus erythematosus, and 1 with polymyositis in autoimmune and collagen diseases, 4 with colonic polyps, 3 with chronic hepatitis C, 3 with hypercholesterolemia, 3 with peptic ulcers who were also *Helicobacter pylori* positive, and 2 with gastric cancer in non-acute and non-autoimmune state. Twenty healthy subjects who were in the same age range as patients were included as a healthy control group.

Measurement of fibrinogen and cryofibrinogen

Blood samples were obtained in the early morning fasting state. A 10 mL of whole blood was collected for cryoprecipitate measurements. The blood sample was pre-warmed at 37 ℃ in a plastic syringe and then was transferred into 2 plain glass tubes at 37 ℃; one tube contained 3.8% sodium citrate solution (9:1; vol/vol blood/citrate ratio), the other tube contained no anti-coagulant.[20] The tubes were incubated at 37 ℃ for 1 h and then were centrifuged at 1500g for 30 min to separate plasma or serum from cells.[21] Fibrinogen was measured by immunoturbidimetric assay according to a published method.[22] Total fibrinogen concentration in the plasma was measured before refrigeration. Plasma and serum samples were kept at 4 ℃ for up to 72h prior to assay of test substances. This was to allow precipitation of any abnormal fibrinogen into CF. Tubes were further spun at 1500g in a refrigerated centrifuge at 4 ℃ for 30 min. The supernatants were then rewarmed to 37 ℃ and used to measure fibrinogen and CF concentrations. The concentration of CF was measured as concentration differentials of fibrinogen before and after refrigeration.[22,23] The fibrinogen concentration was measured at 24 and 72 h after refrigeration. Sera samples were used for the detection and measurement of cryoglobulins (if present).

Assays of trypsin inhibitor and trypsin antibody

Trypsin inhibitor and trypsin inhibitor antibody (TI-Ab) in plasma and in CF[14] were measured by enzyme-linked immunosorbent assay (ELISA) in 18 patients with IBD (10 with UC, 8 with CD) as previously described.[24] These subjects were selected at random among the patients.

Ethics

All participants provided informed consent to be included in this study after they were informed of the purpose of the study and the nature of the procedures involved. Likewise the study protocol was reviewed and approved by our hospital Committee on the Ethics of Clinical Investigations Involving Humans.

Statistical analysis

Numerical data were presented as the mean ± SD values. Comparisons were made with the Chi square test (χ^2). Correlations between CF, TI and acute phase reactants including C-reactive protein (CRP), white blood cell counts (WBC), and erythrocyte sedimentation rate (ESR) were assessed by simple regression analysis or as indicated otherwise. $P<0.05$ was considered statistically significant.

RESULTS

Incidence and concentration of CF among groups

There was no significant difference in CF concentrations between 24 and 72h refrigeration. Therefore, only the results of 72h refrigeration were presented. A very high incidence of CF (upon storage of plasma at 4 ℃) and high CF concentrations were observed in patients with active IBD (Table 1 and Figure 1). This was most striking in patients with active UC (Figure 1). However, patients with UC who had operations or patients with non-IBD acute colitis (associated with infection) did not have abnormally high CF levels. Indeed, amongst the patients groups, the lowest CF concentration was found in patients with acute colitis; none of the 10 cases was CF positive. Thus in patients with UC, CF was still positive in 55.6% of patients with remitted UC, while patients with active CD had high CF levels, but the level in patients with inactive CD was not high (Figure 1). The only other group with high CF was patients with collagen disease, which is consistent with the literature[25]. In the disease control group, only 40% (12 of 40 patients) were positive for CF. In the healthy control group, only 1, (a 50-year-old man) was positive for CF, but the concentration of CF was not very high in this

Table 1 Plasma cryofibrinogen (CFG), fibrinogen levels and CFG/fibrinogen ratio in 284 subjects who participated in this study. When appropriate, the mean ± SD values are presented

Demography	UC			CD		Disease control			Healthy control
	UCA (n = 61)	UCR (n = 63)	UCPost-OP (n = 45)	CDA (n = 35)	CDR (n = 20)	Acute colitis (n = 10)	Collagen disease (n = 15)	Other diseases (n = 15)	(n = 20)
Age(yr)	35±19	33±12	36±17	32±11	27±11	34±11	38±12	33±16	34±18
Gender(M/F)	30/31	31/32	23/22	19/16	12/8	6/4	8/7	6/9	10/10
CFG Positive(%)	87.8[1a]	55.6[2]	33.3[3]	65.7[2b]	35.0[3]	0.0	66.7[2b]	13.3[3]	5.0
Fibrinogen (mg/dL)	318±109	240±96	303±66	287±137	251±66	254±55	384±107	206±96	246±59
CFG/fibrinogen ratio(%)	18±5[1a]	17±7[1a]	4±3	9±4[2]	4±3	0	7±4[2]	6±3[2]	2.5±0.7

UC: ulcerative colitis; UCA: active UC; UCR: remitted UC; CD: Crohn's disease; CDA: active CD; CDR: remitted CD; M: male; F: female; CFG positive (%): percentage of patients in whom cryofibrinogen was detected upon storage of plasma at 4℃, [1]χ^2<0.001 for active UC *vs* healthy control or other disease control; [2]χ^2<0.01 UCR and CDA *vs* healthy control or other disease control; [3]χ^2<0.05 UC Post OP, CDR *vs* healthy control or other disease; [a]χ^2<0.05 for UCA or UCR *vs* UCPost0OP, CDR, or other disease; [b]χ^2<0.05 for CDA, Collsgrn disease *vs* UCPost-OP, CDR, or other disease control.

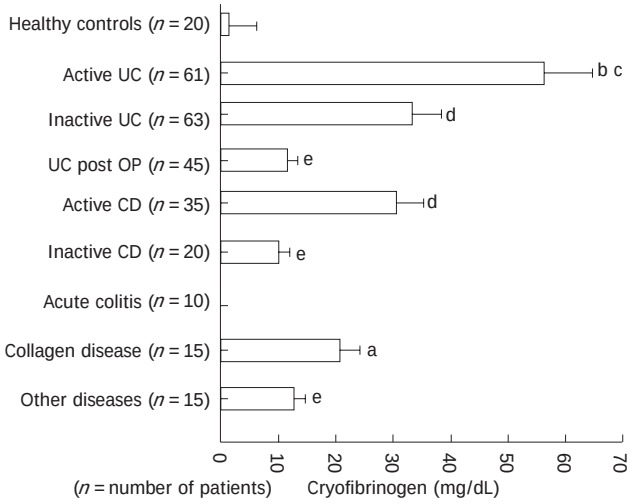

Figure 1 Cryofibrinogen (CF) concentration in subgroups of 284 patients. [b]*P*<0.001 for active UC *vs* healthy control or UC post OP, inactive CD, acute colitis, and other disease control; [d]*P*< 0.01 for inactive UC and active CD *vs* healthy control, UC post OP, inactive CD, acute colitis, and other disease control; [a]*P*<0.05 for collagen disease *vs* healthy control or UC post OP, inactive CD, acute colitis; [c]*P*<0.05 for active UC *vs* inactive UC and active CD; [e]*P*<0.05 for UC post OP, inactive CD, acute colitis, and other disease control *vs* healthy control.

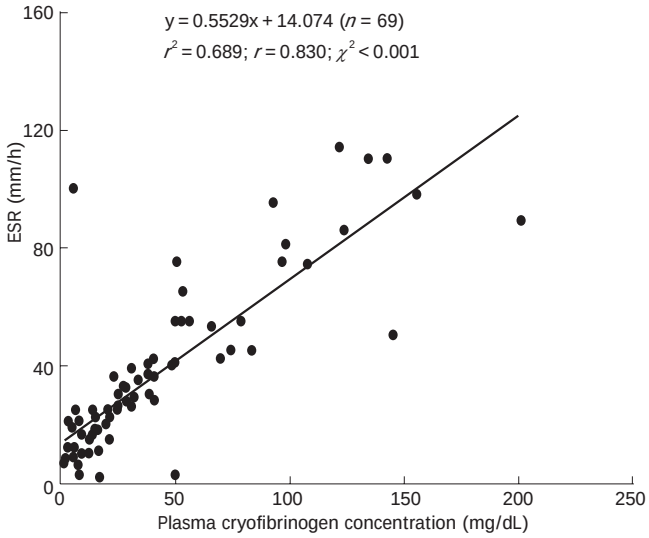

Figure 2 Simple regression analysis for relationship between plasma CF concentration and erythrocyte sedimentation rate (ESR) in 69 patients with active IBD.

patient (15.6 mg/dL). The CF/fibrinogen ratio was also determined for all subgroups of 284 patients. The results are presented in the Table 1. No cryoglobulinaemia was detected in IBD patients or in the healthy control group.

Relationship between CF and acute phase markers

To verify that high plasma CF was not associated with acute inflammation, its concentration was compared with concentrations of acute phase reactants like CRP, ESR, and WBC. Among these, only ESR showed a strong correlation with CF. (r=0.830, χ^2<0.001, Figure 2). There were no significant correlation between CF and CRP or between CF and WBC.

Relationship between CF and IBD clinical course

The relation between CF concentration, severity and duration of IBD were investigated. We divided the patients into 4 subgroups: (1) with more than 50 mg/dL CF; (2) with 10 to 50 mg/dL CF, (3) with less than 10 mg/dL CF; (4) with no CF. Both CAI/CDAI values and the duration of IBD were in this order: 1>2>3 or 4. We followed 44 patients who achieved remission among 61 with active UC and measured CF concentration to see if it follows patients' UC clinical course. Seven patients who became CF negative maintained their remission for over 5 years. In 33 patients in whom, CF concentration decreased by about 50%, 13 relapsed within 12 mo in addition to 4 patients who did not show any change in CF concentration.

Relationship between TI-ab and CF

It is thought that in the plasma, part of the CF complex acts as TI-ab and at 37℃ (the measurement temperature used in this study) and TI-ab dissociates from CF. Accordingly, TI-ab shown in both Figures 3 and 4 are believed to come from the CF complex. This means that a high plasma level of CF can reduce plasma levels of free TI. Figure 4 shows a simple regression analysis for the relationship between plasma CF and TI-ab in patients with

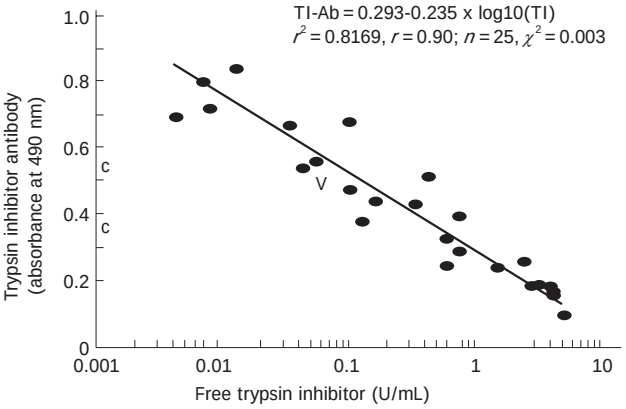

Figure 3 Logistic regression analysis to determine the relationship between TI and anti-TI-ab in patients with IBD.

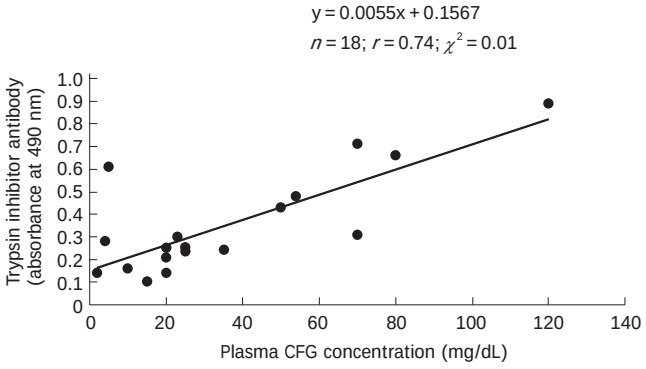

Figure 4 Simple regression analysis for the relationship between plasma CF concentration and TI-ab in 18 patients with active IBD.

active inflammatory bowel disease. As shown, there was a positive correlation between TI-ab and CF in the plasma (r= 0.74, χ^2 = 01).

DISCUSSION

For the first time this study reports the elevated plasma CF in patients with active IBD, with active UC showing the highest plasma CF. We also found that in most patients with active IBD, a marked fall in plasma CF was associated with disease remission. In contrast, persistently elevated CF was associated with medication-resistant and poor prognosis. Further, we could confirm that elevated plasma CF was not associated with typical acute phase reactants like CRP or WBC except showing strong correlation with the inflammatory marker, ESR. To ascertain that high plasma CF was one manifestation of active IBD, we included patient groups with diverse diseases together with a control group. Even in patients with acute colitis of non-IBD type, the concentration of CF was markedly lower compared with active UC of IBD. This observation convinced us that high plasma CF was indeed specific to active IBD. Further, elevated plasma CF was associated with IBD severity. Our impression is that elevated plasma CF is morbigenous; patients have severe and drug-refractory IBD.

Given that high plasma CF is seen during active IBD, and increases with IBD severity, the fundamental question if unequivocally addressed could significantly improve our understanding of the role of CF in IBD and the aetiology of IBD. First, is CF one cause or a consequence of active IBD? Second, exactly how CF contributes to active IBD (if at all)? At present, our data suggest that CF quenches TI thus depriving the immune system of an important natural anti-inflammatory factor (see the introduction section). But we should not forget that CF by precipitating cryoprotein aggregates is a major risk factor for vascular occlusion and impaired microvascular perfusion, which in IBD can delay ulcer healing.[3-8, 20, 25, 26] Additionally, high plasma concentration of CF can activate platelets in the same way as high plasma fibrinogen does[24] and block small vessels with micro-thrombi whereby compromising microcirculatory blood flow within the inflamed intestinal mucosa. Therefore, one mechanism of action of CF and its contribution to tissue injury and the perpetuation of IBD might be via vascular occlusion.

There are reports that treatment with immunosuppressive agents including corticosteroids, azathioprine, and cyclophosphamide is unsuccessful in preventing disease progression (non-IBD)[25-27]. However, the introduction of regular plasmapheresis together with cyclophosphamide therapy depletes plasma CF and this is associated with a marked improvement in the patients' clinical symptoms, Suggesting that extracorporeal circulation therapies such as plasma exchange and plasmapheresis, or cryofiltration[28-30] combined with conventional drug therapy may be effective in patients with refractory UC or CD who present with high plasma concentrations of CF, which we believe is a morbigenous factor in IBD.

Excess CF probably arises either from a misdirected synthesis or from post-synthetic alterations.[25,30,31] In IBD, it is likely that abnormal fibrinogen (CF) reflects a congenital abnormal production or an acquired abnormal production by the liver that is exposed to high cytokinaemia and proteases through portal vein from the intestine. If the elevated CF is caused by pro-inflammatory cytokines and proteases then its concentration in the inflamed intestinal lesions will be much higher compared with plasma.

In conclusion, high plasma CF in patients with active IBD appears to be morbigenous. The elevated plasma CF does not seem to be associated with typical acute phase reactants like CRP and WBC but showing strong correlation with ESR. Currently, our impression is that elevated CF promotes IBD via two main mechanisms quenching of TI (an anti-inflammatory substance) and impaired microvascular perfusion by forming protein aggregates. Further, CF may also serve as a new biomarker of chronic IBD. Clearly additional studies are warranted to fully evaluate the role of CF in IBD and the outcome should contribute to a better understanding of IBD aetiology.

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RAPID COMMUNICATION

Using p53-immunostained large specimens to determine the distal intramural spread margin of rectal cancer

Zhi-Zhong Pan, De-Sen Wan, Chang-Qing Zhang, Jian-Yong Shao, Li-Ren Li, Gong Chen, Zhi-Wei Zhou, Fu-Long Wang

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Abstract

AIM: To determine the distal intramural spread (DIS) margin of rectal cancer.

METHODS: Sixty-one p53-positive specimens of rectal cancer were used. After conventional hematoxylin and eosin (H&E) staining, the DIS margin of rectal cancer in large specimens was examined by immunohistochemistry. The patients were divided into A, B, C, and D groups. After a long-term follow-up, the survival curves of the four groups were estimated using the life table.

RESULTS: Fifty-one of the sixty-one cases (83.6%) had DIS. The extent of DIS ranged 0.11-3.5 cm; meanwhile the mean of DIS measured by H&E staining was 0.13 cm. The significant difference was found between the means (t=5.622, P<0.0001). Only 1 of 51 patients had DIS greater than 3 cm. The DIS was less than 1.0 cm in most rectal cancer patients. The long-term results indicated that the survival rate of the patients whose DIS was greater than 1.0 cm was lower than that of the patients whose DIS was less than 0.5 cm.

CONCLUSION: Rectal cancer patients with DIS greater than 1.0 cm have poor prognosis.

Key words: Rectal cancer; Distal intramural spread; p53; Immunohistochemistry; Large specimen

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INTRODUCTION

Defining the optimal distal surgical margin is very important for surgical oncologists during rectal cancer resection[1]. To ensure complete excision of cancer and maximum protection of normal tissue, we should define the length of DIS. Though a distal margin greater than 5 cm is advocated in the past[2], some studies proposed that a distal excision margin of 1 cm is sufficient[3-5], but the molecular clearance margin of DIS is unknown. Molecular techniques have been used to identify tumor markers and occult tumor cells in recent years[6] and the clear margin determined by molecular methods should be different from that by traditional histopathological methods. The accurate assessment of DIS could guide precise surgical resection, aid in estimating prognosis and predict recurrence site, but the molecular margin of DIS of rectal cancer is unknown. In this study, we used p53-immunostained large specimens of rectal cancer to measure the molecular margin of DIS and to clarify the influence of DIS margin on the long-term survival of patients after the resection of rectal cancer.

MATERIALS AND METHODS

Patients

By using conventional hematoxylin-eosin (H&E) and p53-immunostaining (LSAB), we identified 61 p53-positive cases in 97 rectal cancer patients who were surgically treated in our hospital between August 1996 and October 1997. The characteristics of the 61 patients are listed in Table 1.

Preparation of large specimens

Fresh specimens were opened longitudinally, straightened without stretching and pinned to a cork board. The distal margin (A1) was measured and fixed by 10% formalin for more than a week. The whole specimen including oral and anal edges of the tumor was cut longitudinally into 5-mm-thick giant sections. The sections were embedded in paraffin wax and then serial 8-μm-thick sections were cut from the giant sections on a large microtome. Routine H&E staining and p53-immunostaining (LSAB, 1:100 diluted mouse monoclonal antibody to p53, DO-7; DAKO Ltd, Denmark) were performed respectively. The distal margin was measured on large glass specimens (B1) and the extent of DIS was measured microscopically (b1). The microscopic extent of DIS was converted as the distance

Table 1 Clinicopathological characteristics of rectal cancer patients studied

Male	33
Female	28
Age (yr)	54 (22–79)
Gross morphology	
Fungating	14
Ulcerative	46
Infiltrative	1
Histologic type	
Papillary adenocarcinoma	8
Tubular adenocarcinoma	52
Mucinous adenocarcinoma	1
Histologic grade	
Well differentiated	8
Moderately differentiated	39
Poorly differentiated	14
Dukes' stage	
A	19
B	16
C	22
D	4

Table 2 Comparison of p53 immunostaining (IHC) and H&E staining in measuring the mean of DIS extents

DIS	IHC	H&E
0 cm	10	32
0.01 – 0.50 cm	26	26
0.51 – 1.00 cm	14	2
>1.00 cm	11	1
x	0.59 cm	0.13 cm
t	5.622	
P	<0.0001	

Table 3 Survival curves of groups A, B, C, and D

Group	Gehan value	*P*
A-B	1.204	0.273
A-C	1.685	0.194
A-D	5.359	0.021
B-C	0.814	0.367
B-D	4.627	0.032
C-D	1.124	0.289

of DIS in fresh specimen (a1) by the tissue shrinkage ratio by comparing the distal margin measured in fresh specimens before fixation (A1) to that measured on large sections macroscopically (B1) in each case (A1/B1=a1/b1). The data were converted data.

Statistical analysis

Both means of the DIS obtained by H&E staining and p53-immunostaining were compared by the paired sample statistic *t* test. According to the extent of DIS by p53-immunostaining, we divided the patients into four groups: A (DIS = 0.00 cm), B (DIS = 0.01-0.50 cm), C (DIS = 0.51-1.00 cm) and D (DIS > 1.00 cm) .The survival curves of the groups were generated by the life table and compared by Gehan test. SPSS software (8.0) was used for all statistical analyses. $P < 0.05$ was considered statistically significant.

RESULTS

Modalities of DIS in rectal cancer

Under microscope, no cancer cells were found on the edge of resection in the large specimens. By p53 immunostaining, DIS of p53-positive tumor cells was found in normal glandular epithelial tissue next to the tumor in 23 cases under the mucosa away from the tumor in 40 cases. Emboli of cancer cells were found in micro-veins and/or micro-lymph vasculature under the mucosa in 35 cases. Nest formation of cancer cells was found under the mucosa away from the tumor and a clear line between the tumor and the normal tissue was noted in 21 cases. Spread of cancer cells through the above four pathways was observed but there was a clear large space between the metastatic cells and the main tumor.

Comparison between the extents of DIS by H&E staining and p53 immunostaining

By H&E staining, no DIS was found in 32 cases, 29 cases had DIS (range: 0.10-1.39 cm, mean: 0.13 cm, 95%CI: 0.1-0.16 cm). By p53 immunostaining, only 10 cases had no DIS, 51 cases had DIS (range: 0.11-3.5 cm, mean: 0.59 cm, 95%CI: 0.5-0.67 cm). There was a significant difference between the means obtained by p53-immunostaining and H&E staining ($t = 5.622$, $P < 0.0001$, Table 2).

Follow-up results

By December 2003, the follow-up time ranged 266-2 485 d with a mean of 1 621.4 d. All cases were followed up. Twenty-nine patients died of cancer recurrence. There were 32 disease-free cases in the study.

Survival analysis

The 5-year survival rate of 61 patients was 57.78%. The 5-year survival rate of groups A, B, C, and D was 79.41%, 60.93%, 55.70%, and 31.88%, respectively. There was no statistical difference among the survival rates of groups A, B, and C. The survival rate of group D was significantly lower than that of groups A and B (Table 3 and Figure 1).

DISCUSSION

Different from other studies, the whole specimen was expanded with a similar pull force by surgeons during operation and pinned on a flat board. Then the specimen was fixed by 10% formalin for more than 7 d. The extent of DIS was measured on large specimens microscopically. A tissue shrinkage ratio between the distance measured in fresh specimens from surgical margin to distal tumor edge was used to convert the extent of DIS microscopically to the extent of DIS *in situ*[7]. The error of DIS measured microscopically caused by the shrinkage of tissue in formalin may be avoided by the above method. Moreover, missed diagnosis of DIS should be reduced because the continuous intramural spread can be observed on large specimens.

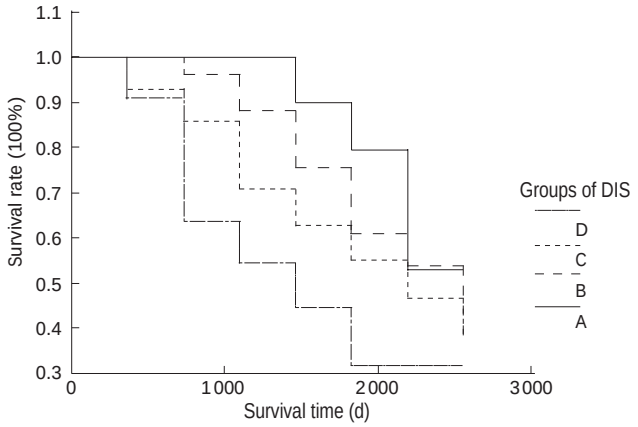

Figure 1 Survival curves of 61 rectal cancer patients.

Mutation of p53 gene is a late event of colorectal carcinogenesis[8]. Using molecular biological techniques, Brennan *et al*[9] and Hayashi *et al*[10] assessed the p53 gene mutation in clearance margins of 25 primary squamous cell carcinomas of the head and neck and local lymph nodes of 120 colorectal cancers. p53 gene mutation was found in some specimens without tumor residue, 40-70% patients with p53 gene mutation in clearance margins or lymph nodes had local recurrence and regional lymph node metastasis after the operation. No metastasis occurred in patients without p53 gene mutation. Their studies indicate that molecular biological analysis is more sensitive in finding hidden tumor cells by identifying tumor markers and can predict the site of local recurrence and the prognosis of patients.

Mutation of p53 gene results in the accumulation of p53 protein and p53 protein is regarded as a useful tumor marker[11]. p53-positive tumor cells that cannot be detected by H&E staining can be identified by immunohistochemical staining. Furthermore, hidden tumor cells usually are too small to be found by microscopy after conventional H&E staining.

In our study, we compared the extent of DIS by p53-immunostaining to that by H&E staining. The results between these two methods were similar, when DIS was less than 0.5 cm. In some cases, when DIS was greater than 0.5 cm, spread of single satellite cells in normal tissue was difficult to be identified by H&E staining. Therefore, H&E staining tends to cause false negative results compared to immune method. Because immunostaining is more sensitive than H&E staining, more positive diagnoses were made. The mean of DIS results was also higher by immunostaining ($P<0.05$, Table 2.).

Accurate identification of DIS of rectal cancer is very important for determining the clearance margin during the operation and preventing anastomosis recurrence[12]. According to the literatures published, DIS detected by H&E staining usually exists in 14.5-65.6% rectal cancer patients[3,7,8]. Because the results of DIS in rectal cancer are different in different studies[13], there is no universal agreement on the length of distal normal rectum that should be removed during radical resection[14]. Some authors suggested that 1-2 cm of distal removal of normal rectum is enough[4]. On the other hand, some cellular and molecular studies suggested that 2 cm is not safe[9]. In our study, though 83.6% (51/61) of the patients had DIS, the length of DIS in 78.4% (40/51) was less than 1 cm, in 6% (3/51) greater than 2 cm and only in 1.6% (1/51) greater than 3 cm, suggesting that 2 or 3 cm of the distal clearance margin is safe enough for most (95% or above) rectal cancers during radical resection.

It was reported that DIS is correlated to depth of cancer invasion and distal metastasis. Patients with positive DIS have a higher risk for developing metastasis after radical resection and their disease-free survival rate is lower than that of those with negative DIS[10]. In our study, patients with longer DIS had a lower survival rate (Figure 1). If DIS is greater than 1 cm, patient survival rate is markedly decreased regardless of distal normal rectum excision and negative tumor residue in distal surgical edge. The relationship between DIS and survival rate indicates that DIS is a prognostic factor of rectal cancer[15].

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CASE REPORT

Duodenal duplication cyst causing severe pancreatitis: Imaging findings and pathological correlation

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Abstract

We here report a case of a 18-year-old man with a history of recurrent abdominal pain and a previous episode of severe acute pancreatitis. Abdominal ultrasonography, contrast enhanced multislice computer tomography, endoscopic retrograde cholangiopancreatography, endoscopic ultrasonography and magnetic resonance imaging demonstrated a cystic mass lesion. Only on delayed phase magnetic resonance images after Gadolinium-BOPTA injection, it was possible to demonstrate the lesion's relationship with the biliary tree, differentiating the lesion from intraluminal duodenal diverticulum, and to achieve the diagnosis of duodenal duplication cyst, a recognized rare cause of acute pancreatitis. The diagnosis was confirmed by histology.

Key words: Pancreatitis; Congenital anomalies; Duodenal duplication cyst; Ultrasonography; Computed Tomography; Cholangiopancreatography; Magnetic Resonance Imaging

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INTRODUCTION

Duodenal duplication cyst (DDC) is a benign congenital anomaly acquired during the digestive system embryonic development. It is a recognized rare cause of acute pancreatitis, usually diagnosed in the first two infancy years. In adults, the diagnosis is difficult because of symptom variety and non-specific nature[1,2]. DDC represents 5-12 % of all gastrointestinal tract duplications and often communicates with either the small bowel or the pancreatic duct, rarely with the biliary system. Duplication anomalies are usually adjacent to the involved bowel. The morphology is spherical or occasionally tubular and may communicate with the lumen[1]. Moreover, they are composed of a smooth muscle wall and an inner mucosal lining. The type III choledochocele by Todani *et al*[3] is an isolated cystic dilatation of the distal portion of the choledochus, eventually protruding into the duodenal lumen, whose imaging findings are similar to those of duodenal duplication communicating with the bile duct[4,5]. The differential diagnosis between these two entities is often preoperatively impossible. Once the lesion is excised, only the different inner mucosa permits to perform a diagnosis. According to the magnetic resonance cholangiopancreatography (MRCP) findings, we report a case for which we suggest two possible final diagnoses: duodenal duplication cyst or type III choledochocele both communicating with the bile duct. In both computer tomography (CT) and endoscopic ultrasonography (EUS), previously performed, the relationship between the cystic lesion and the biliary tree was not clear. As far as we know, this is the first case where magnetic resonance (MR) with Gd-BOPTA (a contrast medium partially excreted by the biliary system) is able to demonstrate the relationship between the cystic lesion and the bile duct, which presents an anomalous pancreatic-biliary junction.

CASE REPORT

A 18-year-old man was referred to emergency room for an episode of severe acute pancreatitis. The patient was already known for previous recurrent abdominal pain. Laboratory values indicated an acute pancreatitis (increase of serum amylase and lipase activity). Other laboratory results including liver enzymes and peripheral blood count, serum protein and creatinine concentration were within the reference ranges. The patient did not have any primary cause of pancreatitis (e.g. alcohol consumption or choledocholithiasis). Ultrasonography (US) showed a hypoechoic area at the head of the pancreas, finding compatible with focal necrosis in pancreatitis. Contrast-

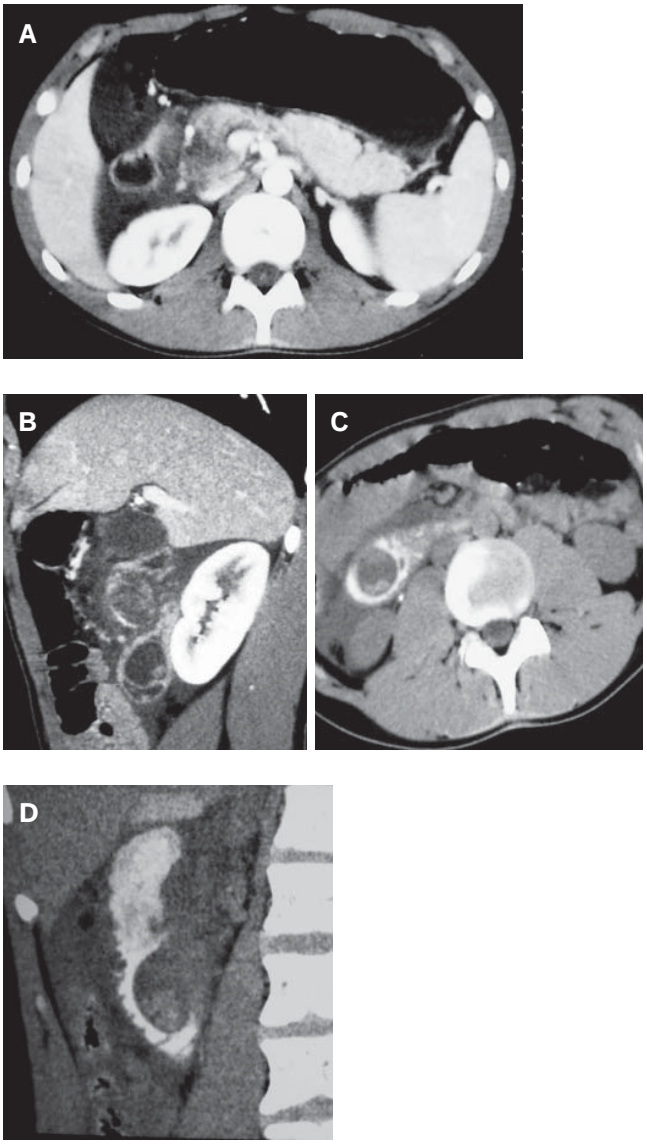

Figure 1 MSCT findings. **A** and **B**: On axial plane after i.v. injection of contrast medium the head of the pancreas presents a necrosis area, and on oblique plane the duodenum shows a cystic lesion filled with small stones; **C** and **D**: Oral contrast medium (diluted Gastrografin) fails to opacify the cystic lesion.

enhanced multislice CT (16 rows) confirmed the presence of necrotizing pancreatitis at the head (Figure 1A) and revealed a cystic lesion containing filling defects within the third portion of the duodenum (Figure 1B). After oral contrast agent (Gastrografin) administration, the cystic lesion was not filled up, suggesting the diagnosis of DDC (Figures 1C and D). The upper gastrointestinal series showed a four-centimeter intraluminal defect located in the medial wall between the second and the third portions of the duodenum. Endoscopic retrograde cholangiopancreatography (ERCP) was impossible to perform because the large submucosal mass was close to the major papilla, obstructing the bile ostium and pancreatic duct (Figure 2A).

EUS confirmed the presence of a submucosal cystic lesion at the inferior duodenal genu filled by calculi (Figure 2B), which was peculiar of choledochocele. Because of the site (duodenum medial wall) and the large size of the cystic

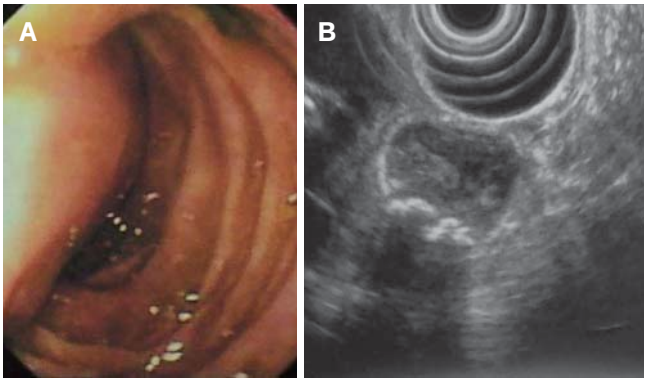

Figure 2 Endoscopic (**A**) and EUS (**B**) findings. By endoscopy the lesion appears as a submucosal mass obstructing the bile ostium and protruding into the duodenal lumen; EUS procedure was impossible to obtain further information because of the cystic lesion large size. The presence of calculi in the lesion was confirmed.

lesion it was impossible to demonstrate a linkage with the biliary system.

MR imaging was performed to solve the diagnostic discrepancy between CT and EUS and to better demonstrate the relationship between the cystic lesion and the bile duct. A 1.5 Tesla MR system with the following protocol was used: Breath Hold T1 GRE fat sat sequence (TR/TE 160/4,2), HASTE sequence TR/TE ∞/120 in the axial and coronal plane, RARE thick slab sequence (pre and post oral superparamagnetic contrast agent administration - Lumirem - Guerbet) and VIBE sequence in the coronal plane pre- and post-contrast agent administration (Gd-BOPTA), followed by a delayed phase at 120 min. The Gd-BOPTA is a mixed (interstitial and hepatocellular) paramagnetic contrast agent partially excreted through the biliary system, giving the opportunity like 99mTc-HIDA scintigraphy, to functionally evaluate the biliary excretion. All MR images showed a four-centimeter well circumscribed mass in the duodenal wall.

MRCP performed before and after a negative contrast agent oral administration, demonstrated that the cystic lesion was not directly in connection with the duodenal lumen. HASTE axial and coronal sequences, showed filling defects in the gravity-dependent position of the cystic lesion (Figure 3A). Heavily T2-weighted sequences (RARE thick slab) obtained in the coronal plane confirmed that the fluid content of the lesion was changing position with the peristalsis, without any definitive information about the relationship with the bile duct (Figure 3 B). Furthermore, on the delayed 3D VIBE images after intravenous (i.v.) injection of Gd-BOPTA, it was possible to demonstrate the direct passage of hyperintense bile fluid into the cystic lesion, confirming the existence of a linkage (Figure 4).

Once the patient recovered from the acute pancreatitis, one month after his first hospital admission, he underwent surgery. During laparotomy (Figure 5), after cholecystectomy and duodenotomy, two probes were inserted into the biliary tree through both cystic duct and ampulla: both the probes reached the diverticulum confirming the linkage between these anatomical structures. Then the diverticulum was opened, multiple stones were found and the wall

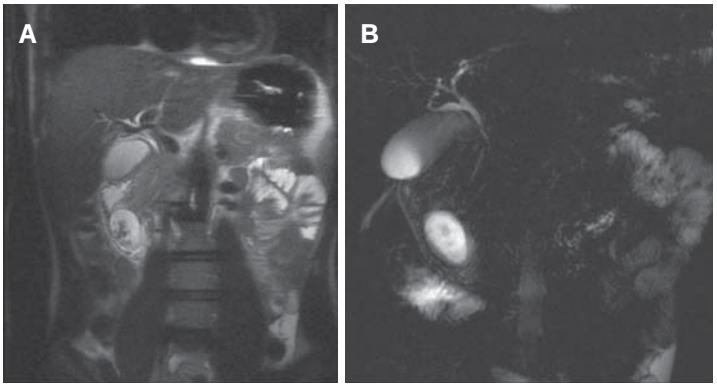

Figure 3 MRCP findings. **A**: Axial HASTE sequences demonstrate the intraluminal site of the duodenal cystic lesion with filling defects in the gravity dependent position; **B**: After superparamagnetic oral contrast medium administration the hyperintense cystic lesion does not change signal intensity.

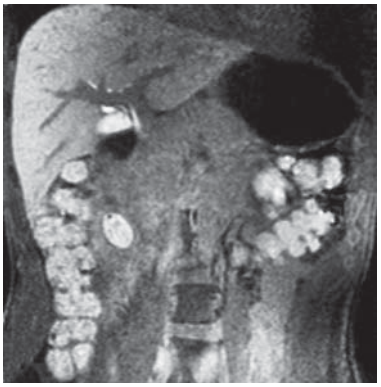

Figure 4 MRCP findings after Gadolinium BOPTA injection. Coronal VIBE sequences obtained in the delayed phase (2h) reveal the presence of hyperintense bile simultaneously in the biliary tree and in the cystic lesion, confirming the relationship between these two structures.

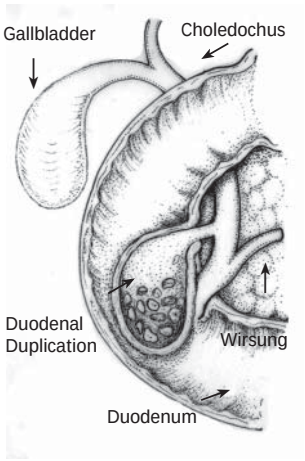

Figure 5 Schematic drawing of operative findings.

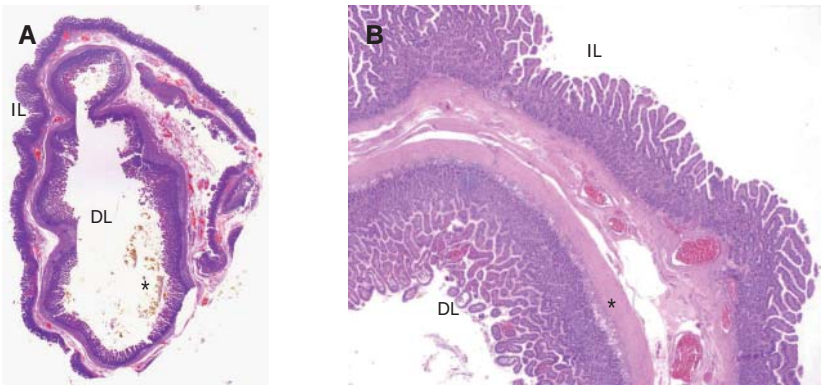

Figure 6 Mucosa, submucosa and muscle coats found in pathological ehamination. **A**: Gross transversal section of the specimen "in toto": inside it is possible to see the duplication lumen (DL) while the intestinal lumen is outside (IL). In the inner lumen there are bile plugs (*); **B**: Histology of the duodenal duplication. Microscopic low power view of the duplication wall (2x). The wall is composed of muscolaris propria extending into the septum between the duplicated segment and the mucosal lining. Intestinal mucosa is on both sides of the duplication, with a lot of macrophage cells (*) in the lamina propria of the internal one.

was completely excised up to the duodenal plane. The hole was closed with single layer stitches. Six days after surgery the patient was discharged.

Pathological examination of the excised cyst wall showed duodenal mucosa, submucosa and muscle coats. The pathologist made a diagnosis of duodenal duplication (Figures 6A and 6B) and found cholesterolosis in the cyst, in accordance with MR findings.

DISCUSSION

Clinical findings of DDC are either non-specific, such as mild abdominal pain, or specific, recalling acute or chronic pancreatitis. Two possible mechanisms might be responsible for pancreatitis: a transitory and mobility-related duodenal obstruction of the major papilla outflow by the cyst, and the migration of biliary sludge and/or microstones from the cyst to the biliary tree as observed in biliary pancreatitis[1]. As regards bile in- and out-flow, what we can imagine is a dynamic mechanism like gallbladder: the DDC wall is made of muscolaris propria, so there is peristalsis that outflows the bile. It is present a certain degree of stasis and demonstrated by the presence of stones. The pathogenesis of stones can be related more to the bile stasis in the cyst than to direct calculi drop down. Nevertheless the stasis, being intermittent due to peristalsis, does not automatically lead to a liver enzymes elevation, as in the literature[2,5,6]. The intraluminal mass has to be differentiated from a peduncled neoplastic lesion or an intraluminal duodenal diverticulum[6,7]. The cystic lesion is not an intraluminal duodenum diverticulum because the signal did not change after superparamagnetic contrast medium administration, remaining hyperintense in T2 weighted sequences. On opposite, the intraluminal duodenal diverticulum should become hypointense, due to the connection with the lumen. The differential diagnosis between these two different lesions is possible without administration of i.v. contrast medium[1]. Moreover MRCP alone cannot reveal the linkage between the cystic lesion and the bile duct, in particular if biliary anomalies are present. Following Gd-BOPTA injection, the presence of hyperintense biliary fluid on VIBE sequences can be demonstrated. The contrast agent fills both biliary tree and cystic duodenal duplication. In this case report the imaging findings of

duodenal duplication cyst communicating with the bile duct were comparable to that of choledochocele type III by Todani *et al*[3]. Considering the intraluminal site of the cystic lesion, it is possible to exclude other cystic diseases such as duodenal wall cystic dystrophy, pancreatic pseudocyst and other cystic masses belonging to the duodenal-choledochal-pancreatic area.

Regarding therapy, any surgical intervention should ensure complete resection of the duplication, in addition to its mucosa, in order to prevent malignant transformation[6].

CONCLUSION

Using both a negative oral contrast agent and an i.v. hepatospecific contrast agent with biliary excretion, MRI has a pivotal role in preoperative diagnosis to characterize duodenal cystic lesions eventually communicating with the biliary system and to differentiate duodenal duplication cyst from intraluminal duodenal diverticulum. In the case herein reported, distinction between choledochocele (type III) and duodenal duplication was only possible at pathological examination.

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CASE REPORT

Jumbo biopsy is useful for the diagnosis of colonic prolapsing mucosal polyps with diverticulosis

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Abstract

We report here a case of multiple prolapsing mucosal polyps with diverticulosis in the sigmoid colon. A 52-year-old man was admitted to our hospital because of bloody diarrhea. Colonoscopy and barium enema showed multiple diverticula, markedly thickened mucosal folds and polypoid lesions with mucus on the top of them in the sigmoid colon. Endoscopic ultrasonography showed thickening of the mucosal and submucosal layers. Several endoscopic biopsy specimens were taken from the polypoid lesions. Histological examination revealed only chronic inflammatory cell infiltration. In order to obtain a definite diagnosis, we performed endoscopic jumbo biopsy for the polypoid lesions after obtaining informed consent. Histological examination revealed marked lymphocyte infiltration, hemosiderin deposits and fibromuscular obliteration in the lamina propria, features similar to those of mucosal prolapsing syndrome. After anti-diarrhetic treatment, clinical findings were improved. Thus, jumbo biopsy is useful for diagnosis and treatment of prolapsing mucosal polyps.

Key words: Colon; Diverticulum; Jumbo biopsy; Prolapsing mucosal polyp

Kato S, Hashiguchi K, Yamamoto R, Seo M, Matsuura T, Itoh K, Iwashita A, Miura S. Jumbo biopsy is useful for the diagnosis of colonic prolapsing mucosal polyps with diverticulosis. *World J Gastroenterol* 2006; 12(10): 1634-1636

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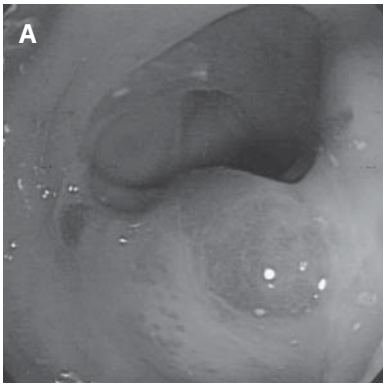
INTRODUCTION

Cases of colonic prolapsing mucosal polyps associated with diverticulosis have recently been reported in literature[1-3]. These polyps are histologically unclassifiable polyps resembling those in mucosal prolapse syndrome (MPS). Accurate differential diagnosis between neoplastic and non-neoplastic polyps is often difficult in these disease conditions. Many cases of colonic prolapsing mucosal polyps have been diagnosed retrospectively because of the low rate of pathological diagnosis of specimens obtained by the use of usual biopsy forceps. We report herein a case of unique non-neoplastic colonic mucosal prolapsing polyps with diverticulosis successfully diagnosed by using the jumbo biopsy method.

CASE REPORT

A 52-year-old man was admitted to our hospital in April 2003 because of abdominal discomfort, mucous discharge and bloody diarrhea. He had a history of frequent diarrhea from his twenties. Laboratory tests showed an increased number of peripheral leukocytes (13 800/mm^3). Barium enema showed polypoid lesions and multiple diverticula in the sigmoid colon. Colonoscopy showed multiple congested hyperemic mucosal folds and polypoid lesions with mucus on the top of them in the sigmoid colon. Slightly elevated red round patches were considered to be early minimal lesions of prolapsing mucosal polyps (Figure 1A). The surfaces of the polypoid lesions, considered to be advanced lesions, were smooth, and congested hyperemic mucosa contrasted sharply with the surrounding intact mucosa. Endoscopic examination with indigo-carmin dye spraying showed an intact mucosal pit pattern (Figure 1B). Endoscopic ultrasonography (EUS) of the early minimal lesions showed thickening of the first and second layers (mucosa) and irregular echoic lesions in the third layer (submucosa) with hypertrophy of the fourth layer (muscularis propria) (Figure 2A). EUS of the advanced lesions showed irregular low echoic lesions throughout the colonic wall (Figure 2B). Endoscopic



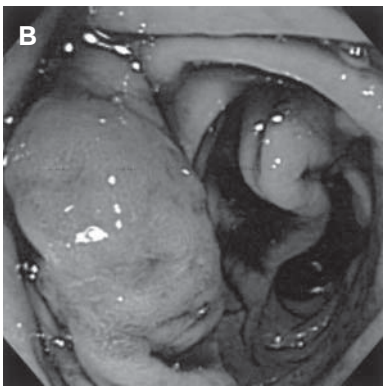
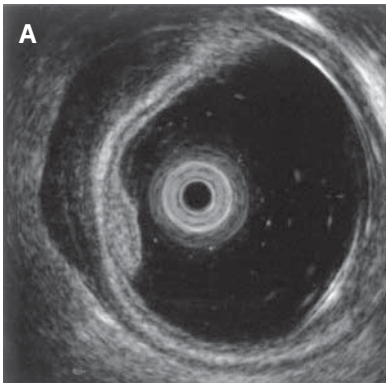


Figure 1 Colonoscopic examination of early minimal lesion (**A**) and advanced lesion (**B**) showing slightly elevated bright red round patches and congestive hyperemic polypoid lesion contrasted sharply with the surrounding mucosa (**B**: indigo carmine dye spraying view).



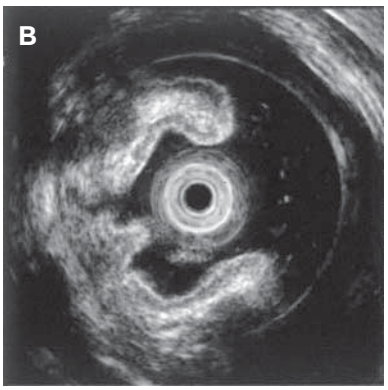
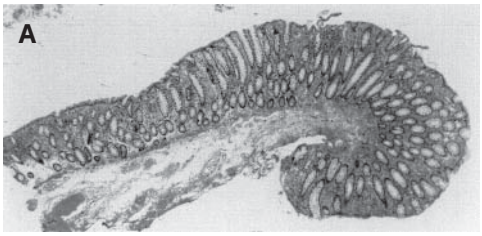


Figure 2 Endoscopic ultrasonographic examination of early minimal lesion (**A**) and advanced lesion (**B**) showing thickening of the first and second layers (mucosa) and irregular echoic lesion in the third layer (submucosa) with hypertrophy of the fourth layer (muscularis propria), and irregular low echoic lesion throughout colonic wall, respectively.



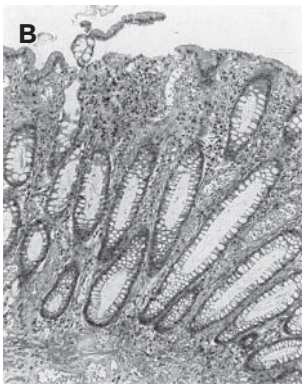


Figure 3 Hisological examination of prolapsing mucosal polyp showing mucosal hyperplasia (**A**) and chronic inflammatory cell infiltration, crypt elongation, congested vessels and fibromuscular obliterations observed in the lamina propria (**B**) (H&E, a: x40, b: x200).

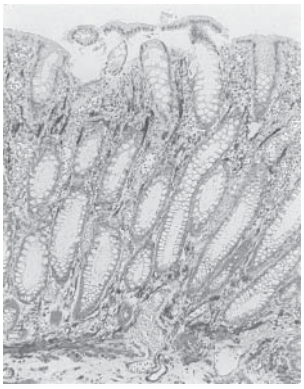


Figure 4 α-Smooth muscle actin (α-SMA) staining of prolapsing mucosal polyp showing smooth muscle fibers radiating from the thickened muscularis mucosa into the lamina propria (x200).

biopsy specimens were taken several times. Histology of biopsy specimens revealed no specific findings other than chronic inflammation. In order to obtain accurate diagnosis, we performed endoscopic jumbo biopsy for the polypoid lesions after obtaining informed consent. The jumbo biopsy specimen was taken by using a saline-solution-assisted snare resection technique that involved cutting with an Olympus SD-210-25 snare and an electro-surgical unit, OLYMPUS UES-20. Histology of the polypoid lesion showed mucosal hyperplasia (Figure 3A). Crypt elongation, chronic inflammatory cell infiltration, congested vessels and hemosiderin deposits were observed in the lamina propria (Figure 3B). Immunohistochemistry using a monoclonal antibody against anti-α smooth muscle actin showed smooth muscle fibers radiating from the thickened muscularis mucosa (fibromuscular obliteration) (Figure 4). Based on these findings, these lesions were diagnosed as prolapsing mucosal polyps with diverticulosis of the sigmoid colon. The patient was treated with anti-diarrhetic drugs and a fiber-enriched diet. Diarrhea was improved and the white blood cell count in peripheral blood decreased to within the normal range.

DISCUSSION

We experienced a patient who was diagnosed by using the jumbo biopsy method as having prolapsing mucosal

polyps with diverticular diseases. Cases of prolapsing mucosal polyps with diverticular disease have recently been reported by Franzin *et al*[1] and Mathus-Vliegen *et al*[2] These polyps are histologically unclassified polyps resembling those in mucosal prolapse syndrome (MPS). MPS including solitary rectal ulcer syndrome is characterized by the presence of polypoid or ulcerative lesions with the histological changes of glandular epithelium, fibromuscular obliteration and teleangiectatic blood vessels in the lamina propria. MPS is thought to result from chronic intermittent ischemia caused by overt or occult mucosal prolapse. Histological features of these polyps are crypt elongation, up-growth of muscle fibers from the muscularis mucosa (fibromuscular obliteration), and mucosal and submucosal vascular congestion, hemorrhage and hemosiderin deposition[3]. These polyps resemble 'cap polyposis'; however, there is a large amount of granulation tissue formation called fibrin-cap on the surface and less teleangiectactic changes in 'cap polyposis' than in MPS.

Endoscopy in our case showed slightly elevated red round patches, considered to be initial lesions, and smooth and congested hyperemic mucosal polypoid lesions. Tendler *et al*[4] reported that all prolapsing polyps occurred in the sigmoid colon usually in association with diverticular disease. Endoscopic ultrasonography (EUS) in our case showed thickening of the first and second layers (mucosa) and mixed hypoechoic lesions in the third layer (submucosa) with hypertrophy of the fourth layer (musclularis propria) in small patches, and moderate irregular hypoechoic lesions extended into the fourth layer (muscularis propria) with destruction of the first and second layers (mucosa) and the third layer (submucosa) in the polypoid lesion. It has recently been reported that EUS of polypoid prolapsing mucosal folds revealed thickening of mucosal and submucosal layers, demonstrating remarkably hyperemic and thickened mucosal folds[5]. These EUS findings resemble those described in previous reports of MPS[6,7]. In our case, the mixed hypoechoic pattern of early minimal lesions was thought to reflect mild fibromuscular obliteration and edema in the mucosal and submucosal layers. This irregular pattern was more apparent in advanced lesions throughout the colonic wall. These EUS findings demonstrated changes in the pathological development of these polyps. However, almost all cases of colonic prolapsing mucosal polyps have been diagnosed retrospectively after treatment. Only one case has been diagnosed by EUS findings and a normal surface pit-pattern before treatment[5].

Diagnosis of prolapsing mucosal polyps by examination of biopsy specimens obtained by using biopsy forceps can be difficult because of the sizes and depths of obtained specimens. Results of examination of biopsy specimens obtained several times from our case only showed chronic inflammation without fibromuscular obliteration. Warren *et al*[8] proposed the usefulness of diagnostic features of 'diamond-shaped' crypts in biopsy specimens. However, there were no such findings in our case. Jumbo biopsy is known to be a safe and useful method for diagnosis and treatment, and it is often used for not only endoscopic cure of early cancers, but also obtaining specimens for accurate pathologic staging[9]. In some cases, inflammatory change induces a "pseudo-malignant status". Colitis cystica profounda, a variant of mucosal prolapse syndrome, is sometimes difficult to distinguish from mucinous-producing carcinoma because of its formation by an aberrantly located gland-forming epithelium[10].

Treatment of prolapsing mucosal polyps is fundamentally conservative. Mathus-Viegen *et al*[2] reported the usefulness of a fiber-enriched diet for treatment of prolapsing mucosal polyps. Improvement was obtained in our case by a fiber-enriched diet and administration of an anti-diarrhetic drug. However, some patients with this disease require surgery because of intractable abdominal pain. It is important for endoscopists and pathologists to be familiar with and recognize the characteristic features of prolapsing mucosal polyps in order to avoid oversurgery for this disease because of its benign nature.

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CASE REPORT

Uveitis in autoimmune hepatitis: A case report

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Abstract

In this case report we describe for the first time an association between autoimmune hepatitis (AIH) and uveitis, without any doubts about other possible etiologies, such as HCV, since all the old reports describe the association of AIH with iridocyclitis before tests for HCV-related hepatitis could be available. A 38-year-old businessman with abnormal liver function tests and hyperemia of the bulbar conjunctiva was admitted to the hospital. Six years before admission, the patient presented with persistent fever, arthralgias, conjunctival hyperemia, leukocytosis and increased ESR, referred to acute rheumatic fever. The presence of systemic diseases, most commonly associated with uveitis, was investigated without results and the patient was then treated with topical corticosteroids. His symptoms resolved. A test for anti-nuclear antibodies was positive, at a titre of 1:320, with a speckled and nucleolar staining pattern. Liver ultrasound showed mild hepatomegaly with an increased echostructure of the liver. Percutaneous liver biopsy was performed under ultrasound assistance. Histological examination showed necroinflammation over the portal, periportal and lobular areas, fibrotic portal tracts, with periportal fibrosis and occasional portal-to-portal bridgings, but intact hepatic architecture. Some hepatocytes showed barely discernible granules of hemosiderin in the lobular area. Bile ductules had not any significant morphological alterations. METAVIR score was A2-F3, according to the modified HAI grading/fibrosis staging. The patient was diagnosed to have AIH with mild activity and fibrosis and was discharged on 25 mg prednisone, entering clinical and biochemical remission, further confirming diagnosis. After discharge the patient continued to have treatment with corticosteroids as an outpatient at a dose of 5 mg. On January 2002 the patient was readmitted to the hospital. A test for anti-nuclear antibodies was positive, at a titre of 1:320, with a speckled and nucleolar staining pattern. Anti-smooth muscle antibody test was also positive (1:160), while anti-LKM antibodies were negative. Ophthalmologic examination revealed inflammatory cells and proteinaceous flare in the anterior chamber of the left eye, and a stromal lesion in the cornea. He was maintained on immunosuppressive therapy (5 mg prednisone plus topical antibiotic therapy for two weeks) and then discharged. A complete remission of the symptoms was registered on follow-up. At present (July 2005), the patient is on prednisone (5 mg) and has no symptoms. Liver function tests are also within the normal range.

Key words: Autoimmune hepatitis; Uveitis

Romanelli RG, La Villa G, Almerigogna F, Vizzutti F, Di Pietro E, Fedi V, Gentilini P, Laffi G. Uveitis in autoimmune hepatitis: A case report. *World J Gastroenterol* 2006; 12(10): 1637-1640

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CASE REPORT

A 38-year-old businessman was admitted to the Azienda Ospedaliero-Universitaria Careggi (Florence, Italy) on May 4th 2000, because of abnormal liver function tests and hyperemia of the bulbar conjunctiva.

The patient had no history of liver disease or jaundice, receipt of blood products, intravenous drug abuse, and exposure to alcohol or hepatotoxic drugs, seafood ingestion, any abdominal pain, excessive fatigue, rashes, or foreign travels. All his children were healthy. Family history of gastrointestinal or liver diseases was negative. His mother was affected by goitre and diabetes mellitus.

Six years before admission, the patient presented with persistent fever, arthralgias, conjuntival hyperemia, leukocytosis and increased ESR, which were referred to acute rheumatic fever, despite no evidence of recent group A streptococcal infection, since throat cultures were negative and streptoccal antibody test was normal. The patient was given salicylic acid up to 4 g for 2 mo. and prophylaxis with intramuscular benzathine penicillin G (1.2 g/mo. for 5 years). In 1998, conjunctival hyperemia recurred and iridocyclitis was diagnosed. The presence of systemic dis-

Table 1 Autoimmune hepatitis: Revised scoring system (1999) (International Autoimmune Hepatitis Group, *J Hepatol* 1999; 31: 929-938. Score Autoimmune Hepatitis)

	May 2000	January 2002
Gender: male	0	0
ALP/AST: 249/960 = *0.25* (admission May 2000); *3.85* (admission Jan 2002)	+2	-2
ALP/AST: 249/160 = *1.55* (discharge May 2000)	0	
ALP/ALT: 249/1 510 = *0.16* (admission May 2000); *4.15* (admission Jan 2002)	+2	-2
ALP/ALT: 249/483 = *0.51* (discharge May 2000)	+2	
IgG Assay: 1 590 (May 2000); 1 670 (January 2002)	+2	+2
ANA, SMA, or LKM1: negative	0	0
AMA: negative	0	0
Hepatitis markers: negative	+3	+3
Drug history: negative	+1	+1
Alcohol intake: negative	+2	+2
Histology: *Chronic hepatitis with moderate activity. Fibrotic portal tracts, periportal fibrosis with portal-portal septa, but intact architecture. Biliary ducts without any significant morphological alterations.*	+3	+3
Response to therapy: complete	+2	+2
Other autoimmune diseases: (thyroiditis)	+2	+2
Score:	21 (definite)	11 (probable)

eases, most commonly associated with anterior uveitis, was investigated without results and the patient was treated with topical corticosteroids. His symptoms resolved. At admission for further studies on systemic alterations other than uveitis, the temperature was 36.6 ℃, the pulse was 84 bpm, and the respiration rate was 18/min. Blood pressure was 105/70 mmHg. At physical examination, the head, the neck and the extremities were normal. Auscultation of the heart and both lungs were normal. The abdomen was soft with normal bowel sounds and not distended; there was tenderness in the right upper quadrant, and also in the lower one, with rebound tenderness in the latter; the liver was enlarged (about 3 cm below the right costal margin); the spleen was not palpable; no hernia was detected. There was no evidence of edema, bleeding diathesis, or spider angiomata.

Main initial laboratory findings were as follows: hematocrit 48.2%, leukocytes 5 400/μL (39.5% neutrophils, 39.7% lymphocytes, 18.2% monocytes, 0.6% basophyls, 2.0% eosinophils), platelets 157 000/μL, reticulocyte count 1.35%, glucose 0.83 mg/dL, BUN 0.25 mg/dL, creatinine 1.0 mg/dL, proteins 72 g/L, albumin 42.3 g/L, globulins 29.7 g/L, IgG 1 590 mg/dL, IgA 224 mg/dL, IgM 83 mg/dL, AST 960 IU/L, ALT 1 510 IU/L, γ-glutamiltranspeptidase 112 IU/L, alkaline phosphatase 249 IU/L, total bilirubin 1.57 mg/dL, conjugated bilirubin 0.4 mg/dL, total cholesterol 116 mg/dL, serum calcium 8.4 mg/dL, serum phosphorus 2.6 mg/dL, serum iron concentration 276 μg/dL, serum ferritin 3 020 ng/mL, and haptoglobin 54 mg/dL; international normalized ratio for prothrombin time was 1.3, the activated partial thromboplastin time 39.6 s, and TSH 2.22 mU/L. Urinanalysis was normal. Hepatitis B surface antigen, anti hepatitis A, B and C virus (IgG) were undetectable. Anti-CMV IgG were positive (30 UA/mL), but IgM resulted negative. Serologic tests for brucella and mycobacterium sp (IgG and IgM) were negative. The direct and indirect Coombs tests were also negative. Serum circulating immune complexes were within the normal range (IgG binding C1q 2.6 μEq/mL and IgG binding C3 5.2 μEq/mL), C3 was 111, C4 22 mg/dL. A test for anti-nuclear antibodies was positive, at a titer of 1:320, with a speckled and nucleolar pattern of staining. Test for anti-smooth muscle antibodies was also positive (1:160), anti-LKM antibodies were negative. Titres of antithyroid peroxidase autoantibodies and anti-thyreoglobulin autoantibodies were 420 and 85.6 IU/mL, respectively.

Liver ultrasound showed mild hepatomegaly with an increased echostructure of the liver. Percutaneous liver biopsy was performed under ultrasound assistance. Liver biopsy showed necroinflammation over the portal, periportal and lobular areas, fibrotic portal tracts, with periportal fibrosis and occasional portal-to-portal bridgings, but intact hepatic architecture. Some hepatocytes showed barely discernible granules of hemosiderin in the lobular area. Bile ductules were without any significant morphological alterations. METAVIR score resulted A2-F3, according to the modified HAI grading and fibrosis staging as proposed by Ishak *et al*[1]. No stainable iron or copper deposits were found.

Ophthalmologic examination, performed to rule out Wilson's disease, revealed no diminished visual activity in both eyes; eye movements were normal, without diplopia; the globes were intrinsically normal, so as papillary light reflexes. The fundus oculi was normal, without signs of vasculitis. Biomicroscopy of the anterior segment of the eye was negative for iridocyclitis. No Kayser-Fleischer rings were noted.

Considering the clinical presentation, together with increased serum aminotransferase levels, absence of viral markers for hepatitis B, C, and other hepatotropic viruses, evidence on liver biopsy of chronic hepatitis with mild-

Table 2 Extrahepatic associations of AIH

Frequent:	Autoimmune thyroid disease	Rare:	Rheumatoid arthritis
	Ulcerative colitis		Lichen planus
	Synovitis		Diabetes mellitus
	Autoimmune		CREST syndrome
	Hemolytic anemia		Autoimmune
			Thrombocytopenic purpura
			Vitiligo
			Alopecia

to-moderate fibrosis, the patients was diagnosed to have autoimmune hepatitis (AIH) with mild activity and discrete fibrosis. Patient was discharged on prednisone 25 mg and entered clinical and biochemical remission, further confirming diagnosis. After the discharge the patient continued to have treatment with corticosteroids as an outpatient at a dose of 5 mg.

On January 2002 the patient was readmitted to the hospital because of fever (39 ℃), occurring especially in the evening. He also complained photophobia, and blurred vision in both eyes. The blood pressure was 110/65 mmHg, the pulse rate was 87 bpm, and the respirations were 18/minute. Physical findings were unmodified in respect to the previous readmission.

Laboratory investigations showed the following data: hematocrit 39.7%, leukocytes 9 690/μL (65% neutrophils, 19.3% lymphocytes, 14.5% monocytes, 0% basophyls, 1.2% eosinophils), platelets 497 000/μL, reticulocyte count 1.35%, glucose 1.22 mg/dL, BUN 0.24 mg/dL, creatinine 1.2 mg/dL, proteins 84 g/L, albumin 33.9 g/L, globulins 50.1 g/L, IgG 1 670 mg/dL, IgA 273 mg/dL, IgM 64 mg/dL, AST 14 IU/L, ALT 16 IU/L, γ-glutamiltranspeptidase 26 IU/L, alkaline phosphatase 52 IU/L, total bilirubin 0.54 mg/dL, conjugated bilirubin 0.14 mg/dL, total cholesterol 96 mg/dL, serum calcium 9.1 mg/dL, serum phosphorus 2.4 mg/dL, serum iron concentration 82 μg/dL, serum ferritin 263 ng/mL, haptoglobin 1 120 mg/dL, international normalized ratio for prothrombin time 1.0, and the activated partial thromboplastin time 26.1 s. Flogosis indexes (ESR, C-reactive protein, fibrinogen) were elevated. TSH was 1.57 mU/L. Urinanalysis was normal. Direct and indirect Coombs tests were both negative. Serum circulating immune complexes were within the normal range; C3 was 196 mg/dL, C4 43 mg/dL, and serum immunoelectrophoresis showed increased IgG. A test for anti-nuclear antibodies was positive, at a titre of 1:320, with a speckled and nucleolar staining pattern. Test for anti-smooth muscle antibodies was also positive (1:160), anti-LKM antibodies were negative. Titres of antithyroid peroxidase autoantibodies and anti-thyreoglobulin autoantibodies were 420 and 85.6 IU/mL, respectively.

Serologic markers of viral infection (toxoplasma, EBV, mycobacterium sp, CMV, HAV, HBV, HCV and neurotropic viruses) were all negative. Antistreptococcal and anti-staphylococcal antibodies were undetectable (Table 1).

Ultrasound of the liver was normal. Ophthalmologic examination revealed inflammatory cells and proteinaceous flare in the anterior chamber of the left eye (Tyndall + - -), and a stromal lesion in the cornea. The right eye was unaffected.

He was maintained on immunosuppressive therapy (5 mg prednisone) plus topical antibiotic treatment for two weeks and then discharged. A complete remission of the symptoms was registered on follow-up. At present (July 2005), the patient is on prednisone (5 mg) and has no symptoms. Liver function tests are also within the normal range.

DISCUSSION

About 25% of patients with AIH have an acute onset. In most cases, however, the clinical presentation of AIH is characterized by the same signs and symptoms of chronic hepatitis of other aetiology. A specific feature of AIH is the association with extrahepatic immune-mediated syndromes, including autoimmune thyroiditis[2-5], scleroderma, rheumathoid arthritis, Sjögren's syndrome or diabetes mellitus (Table 2). An old report describes the association of chronic autoimmune hepatitis with iridocyclitis[2], but at that time tests for HCV-related hepatitis were not available[6].

The hallmark of acute anterior uveitis is the presence of inflammatory cells and proteinaceous flare in the anterior chamber of the eye. Symptoms include pain, photophobia, and blurred vision in the involved eye(s). Iridocyclitis may be part of a more generalized autoimmune and endogenous uveitis, such as Reiter's syndrome, more commonly observed in patients with HIV infection. Phacogenic uveitis, sympathetic ophtalmia and Vogt-Kaganayi-Harada syndrome can represent ocular autoimmune diseases suitable for a differential diagnosis, even if both phacogenic uveitis and sympathetic ophtalmia are post-traumatic granulomatous uveitis. In this patient, however, there is no history of physical or surgical trauma, ruling out the possibility of an involvement of the above disorders in this case. The Vogt-Kaganayi-Harada syndrome is an idiopathic, bilateral, inflammatory syndrome occurring in middle age and characterized by a typical granulomatous intraocular inflammation. The aetiology of this syndrome is unknown and the disorder is assumed to be an autoimmune hypersensitivity response to pigment. Poliosis (i.e. localized depigmentation of the hair) occurs in more than 90 percent of these patients, together with alopecia and vitiligo. Auditory disturbances occur in more than 75% of patients, and many other neurologic findings, including psychosis, have been sometimes reported. However, this disorder is very rare, and most cases have been associated with a previous penetrating ocular injury, which is absent in our patient.

We also considered, for the differential diagnosis, a possible ophtalmic localization of systemic diseases, either infectious or not[7]. Wegener's granulomatosis is characterized by fever, weight loss and other systemic manifestations, together with ocular and oropharyngeal signs. Moreover, ankylosing spondylitis and chronic inflammatory bowel diseases can both be complicated by anterior uveitis. Furthermore, viral infections can cause uveitis: toxoplasmosis is generally the most common cause of infective uveitis, and AIDS is also mostly associated with cryptococ-

cal chorioretinitis or posterior uveitis. A possible cause of uveitis can, finally, be represented by systemic lupus erythematosus, which is typically characterized by arthralgia, fever and ocular involvement, the presence of circulating antinuclear autoantibodies and no substantial remission after corticosteroid therapy.

AIH type I is the most common form of AIH worldwide and is associated with antinuclear antibodies and/or smooth muscle antibodies. This is a disorder characterized by hepatic and extrahepatic involvement; the most frequent extrahepatic associations of AIH are mentioned in the table above *(*Table 2*)*. In this case report, we have described, for the first time, an association between AIH and uveitis, without any doubts about other possible etiologies, such as HCV infection[8], since all the old reports describe the association of AIH with iridocyclitis before tests for HCV-related hepatitis could be available. Furthermore, this report concentrates on clinical attention to patients who may be at increased risk for potentially serious ocular disorders and for whom slit-lamp examination may be warranted on a regular basis.

The majority of patients affected by AIH require a long-term maintenance therapy, and the milder disease is associated with a better response to therapy[9]. Corticosteroid treatment is the best choice therapy for AIH and, even if it is usually followed by immunosuppression, thereafter[10], we preferred a low-dose steroid treatment, registering both remission of hepatitis and absence of any significant side effects. This is well documented in the literature[11]. Moreover, azathioprine has a significant early adverse reaction (EAR) profile, which includes an acute syndrome of constitutional symptoms, fever, rash, and acute pancreatitis and often requires discontinuation of drug. Some Authors report that EAR (early adverse reactions) precludes azathioprine use in patients with Crohn's disease (CD) and autoimmune hepatitis (AIH)[12]. Finally, we skip immunosuppressive treatment since it has been described an increased tumor risk under azathioprine therapy, especially in Vogt-Kaganayi-Harada (VKH) syndrome. Vogt-Kaganayi-Harada (VKH) syndrome was excluded in the differential diagnosis of our patient. Azathioprine treatment has been registered associated with increased risk of malignancies[13,14].

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CASE REPORT

Dermatomyositis associated with hepatocellular carcinoma in an elderly female patient with hepatitis C virus-related liver cirrhosis

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Abstract

A 79-year-old female patient with hepatitis C virus-related liver cirrhosis was diagnosed as having hepatocellular carcinoma (HCC) with a diameter of 2.0 cm. She refused therapy for HCC. Nine months after the diagnosis, she developed dermatomyositis when the HCC enlarged to a diameter of 6.0 cm. She underwent therapy for dermatomyositis, and then transcatheter arterial chemoembolization for HCC. Although the manifestations of dermatomyositis improved and entire tumor necrosis was achieved, she died of pneumonia 2 mo after the treatment of HCC. HCC and/or chronic hepatitis C virus infection might be involved in the pathogenesis of dermatomyositis.

Key words: Dermatomyositis; Hepatocellular carcinoma; Hepatitis C virus

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INTRODUCTION

The cause of dermatomyositis remains unknown. However, some factors have been implicated in the pathogenesis of dermatomyositis. Many reports on the relationship between dermatomyositis and cancer or viral infection have been published[1-17]. We report herein a rare case of dermatomyositis that occurred during the natural course of hepatocellular carcinoma (HCC) in an elderly female patient with hepatitis C virus (HCV)-related liver cirrhosis.

CASE REPORT

A 79-year-old woman was treated for HCV-related liver cirrhosis at our hospital. She received medication for the liver disease (ursodeoxycholic acid) and for epilepsy (clonazepam) due to a past head injury. There was no family history of autoimmune disease or malignancy. In October 2003, a hepatic tumor with a diameter of 2.0 cm in the right anterosuperior segment was detected on dynamic computed tomography (CT): the tumor was shown to be a hypodense lesion in the basal study which was enhanced during the arterial phase and became hypodense during the delayed phase. The serum alpha-fetoprotein (AFP) and des-gamma-carboxy prothrombin (DCP) levels were 3.4 ng/mL (normal, <10 ng/mL) and 431 mAU/mL (normal, <40 mAU/mL), respectively. Based on these findings, the tumor was diagnosed as HCC. However, the HCC was not treated at that time because the patient refused therapy.

In July 2004, she began to develop progressive muscle weakness in the extremities and systemic erythema. She was admitted to the Department of Dermatology at our hospital. On admission, her height was 146 cm and her body weight was 60 kg. A heliotrope rash in the periorbital skin, Gottron papules on the metacarpophalangeal joints, and poikiloderma on the anterior neck and upper chest were observed. There was no skin thickening of the extremities or joint swelling. Muscle examination revealed symmetrical proximal weakness: she could not rise from a chair or raise her hands up to the level of her shoulders. Table 1 shows her laboratory data on admission. Elevated serum muscle enzymes were observed. HBsAg (enzyme immunoassay [EIA], Dinabot, Tokyo, Japan) was negative. Anti-HCV antibody (second generation, EIA, Dinabot) was positive with a high titer, and HCV RNA (reverse transcription-PCR, Sionogi, Osaka, Japan) was also positive. Hypergammaglobulinemia was observed. Antinuclear antibodies were positive (1:640, homogeneous, speckled), while rheumatoid factor, myeloperoxidase antineutrophil cytoplasmic antibodies, anti-DNA, anti-

Table 1 Laboratory data on admission

			Range				Range
WBC	3190	/mm^3	3500-8500	CPK	2092	IU/L	46-160
RBC	401×10^4	/mm^3	380×10^4-480×10^4	aldolase	13	IU/L	2.5-6.2
Hb	13.7	g/dL	11.8-15.0	myoglobin	293	ng/mL	≤60
Plt	9.0×10^4	/mm^3	13.0-30.0×10^4				
PT	11.6	s	10.0-13.0	IgG	2027	mg/dL	870-1700
				CRP	0.08	mg/dL	0-0.4
AST	150	IU/L	≤35				
ALT	39	IU/L	≤35	HBsAg	(-)		
LDH	583	IU/L	≤230	anti-HCV	(+)		
ALP	200	IU/L	≤340	HCV RNA	(+)		
Γ-GTP	20	IU/L	≤55				
T-Bil	0.8	mg/dL	0.2-1.2	ANA	× 640		
ChE	192	IU/L	185-431		HO, SP		
T-CHO	170	mg/dL	≤220	RF	(-)		
TP	5.7	g/dL	6.5-8.0	MPO-ANCA	(-)		
Alb	2.7	g/dL	4.2-5.0	anti-Jo-1	(-)		
BUN	15.3	mg/dL	9.0-20.0	anti-DNA	(-)		
Cr	0.6	mg/dL	0.4-1.1	anti-RNP	(-)		
FBS	96	mg/dL	≤110	anti-SSA	(-)		
Ca	8	mg/dL	8.5-10.2	anti-SSB	(-)		

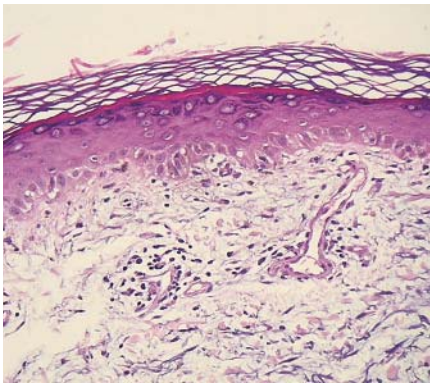

Figure 1 Hematoxylin-eosin staining of skin biopsy of the left forearm showing flattened epidermis and scattered inflammatory cell infiltration around the small vessels of the dermis (200 × magnifications).

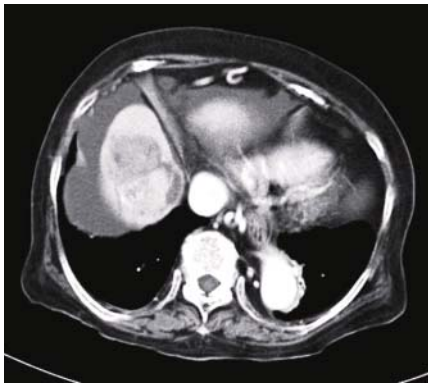

Figure 2 Contrast-enhanced computed tomography showing a hypervascular, in part, necrotic tumor in the right anterosuperior segment of the liver and ascites.

Jo-1, anti-RNP, anti-SSA, and anti-SSB were all negative. Electromyography of the right biceps brachii muscle showed fibrillation and low amplitude voltage of a short duration (less than 200 mV). Skin biopsy of the left forearm showed flattened epidermis, partial liquefaction of the basal layer, scattered inflammatory cell infiltration around the small vessels, and degeneration of collagen fiber of the dermis (Figure 1). Muscle biopsy of the left triceps failed to show remarkable findings. On the basis of these findings, the diagnosis of dermatomyositis was made. Initially, 50 mg of prednisolone per day was administered, and then the dosage was gradually decreased to 15 mg/d. Skin eruption almost disappeared completely and muscle power slowly improved. Muscle enzymes also improved to the normal range. During the treatment of dermatomyositis, the patient decided to undergo therapy for the HCC.

When she was referred to the Department of Internal Medicine in August 2004, the HCC nodule was enlarged to a maximal diameter of 6.0 cm, and ascites was observed (Figure 2). The serum AFP and DCP levels also increased to 2 130.2 ng/mL and 7 423 mAU/mL, respectively. Other serum tumor markers, including carcinoembryonic antigen, carbohydrate antigen 19-9, and carbohydrate antigen 125, were all negative. No other malignancies were identified by imaging studies, such as ultrasonography and CT or esophagogastroduodenal endoscopy. In August 2004, she underwent transcatheter arterial chemoembolization for the treatment of the HCC after the ascites was controlled. After transcatheter arterial chemoembolization, entire tumor necrosis was achieved; the serum AFP and DCP levels decreased to 46.7 ng/mL and 54 mAU/mL, respectively. The clinical course of dermatomyositis was not affected by the embolization. Unfortunately, the patient died of bacterial pneumonia in October 2004.

DISCUSSION

The relationship between cancer and dermatomyositis has been widely reported in the literature[1-9]. Recent

population-based studies have demonstrated that 15-32% of patients with dermatomyositis are associated with various types of cancers, such as ovarian, lung, pancreatic, stomach, and colorectal cancers[1,2]. However, there have been few reports on the association between dermatomyositis and HCC[3-9]. Although the rare association might be fortuitous, a recent report described the improvement of dermatomyositis without the need of corticosteroids after the resection of HCC, supporting the hypothesis that HCC can cause dermatomyositis through a paraneoplastic mechanism[8]. In the present case, dermatomyositis occurred when the HCC increased from a baseline diameter of 2.0 cm to 6.0 cm at 10 mo. In most other reported cases, advanced HCC, more than 6.0 cm in diameter or multiple, has been found at the diagnosis of dermatomyositis[4-9]. Those reports indicate that advanced HCC might induce an autoimmune response as the trigger of dermatomyositis.

Recent studies have demonstrated that antibodies to Mi-2, a component of the nucleosome remodeling-deacetylase complex, are strongly associated with dermatomyositis (frequencies up to 31%)[18,19]. An *in vitro* study has shown that a 169-bp cDNA product, which is 88.8% homologous to the human Mi-2beta antigen, is identified in H4IIE rat hepatoma cells[20]; and 100% homology is found at the protein level. Based on these findings, anti-Mi-2 antibodies might be cross-reactive with HCC. Unfortunately, anti-Mi-2 antibodies were not examined in the present case. Further studies on autoantibodies cross-reacting with HCC should be undertaken to shed light on the pathogenesis of dermatomyositis associated with HCC.

Occasional associations between dermatomyositis and HCV infection have been reported[6,7,9-14]. Thus far, there has been no direct evidence that HCV infection can cause dermatomyositis. A recent preliminary study has shown no significant higher incidence of HCV in patients with dermatomyositis as compared with the control population[10]. However, chronic HCV infection has been known to be associated with the presence of autoantibodies and various types of autoimmune diseases, leading to the hypothesis that chronic HCV infection could cause dermatomyositis through an autoimmune mechanism[21]. In the present case, antinuclear antibodies were strongly positive, suggesting that there might be some immune disorders related to the development of dermatomyositis. We speculate that antibodies against HCV or HCV-enzyme complex might cross-react with homologous area of host proteins and thus cause autoimmune diseases including dermatomyositis. Crowson *et al*[22] have reported that HCV RNA is expressed in a focal, weak fashion in endothelia and perivascular inflammatory cells in some HCV-infected patients associated with cutaneous eruptions. They postulated that parasitism by HCV can render endothelia autoantigenic through exposure of cryptic antigens via virally induced endothelia cell injury. Histopathologic examinations of HCV expression using skin biopsy specimens from patients with dermatomyositis associated with HCV infection might provide information on the role of HCV in the pathogenesis of dermatomyositis.

In the present case, other infectious agents including echovirus, adenovirus, and coxsackievirus, which are known to cause myositis, were not examined[15-17]. Therefore, there remains the possibility that dermatomyositis of the present case might be caused by such viruses.

In summary, this report suggests that HCC, in particular advanced HCC, and/or chronic HCV infection might be factors in the pathogenesis of dermatomyositis. The mechanisms of autoimmune responses induced by such factors should be studied in the future.

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CASE REPORT

Spontaneous regression of hepatic inflammatory pseudotumor with primary biliary cirrhosis: Case report and literature review

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Abstract

Hepatic inflammatory pseudotumor (IPT) is a rare benign non-neoplastic lesion characterized by proliferating fibrous tissue infiltrated by inflammatory cells. The exact etiology of IPT remains unclear. Although the association of IPT with systemic inflammatory disorders has been well established, a specific relationship with cholangitis is distinctly rare. We report a case of spontaneous regression of hepatic IPT with primary biliary cirrhosis (PBC). To date, only two cases of IPT with PBC have been reported. In our case, however, IPT developed during the course of improvement of cholangitis of PBC induced by effective treatment, differing from two previously reported cases. Our case indicates that the development of IPT does not also relate to the activity of cholangitis and/or hyper gamma-globulinemia, since our case was confirmed radiologically to be free of IPT when biliary enzymes and immunoglobulins were much higher than the corresponding values on admission. Comparison of our case with the two previously reported cases suggests that IPT occurring with PBC does not represent the same disease entity or be a bystander for PBC.

Key words: Hepatic inflammatory pseudotumor; Primary biliary cirrhosis; Spontaneous regression; Ursodeoxycholic acid; Bezafibrate

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INTRODUCTION

Hepatic inflammatory pseudotumor (IPT) is a rare benign non-neoplastic lesion characterized histopathologically by proliferating fibrous tissue infiltrated by inflammatory cells. The exact etiology of IPT remains unclear. Although the association of IPT with systemic inflammatory disorders has been well described, a specific relationship with cholangitis is distinctly rare[1-4]. Primary biliary cirrhosis (PBC) is a chronic cholestatic disease with cholangitis. We report a case of spontaneous regression of IPT associated with PBC. Our case was peculiar in view of detection of IPT during improvement of cholangitis of PBC, thus differing from the two previous reports of IPT with PBC[5,6].

CASE REPORT

The patient was a 71-year-old man. He was diagnosed in 2001 with PBC (Scheuer's histological stage II) based on histopathological findings of liver biopsy specimens and positive anti-mitochondrial antibody (AMA) and was followed with ursodeoxycholic acid (UDCA) in our department. He requested a check up for cancer and fluorodeoxyglucose positron emission tomographic study (FDG PET) was performed on his request on his 70th birthday. The whole-body images demonstrated a 3-cm-diameter spherical mass in the liver (Figure 1A, B). One year before FDG PET, no liver tumor was noted on enhanced computerized tomography (CT). He was admitted to our department for full examination and assessment. We had started treating the patient with bezafibrate, which was recently reported to be effective in improving PBC patients with elevated biliary enzymes[7], two months before detection of the liver mass because UDCA had not been fully effective. On admission, he was asymptomatic and physical examination was negative. Laboratory data on admission (Table 1) showed elevated

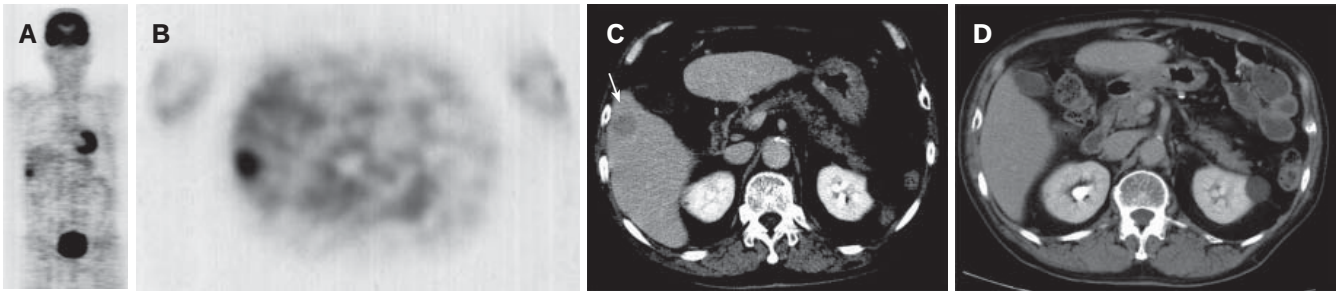

Figure 1 A, **B**: Whole body F-18 fluorodeoxyglucose (FDG) positron emission tomography (PET) scan showed a 3-cm spherical mass in the right lobe of the liver. **A**: Coronal sectional view, **B**: horizontal sectional view. **C**: Enhanced CT showed a 3-cm spherical low-density mass (arrow) in the right lobe of the liver. **D**: Another enhanced CT performed 3 mo after targeted liver biopsy showed complete resolution of the mass.

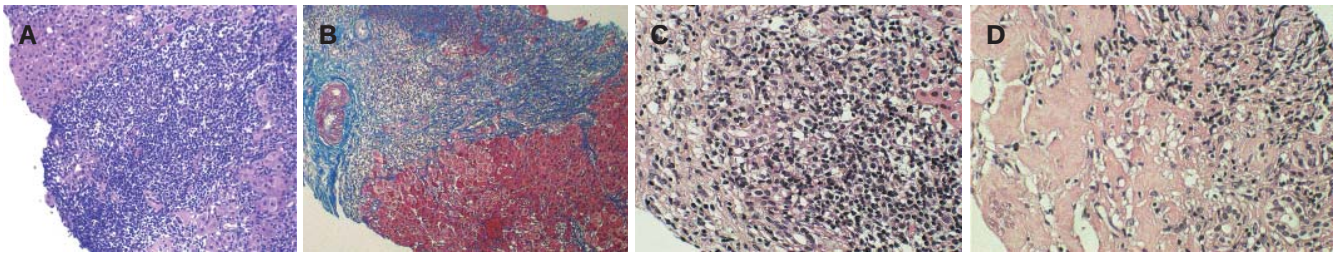

Figure 2 A: Liver biopsy specimen from the non-tumorous liver. Histopathological findings are consistent with primary biliary cirrhosis. The enlarged portal tract with damaged bile ducts in florid lesion of non-suppurative destructive cholangitis is infiltrated by inflammatory mononuclear cells. H&E, X100. **B**: Liver biopsy specimen shows clear-cut boundary between the "tumor" and liver parenchyma. Azan-Mallory, ×100. **C**:The "tumor" is composed of chronic inflammatory cells including lymphocytes and plasma cells. H&E, × 400. **D**: The "tumor" is composed of fibrous tissue, thick hyalinized collagen bundles with disappearance of liver parenchyma. H&E, ×400.

Table 1 Changes of Biochemical and immunologic Profile

	Before treatment of UDCA without detection of IPT	On admission	At the time of regression of IPT
Total bilirubin (mg/dL)	0.7	0.8	0.6
AST (IU/L)	46	22	25
ALT (IU/L)	391	16	15
ALP (IU/L)	682	159	141
γGTP (IU/L)	248	52	45
IgM (mg/dL)	1010	303	281
IgG (mg/dL)	2010	1360	1170
ANA	×5120	×160	×160
AMA (index)	181	205.6	173.2
CRP	<0.1	<0.1	<0.1

AMA (205.6 arbitrary unit), anti-nuclear antibodies (ANA, ×160), gamma-glutamyl transpeptidase (gamma-GTP, 52 IU/L) and IgM (303 mg/dL) levels and a normal value of alkaline phosphatase (ALP). The results of these tests showed satisfactory improvement compared with the data recorded when treatment was limited to UDCA only, thus reflecting the effectiveness of bezafibrate. Total bilirubin, aspartate aminotransferase, alanine aminotransferase, C-reactive protein, IgG levels, leukocyte count and gamma-globulin ratio of serum protein were normal. Serology for hepatitis B and C, alpha-fetoprotein, protein levels induced by the absence of vitamin K or antagonist-II and carcinoembryonic antigen were normal. Magnetic resonance cholangiopancreatography (MRCP) findings were normal. Enhanced CT showed a 3-cm spherical low-density mass in the right lobe of the liver (Figure 1C) corresponding to the mass on FDG PET. To rule out hepatocellular carcinoma, metastatic liver tumor, and cholangiocarcinoma, percutaneous needle biopsy was performed under CT because of the isoechoic lesion by ultrasonography. Histopathological examination showed marked infiltration of lymphocytes, plasma cells, fibrosis and loss of hepatic cells, findings consistent with IPT (Figure 2). Immunohistochemical staining for kappa- and lambda-light chains of immunoglobulin demonstrated the plasma cells contained both chains almost equally. The patient was followed up conservatively, and a repeat CT scan 3 mo after biopsy showed complete regression of IPT (Figure 1D). At this point, laboratory data (Table 1) showed normalization of gamma-GTP level and improvement of AMA (173.2 arbitrary unit) and IgM (281 mg/dL) levels except the same level of ANA (×160).

DISCUSSION

IPT is acute benign lesion that develops throughout the body. Hepatic IPT is relatively unique but has been recognized with increased frequency. It is difficult to make a specific diagnosis based on the findings of laboratory or imaging techniques because there is no specific laboratory marker and radiographic appearance. Therefore the vast majority of reported cases of IPT of the liver have been diagnosed after surgery or at autopsy[8-11]. Recently ultrasonography-guided percutaneus liver biopsy is reported to be useful[10,12].

The CT or MRI appearance of abdominal IPT are variable[13]. Moreover it has been reported that there are no specific signs of the disorder, in spite of the advances in imaging techniques (ultrasonography, endoscopic

retrograde cholangiography and angiography)[14-16], Yoon *et al*[17] reported that the area of low attenuation indicated the presence of chronic inflammatory infiltrations with foamy histiocytes, plasmacytes, and lymphocytes, while areas of iso- or high attenuation representes fibroblastic proliferation. Therefore the low attenuation of IPT on CT findings in our case may indicate a lower component of fibrosis and a higher component of cellular infiltration. Interestingly, to our knowledge, there is only one case of IPT detected by FDG PET, resulting from foreign body in abdominal cavity[18]. The tumor mass was identified as an inflammatory granuloma[18]. FDG PET may reflect inflammatory cell infiltrations in IPT in our case. The protean radiologic manifestations of IPT in our case may be the result not only of the variable morphologic structure including hyalinized collagen bundles or inflammatory cell infiltrations but the dynamic, rapidly changing nature of an inflammatory process.

While the exact etiology of hepatic IPT remains unclear, several mechanisms have been postulated such as infection, immune reaction, intraparenchymal hemorrhage and necrosis, occlusive phlebitis of intrahepatic veins, and secondary reaction to intrahepatic rupture of a biliary radical[19]. Cholangitis arising from various causes including infection with microorganisms, immunological or allergic reactions, and primary sclerosing cholangitis (PSC) has been also considered responsible for the development of hepatic IPT[1-4]. In only two recent reports[5,6], PBC was proposed as a possible cause of hepatic IPT. In both of these cases, hepatic IPT was detected with the simultaneous discovery of PBC or untreated PBC, associated with extremely high levels of biliary enzymes and autoimmune antibodies. Hyper gamma-globulinemia has been also suggested to contribute to the development of IPT[20-22]. The activity of cholangitis or hyper gamma-globulinemia as the etiological factor is, however, cannot be applied to our case because ANA (X5120), ALP (682 IU/L), gamma-GTP (248 IU/L), IgG (2010 mg/dL) and IgM levels (1010 mg/dL), measured when hepatic IPT had not been detected by CT before treatment of UDCA, were much higher than the corresponding laboratory data on admission (Table 1). At the time of regression of IPT, some of the laboratory data (Table 1) improved compared to those on admission probably due to effectiveness of UDCA add-on bezafibrate. However causal association between development or regression of IPT and improvement of laboratory data remains uncertain. Importantly, our case suggests IPT can develop at relatively inactive or recovery phase of PBC differing from previous reports[5,6] and on the contrary, IPT with PBC is probably not the same disease entity or a bystander for PBC.

The other finding exemplifying disease diversity is that the rapid resolution of some cases[4], including our case, may be related to the effectiveness of treatment of the underlying disease whereas in the other reported case[5] IPT regressed even though UDCA did not effectively improve the laboratory data. With regard to treatment of IPT, most reported patients underwent surgical resection because it is difficult to distinguish IPT from malignant liver tumors using only imaging modalities[9,23]. However, the prognosis of hepatic IPT is usually benign. Based on its spontaneous regression property, it is suggested that treatment of IPT should be conservative or noninvasive in patients diagnosed by imaging or needle biopsy[24]. As a treatment protocol, we recommend the use of medications first, such as UDCA and/or bezafibrate for patients with IPT and PBC or PSC after making a definite diagnosis. Once PBC is diagnosed, it is advised to follow such patients regularly and carefully by imaging studies to rule out the development of hepatic IPT during the clinical course, like our case. Since IPT rarely arises in PBC, but hepatocellular carcinoma often develops in cirrhotic patients with PBC, IPT may be one of hepatic tumors arising in PBC patients as a differential diagnosis during the clinical course. The coexistence of hepatic IPT and PBC, however, may be an accidental event. Further cases need to be diagnosed and studied in more detail.

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CASE REPORT

Malignant phyllode tumor metastatic to the duodenum

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Abstract

Phyllode tumor (PT) is extremely rare tumor of the breast. Distant metastasis occurs in 10-20% of patients with malignant phyllode tumor. The most common sites of metastases are the lungs and bones. Although theoretically any organ may have metastasis, an isolated duodenum metastasis has not been documented as yet in the English-language literature. We report herein a case with a isolated duodenal metastasis from PT of breast in a 31 year-old-woman who underwent right mastectomy 4 years before because of the recurrent malignant PT. She presented to our hospital with massive upper gastrointestinal bleeding. Clinical evaluation revealed a huge mass originated from duodenum. Urgent laparotomy and pancreaticoduodenectomy were carried out in order to remove the bleeding duodenal mass. The pathologic examination of the resected specimen showed a malignant spindle cell tumor consistent with metastatic malignant PT. Our case of gastrointestinal bleeding due to an isolated duodenal metastasis as a result of hematogenous spread from malignant phyllode tumor of breast is unique in the English literature and pancreaticoduodenectomy is a curative treatment for patients with isolated duodenal involvement.

Key words: Malignant phyllode tumor; Duodenal metastasis; Pancreaticoduodenectomy

Asoglu O, Karanlik H, Barbaros U, Yanar H, Kapran Y, Kecer M, Parlak M. Malignant phyllode tumor metastatic to the duodenum. *World J Gastroenterol* 2006; 12(10): 1649-1651

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INTRODUCTION

Duodenal metastases of malignant tumors are rare. Lung cancer, renal cell carcinoma, breast carcinoma, and malignant melanoma are the most common primary tumors that metastasize to the pancreaticoduodenal region[1]. The proportion of duodenal metastases from breast carcinoma has been reported in autopsy series as occuring in more than 15% patients, generally associated with extensive systemic spread, and clinical presentations from such metastases have been described in less than 1% of cases[2]. Lobular infiltrating carcinoma of the breast is the histological type that most frequently metastasizes to the duodenum[2]. Infiltrating ductal carcinoma of the breast has also been reported to metastasize to the duodenum[2,3]. However, gastrointestinal metastases from phyllode tumor of breast have rarely been reported in the literature[3,4]. To our knowledge, this report is the first case in the English literature of isolated duodenal metastases of phyllode tumor.

CASE REPORT

A 31-year-old female presented to the surgical clinic with severe upper gastrointestinal bleeding (hematemesis and melena). She was diagnosed of right breast phyllode tumor for which an excision of 4 cm mass was performed 6 years ago. Two years later she had a recurrence, and the excisional biopsy revealed malignant phyllode tumor, and hence she underwent a right mastectomy. She refused postoperative radiation therapy for malignant PT.

At the time she presented to our hospital, she was hypotensive with a blood pressure of 80/60 mm/Hg, and had temperature 36.2℃ (97.1°F), pulse 106 bpm, and respiratory rate 18 breaths/min. She appeared pale, weak, and in distress, with a dry oral mucosa. A huge firm mass was palpable on epigastrium and upper right side on physical examination. Results of laboratory testing on admission showed 8g/dL hemoglobin, 23.6% hematocrit, 38 mg/dL blood urea nitrogen, and 1.8 g/dL albumin level. The results of coagulation tests (prothrombin time and partial thromboplastin time) were within normal limits.

Upper gastrointestinal endoscopic examination revealed clotted blood in the stomach and an ulcerated duodenal tumor without active bleeding. Computed tomography scan showed that the huge mass (10 cm ×15 cm in dimension) originated from duodenum, and compressedthe liver, pancreas, kidney, inferior vena cava, and superior mesenteric vein (Figure 1). Throughout testing, the patient remained hemodynamically unstable. A central line was placed, and

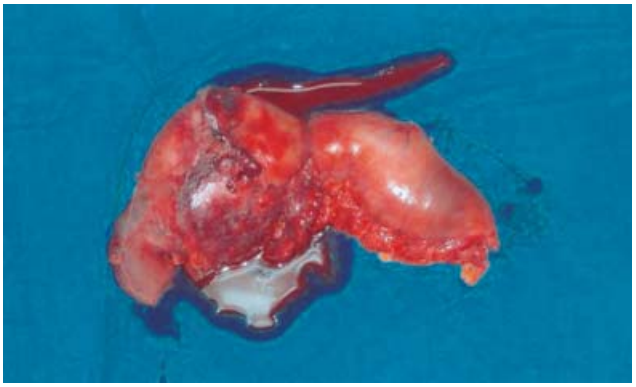

Figure 1 Specimen of the pancreaticoduodenectomy with negative margin.

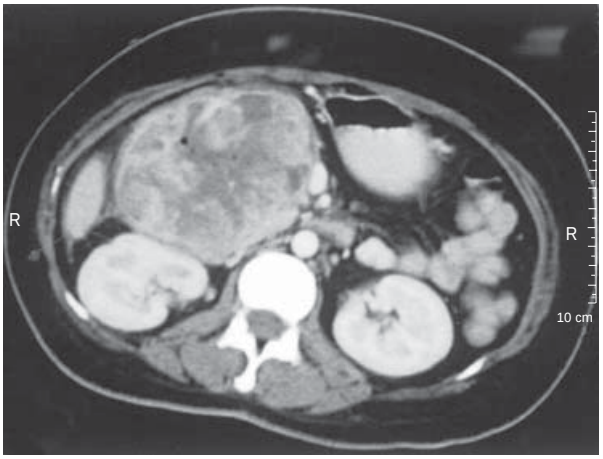

Figure 2 Computed tomography showing a 10 cm x 15 cm oval, mildly hypoechoic tumor with smooth margins.

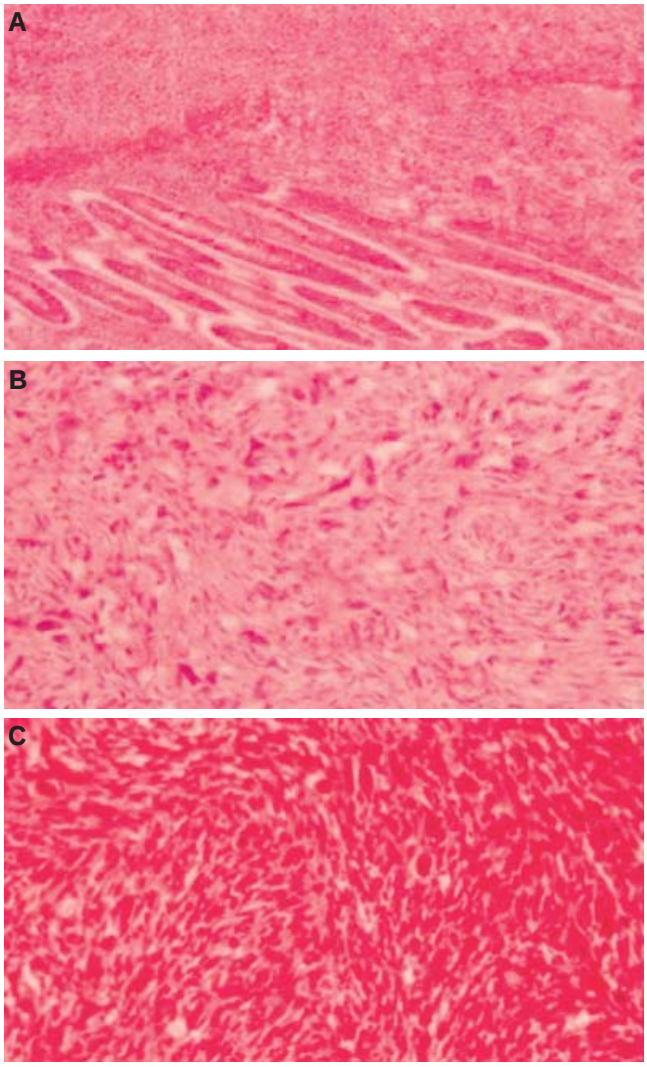

Figure 3 Neoplastic cells showing invasion of duodenal mucosa and submucosa (**A**) (H&E x 100); duodenal tumor consisted of haphazardly arranged pleomorphic spindle cells (**B**) (H&E x 200); breast tumor showing the similar histopathological findings with the duodenal tumor (**C**) (H&E x 200).

she received 8 units of packed erythrocytes. Since it was impossible to stop bleeding by embolisation of the huge mass, an urgent laparotomy and standard pancreaticoduodenectomy were carried out with the suspicion of primary duodenal tumor.

Gross examination of the resected specimen revealed a highly necrotic mass 15 cm×10 cm×10 cm in size within the wall of duodenum and pancreas with tumor-free resection margins (Figure 2). Histopathologically, tumor consisted of spindle cells with prominent nuclear pleomorphism and high mitotic index. Neoplastic cells showed invasion of all layers of duodenum, including mucosa (Figures 3A and 3B). Patient's mastectomy slides were obtained and compared to the duodenal tumor. Both tumors had the similar histopathological findings; atypical spindle cells showing an irregular cellular pattern without having an epithelial component (Figure 3C). Immunohistochemical analysis revealed only vimentin positivity in both tumors. The expressions of pan-cytokeratin, CD-117, smooth muscle actin, S-100, desmin and CD-34, CD-31 and CD-68 were all negative. Thus, a diagnosis of malignant spindle cell tumor consistent with metastatic malignant phyllode tumor was made.

The patient recovered uneventfully, and was discharged on the 10th postoperative day. The patient was alive and in good health 12 mo after surgery, with no evidence of disease at the moment.

DISCUSSION

Phyllode tumor is an uncommon fibroepithelial breast neoplasm that accounts for 0.5-1.0% of female breast tumor cases[5,6]. Phyllode tumors are generally classified into benign, borderline, and malignant tumors[5]. Malignant forms comprise nearly 25% of cases[7]. The rates of local recurrence and distant metastasis in patients with malignant PT range between 20-32% and 25-40%, respectively[5,6]. Positive or close surgical margin and large tumor size are important factors responsible for developing local recurrence[5,8]. They usually metastasize to the lung, pleura, bone, and liver but discrete localizations of metastases such as heart and central nervous system have also been reported[9,11]. The most reliable predictors for development of distant metastasis are stromal overgrowth, nuclear pleomorphism and high mitotic activity[6,7]. Mixed mesen-

chymal components (osteosarcomatosis, chondrosarcom *etc*.) have also been reported to be associated with worse prognosis[7]. Whether both local recurrence and tumor size contribute to the development of distant metastasis is still controversial[5,7,8]. In our case, the tumor size was 4 cm and the patient had a local recurrence 4 years ago.

Distant metastases of PT usually develop within 3 years of initial therapy, they may occur as soon as synchronous presentation or as long as more than ten years[7]. Because lungs are the most common site of metastasis for malignant phyllode tumors, all patients diagnosed with either a borderline or malignant lesion should undergo CT scanning of the chest prior to definitive surgery. This scan should include the liver, although liver metastases are less common. The primary goal for treatment of localized phyllode tumor is to achive wide, negative margin excision, this is generally agreed to include margins of 1 cm or greater.

We would like to stress that the preoperative diagnosis of a secondary gastrointestinal tumor begins with suspicion based on the history of a previously known non-gastrointestinal tumor[12,13]. In the presence of gastrointestinal mass in patients with a history of previous malignant PT, the physician should be aware of the possibility of gastrointestinal metastasis. The second primary tumor is known to be more common than isolated gastrointestinal metastasis. It is crucial to distinguish a metastatic gastrointestinal tumor from a primary one because of the therapeutic and prognostic implications[1,13]. Gastrointestinal metastasis can occur anywhere in the gut and can cause gastrointestinal symptoms, such as intestinal obstruction or bleeding. Radiological and endoscopic evaluations are usually nonspecific. Moreover, even histopathologically it is sometimes very difficult to distinguish a primary gastrointestinal stromal sarcoma or other sarcomas from a metastatic PT. In our case, we applied immunohistochemically a panel of antibodies to both duodenal and breast tumors. Results were not in concordance with other primary tumors. The histopathological findings were very similar to malignant PT, although we could not find any epithelial component, but it is a very well phenomenon that the metastases are commonly of stromal elements only in malignant PT[5].

To our best of knowledge, this is the first report of a PT metastasis to the duodenum. Currently, there are no clinical trials available to assess the best treatment regimen for metastatic PT. Experience with systemic therapy for metastatic PT is anecdotal. Chemotherapy may occasionally afford some palliation[14,15]. Ifosfamide is thought to be the most active agent. There is no demonstrated role for hormonal therapy. Given this limited experience, adjuvant systemic therapy cannot be recommended even for poor prognosis tumors. However, surgical treatment may become an ultimate option for patients who develop complication such as bleeding from the metastatic tumor, as seen in our case. Because of the therapeutic and prognostic implications, it is important to distinguish a metastatic gastrointestinal tumor from a primary one.

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Meetings

MAJOR MEETINGS COMING UP

Digestive Disease Week
107th Annual of AGA, The American Gastroenterology Association
20-25 May 2006
Loas Angeles Converntion Center, California

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
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symposia@falkfoundation.de

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
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7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
Seatlle - Washington
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EVENTS AND MEETINGS IN 2006

10th World Congress of the International Society for Diseases of the Esophagus
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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 Xiaohua Zazhi* 1999; 7: 285-287

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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_2SO_4	0.5 mol/L H_2SO_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_r18 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 $\cdot L^{-1}$	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	A_{260} nm	*A* should be in italic.
	$^bP<0.05$	$^aP<0.05$	In Table, no note is needed if there is no significance instatistics: $^aP<0.05$, $^bP<0.01$ (no note if $P>0.05$). If ther is a second set of *P* value in the same table, $^cP<0.05$ and $^dP<0.01$ are used for a third set: $^eP<0.05$, $^fP<0.01$.
45	**F*=9.87, $^§F$=25.9, $^\#F$=67.4	^1F=9.87,^2F=25.9, ^3F=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	$10\times10\times10cm^3$	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 *et al.*	
72	EcoRI	*Eco*RI	*Eco* in italic and RI in positive. Restriction endonuclease has its prescript form of writing.
73	Ecoli	*E.coli*	Bacteria and other biologic terms have their specific expression.
74	Hp	*H pylori*	
75	Iga	*Iga*	writing form of genes
76	*igA*	IgA	writing form of proteins
77	-70 kDa	~70 ku	

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EDITORIAL

Intestinal sugar transport

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Abstract

Carbohydrates are an important component of the diet. The carbohydrates that we ingest range from simple monosaccharides (glucose, fructose and galactose) to disaccharides (lactose, sucrose) to complex polysaccharides. Most carbohydrates are digested by salivary and pancreatic amylases, and are further broken down into monosaccharides by enzymes in the brush border membrane (BBM) of enterocytes. For example, lactase-phloridzin hydrolase and sucrase-isomaltase are two disaccharidases involved in the hydrolysis of nutritionally important disaccharides. Once monosaccharides are presented to the BBM, mature enterocytes expressing nutrient transporters transport the sugars into the enterocytes. This paper reviews the early studies that contributed to the development of a working model of intestinal sugar transport, and details the recent advances made in understanding the process by which sugars are absorbed in the intestine.

Key words: Glucose; Fructose; SGLT1; GLUT2; GLUT5; Transport; Intestine; Enterocytes; Sugar

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INTRODUCTION

It has been known for decades that two different processes existed for intestinal glucose and fructose absorption. In studies using everted sacs of hamster small intestine, Crane and colleagues found that when the serosal and the mucosal side of the tissue were bathed in glucose, glucose accumulated on the serosal side[1]. This was not the case for fructose, and therefore the absorptive process was labelled as non-concentrating. The involvement of sodium (Na+) in glucose absorption was first proposed by Riklis and Quastel (1958)[2], although studies had previously demonstrated that the decrease in sugar absorption seen in adrenalectomized animals was prevented by adding NaCl to the drinking water[3]. The original Na^+/glucose cotransport hypothesis was presented by Crane in the 1960s[4]. This group showed that active glucose absorption by hamster small intestine required sodium (Na^+) in the bathing medium. Glucose transport was also blocked by ouabain that inhibits the Na^+K^+-ATPase in the basolateral membrane (BLM). This protein is responsible for maintaining the Na^+ gradient in the enterocytes, and driving Na^+ dependent transporters such as the sodium-dependent glucose transporter (SGLT1) in the brush border membrane (BBM).

Crane further developed the model of a mobile carrier in the BBM with two binding sites, one for glucose and one for Na^+[5]. He determined that the continuously maintained outward Na^+ gradient accomplished by the Na^+K^+-ATPase on the BLM was the primary asymmetry providing the driving force for active sugar transport. The phenomenon was considered to be "secondary active transport", as the hydrolysis of ATP was indirectly coupled to glucose transport via this electrochemical gradient. This pioneering work provided the framework for the further characterization of not only glucose transport, but also the transport of other co-transported solutes, and this concept is now considered to be a central tenet in cell physiology.

The pioneering work done by Crane was followed by the electrophysiological studies of Curran and colleagues[6-8] that further characterized transcellular Na^+ transport, and increased the understanding of Na^+ coupled co-transport. Further important advances were made in the 1980s. The method of expression cloning, developed by Wright and colleagues, resulted in SGLT1 being the first eukaryote cotransporter to be cloned. This technique takes advantage of the fact that Xenopus oocytes have the unique ability to translate foreign mRNA, and insert functional transporters into their plasma membrane. The researchers injected rabbit polyA RNA into the oocytes, and observed increases in glucose transport. Utilizing molecular techniques, they were able to isolate a single clone, and use it as a probe to identify human SGLT1[9].

With the continuing development of molecular techniques, the process of intestinal sugar absorption was developed further. The cloning and characterization of the sugar transporters GLUT2[10] and GLUT5[11]

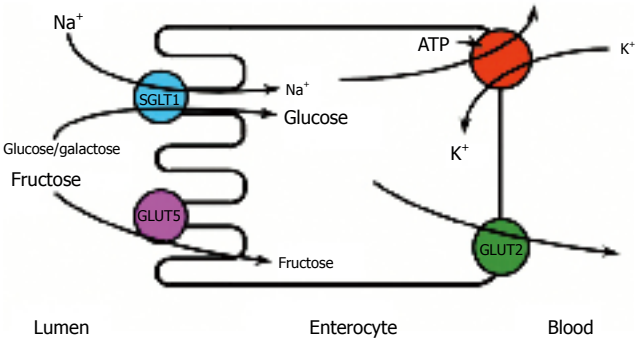

Figure 1 Classical model of intestinal sugar transport (from Wright, 1998). SGLT1 is the sodium dependent glucose/galactose transporter on the brush border membrane (BBM). The Na+K+-ATPase on the basolateral membrane (BLM) maintains the gradient necessary for the functioning of SGLT1. GLUT5 is a facilitative transporter on the BBM which transports fructose into the cell. GLUT2 on the BLM transports glucose, galactose and fructose out of the cell.

Table 1 Affinity constants of sugar transporters

Transporter	Km
SGLT1 (BBM)	Glucose:0.1-0.6 mmol/L (Wright *et al*, 2003)
GLUT2 (BLM)	Glucose:> 50 mmol/L Fructose: 66 mmol/L (Walmsley *et al*, 1998)
GLUT5 (BBM)	Fructose: 6-14 mmol/L (Walmsley *et al*, 1998)

soon followed, and the molecular aspects of the process of sugar absorption across the BBM and BLM were characterized. What is now known as the *"classical model of sugar absorption"* was developed (Figure 1), with SGLT1 actively transporting glucose and galactose across the BBM, and fructose crossing the BBM by facilitative diffusion via GLUT5. GLUT2, a low affinity transporter, was responsible for transporting these sugars across the BLM via facilitative diffusion.

SGLT1

The sodium/glucose cotransporter family (SLCA5) contains more than 200 members found in both animal and bacterial cells. There are 11 human genes expressed in tissues ranging from epithelia to the central nervous system. Hediger *et al* (1987) cloned the SGLT1 gene[9]. The cotransporter is a 73 kDa membrane protein with a Na^+-glucose stoichiometry of 2:1. The transporter has the same affinity for both glucose and galactose (Table 1), and transport is phloridzin sensitive (Ki = 0.1 mmol/L) (Table 2). The membrane topology of SGLT1 was determined using N-glycosylation scanning mutants and hydropathy plots. The transporter contains 14 transmembrane alpha-helices, with an extracellular N and C terminus[12-14]. The transporter contains a single glycosylation site between transmembrane 5 and 6; however, glycosylation is not required for functioning of the protein. Phosphorylation sites have been identified between transmembrane helices 6 and 7, and between transmembrane helices 8 and 9[15]. The importance of SGLT1 phosphorylation will be discussed below. SGLT1 is found in brush border membrane of mature enterocytes in the small intestine, with very small amounts detectable in the kidneys and the heart. Recently, SGLT1 has also been detected in the luminal membrane of intracerebral capillary endothelial cells, where it may participate in the transport of glucose across the blood-brain barrier[16].

Table 2 Inhibitors of sugar transporters

	Inhibitors
SGLT1	Phloridzin
GLUT2	Cytochalasin B Phloretin
GLUT5	Glyco-1, 3-oxazolidin-2-thiones, -ones (Girniene *et al*, 2003)
Na^+K^+-ATPase	Oubain

The process of intestinal sugar transport has been reviewed by Wright *et al* (2003)[17]. Initially, on the luminal side of the BBM, two Na^+ ions bind to SGLT1 and produce a conformational change that permits sugar binding. Another conformational change allows the substrates to enter the enterocyte. The sugar, followed by the Na^+, dissociates from SGLT1 because the affinity of the cytosolic sites is low, and also because the intracellular concentration of Na^+ is low (10 *vs* 140 mEq/L). Sodium can be replaced by H^+ or Li^+, but the affinity for glucose then decreases (apparent Michaelis affinity constant (Km) = 4-11 mmol/L).

The Na^+K^+-ATPase in the BLM is responsible for maintaining the Na^+ and K^+ electrochemical gradients across the cell membrane. The Na^+K^+-ATPase contains a 110 kDa α_1 catalytic subunit, as well as a highly glycosylated 55 kDa β_1 subunit[18,19]. The Na^+K^+-ATPase is up-regulated in experimental diabetes[20] and experimental ileitis [21], with post-translational modifications playing an important role in its regulation. This up-regulation may influence the functioning of SGLT1 and subsequently alter intestinal sugar uptake in these conditions.

Panayotova-Heiermann and Wright (2001) expressed various cDNA constructs of rabbit SGLT1 in Xenopus oocytes in order to determine the helices involved in sugar transport[22]. They found that helices 10-13 form the sugar permeation pathway for SGLT1, and they speculated that the N terminal region of SGLT1 (helices 1-9) may be required to couple Na^+ and glucose transport.

A number of factors influence the transport function of SGLT1 (Table 3). For example, the regulation of SGLT1 by dietary sugars was examined by Miyamoto *et al* (1993)[23]. Using Northern blotting, they showed that SGLT1 mRNA was increased by feeding rats 55% sugar diets containing glucose, galactose, fructose, mannose, xylose, or 3-O-methylglucose. Because 3-O-methylglucose is transported by SGLT1, but is not metabolized, and because SGLT1 does not transport fructose, mannose or xylose, the up-regulation of SGLT1 does not appear to

Table 3 Factors influencing transporter function

Factors influencing SGLT1 function	Factors influencing GLUT2 function	Factors influencing GLUT5 function
Foxl1 (Katz *et al*, 2004)	PKCbII(Helliwell *et al*, 200b)	cAMP (Mahraoui *et al*, 1994)
AMPK (Walker *et al*, 2004)	p38(Helliwell *et al*, 200b)	p38 (Helliwell *et al*, 200b)
PKA (Wright *et al*, 1997)	ERK(Helliwell *et al*, 200b)	ERK (Helliwell *et al*, 200b)
PKC (Wright *et al*, 1997)	PI3K(Helliwell *et al*, 200b)	PI3K (Helliwell *et al*, 200b)
RS1 (Veyhl *et al*, 1993)	mTOR (Helliwell *et al*, 2003)	TNF-α (Garcia-Herrera, 2004)
HNF-1 (Martin *et al*, 2000)	AMPK (Walker *et al*, 2004)	
Sp1 (Martin *et al*, 2000)		
Hsp 70 (Ikari *et al*, 2002)		
TGF-b (Ikari *et al*, 2002)		

depend on either metabolism or transport of the sugar in question (Table 3).

Wright *et al* (1997) evaluated the role of SGLT1 phosphorylation[24]. They expressed rabbit SGLT1 in Xenopus oocytes, and activated protein kinase A (PKA) or protein kinase C (PKC) using 8-Br-cAMP and *sn*-1, 2-dioctanoylglycerol (DOG), respectively. PKA activation increased glucose transport by approximately 30%, while PKC activation reduced transport by 60%. The change in maximal transport rates (Vmax) was accompanied by alterations in the number of transporters in the plasma membrane, as well as changes in the surface area of the membrane. Since endocytosis and exocytosis alter the membrane surface area, the findings of the effects of PKA and PKC on SGLT1 suggest that these proteins may be involved in the regulation of glucose transport.

Similar increases in Vmax were obtained with activation of PKA in oocytes expressing rabbit, human, and rat SGLT1 isoforms. The effects of PKC, however, may depend on the sequence of the co-transporter, as there are conflicting reports of the effect of PKC on glucose transport. For example, PKC decreases sugar transport in Xenopus oocytes expressing rabbit and rat SGLT1[25,26], and increases sugar transport when human SGLT1 is expressed[27].

Veyhl *et al* (1993) demonstrated the presence of an intracellular regulatory protein (RS1) that may modify the activity of SGLT1[28]. Co-expression of RS1 and SGLT1 in Xenopus oocytes reduced both the Vmax for glucose transport as well as SGLT1 protein levels[26]. Plasma membrane surface area was also reduced, suggesting alterations in the endo- and/or exo-cytosis of membrane vesicles.

To investigate the role of intracellular trafficking in sugar transport, oocytes were injected either with cRNA of wild type, or mutant dynamin. Dynamin is a motor protein involved in receptor-mediated endocytosis, vesicle recycling, caveolae internalization and vesicle trafficking from the Golgi[29]. The inhibition of glucose uptake by RS1 was largely reduced after co-expression of the mutant dynamin protein. The investigators concluded that RS1 modulates dynamin-dependent trafficking to the BBM of intracellular vesicles containing SGLT1.

In order to further characterize the role of the RS1 protein in the regulation of intestinal glucose transport, a knockout mouse lacking the RS1 protein was recently developed: Osswald *et al* (2005) showed that RS1-/- mice developed obesity associated with increases in food intake, glucose transport and SGLT1 expression in the small intestine[30]. The effect of RS1 deficiency was tissue-specific and occurred through post-transcriptional mechanisms, as SGLT1 mRNA abundance was unchanged. These researchers speculated that therapeutic strategies aimed at reducing glucose uptake by increasing RS1 might potentially be used to treat obesity.

Heat shock proteins (hsp) may also play a role in regulating SGLT1 function (Table 3). A study done in renal epithelial cells showed that treatment with hsp70 increased glucose transport, but not the abundance of SGLT1 protein[31]. The increase in sugar transport was inhibited by an antibody directed against transforming growth factor β (TGF- β), leading the investigators to explore the effect of TGF-β on SGLT1: there was an increase in SGLT1 activity, as well as an increase in hsp70 protein when TGF-β was added to the culture media. The researchers speculated that hsp70 might stabilize SGLT1 expression in the membrane. This concept was supported by confocal microscopy studies, which demonstrated that TGF-β appears to move both SGLT1 and Hsp70 near the apical membrane site. However, the mechanism by which TGF-β exerts these effects on Hsp70 and SGLT1 is not known.

It is not known if SGLT1 is localized to specific microdomains within the BBM. In renal proximal tubular cells, SGLT1 was found in detergent-resistant membrane microdomains, also referred to as "lipid rafts"[32]. In this model, the absence of vimentin, an intermediate filament component, decreased glucose transport and caused a reduction in the amount of SGLT1 protein in these membrane microdomains. Furthermore, fluidization of the plasma membrane, or depleting the membrane of cholesterol, dramatically decreased glucose transport. This suggests that the activity of SGLT1 is optimal in a microenvironment characterized by low fluidity. Further research is required to determine if SGLT1 is localized to lipid rafts in the intestinal BBM, if this localization is mandatory for the functioning of SGLT1, and what are the factors that may regulate the localization of SGLT1 to these specialized microdomains.

The transcription factors hepatocyte nuclear factor-1 (HNF-1) and Sp1 may also regulate SGLT1. Martin *et al*, (2000) characterized the promoter region of the human SGLT1 gene by transfecting reporter constructs into

Caco-2 cells[33]. They demonstrated that three cis-elements, HNF-1, and two sites on the SGLT1 promoter ("GC boxes" to which Sp1 binds are required for maintaining basal transcription of SGLT1. Members of the Sp1 family bind to the GC boxes, and in the presence of HNF-1, synergistically activate the promoter. Some members of the Sp1 family have been implicated in tissue- and developmental- specific regulation of genes[34,35]. HNF-1 alters the expression of many small intestinal genes, including sucrase-isomaltase (SI) and lactase. It has also been implicated in the diurnal regulation of SGLT1 in rodents[36]. If HNF-1 was required for basal SGLT1 expression, glucose-galactose malabsorption would have been expected to be observed. Of interest, HNF-1 knockout mice experience life-threatening effects on the hepatic and renal systems, but no adverse effects on the gastrointestinal tracts were reported.

Katz *et al* (2004) identified a link between a mesenchymal factor and the regulation of a specific epithelial transport process[37]. *Foxl1* is a winged-helix transcription factor expressed in the mesenchymal cells bordering the crypts in the small intestine. Using the everted sleeve method coupled with Western blotting, the researchers showed that homozygous *Foxl1* null mice had decreased intestinal glucose uptake and decreased levels of SGLT1 protein. Growth retardation and abnormal small intestinal architecture were observed, characterized by short, broad and irregular villi. The effect of the loss of Foxl1 on SGLT1 was specific, as no changes in the expression of SI, lactase, GLUT2 or Na^+K^+ATPase were observed.

The transport of water across the intestinal epithelia has always been a subject of curiosity. The discovery of aquaporins by Preston *et al* (1992) renewed interest in this topic[38]. Although aquaporins may account for a portion of water absorption in the intestine, Wright and colleagues investigated the coupling of water transport to active Na^+-glucose cotransport. Overexpression of human or rabbit SGLT1 in Xenopus oocytes revealed that activation of the transporter was associated with an increase in volume of the cell (reflecting water transport), and this effect was blocked by phlorizin. If oocytes expressing SGLT1 were incubated in a sugar-free solution, no change in oocyte volume was observed. The increase in volume could be accounted for by a stoichiometry of two Na+ ions, one glucose molecule, and 249 water molecules[39]. The transport of water was independent of the osmotic gradient across the membrane, and may be a consequence of the conformational changes in SGLT1 that occur during Na^+/glucose transport. A channel formed by five C-terminal transmembrane helices of SGLT1 is thought to transport not only water, but also urea[22,40].

It is important to note, however, that others have suggested that local osmotic gradients fully account for water fluxes. Lapointe *et al* (2002) present evidence from experiments using Xenopus oocytes expressing human SGLT1 that contradicts the water cotransport hypothesis and suggests the passive movement of water across the plasma membrane[41].

Oral rehydration therapy (ORT) was developed in the 1970's to treat diarrheal dehydration[42]. The introduction of this very simple treatment has reduced mortality due to diarrhea in children under five years of age from 5 million in 1978, to 1.3 million in 2002 (http: //www.who.int/child-adolescent-health.2002; Victora *et al*, 2000). This success led to the proclamation that ORT was the most important medical advance of the 20th century, and earned Dr Hirschhorn and colleagues the first Pollin prize for Pediatric Research.

The goals of ORT are to replace fluids and minimize malnutrition. Starting in 1978, solutions containing a mixture of glucose, sodium, chloride, potassium and citrate were being commonly distributed by the World Health Organization. In fact, 800 million packets of ORT were distributed worldwide in 1991-1992[43]. Interestingly, controversy now exists over the optimal formulation, with reduced osmolarity formulas, rice-based formulas, or formulas containing amylase-resistant starch being favored by some researchers. For example, hypoosmolar rice-based formulas produced better results in cholera patients when compared to standard formulas[44]. The advantages of this rice-based formula are that it is cheap, offers more calories than standard ORT, and rice is readily available in many cholera-stricken regions. ORT formulas containing amylase-resistant starches may be favored due to the production of short chain fatty acids, which increase colonic Na+, Cl- and fluid absorption, and reduce colonic secretions[45-47]. These effects counteract the fluid losses and hypersecretion seen with infectious diarrhea.

Several features of carbohydrate digestion contribute to the efficacy of ORT. This life-saving therapy is based on the ability of SGLT1 to co-transport water. Na^+-dependent glucose absorption is not affected by the increased cAMP levels commonly seen with infections such as Cholera, and therefore this physiological fact can be exploited as a means to achieve glucose, Na^+ and water absorption, even in the presence of chloride and water secretion. Also, the oral administration of glucose or carbohydrates up-regulates SGLT1, thereby further increasing the intestinal transport of glucose, Na^+ and water. Since ORT is commonly administered to infants, it is important to utilize a transport system that is expressed and functional early in life. SGLT1 is expressed prenatally[48], and is functional at birth, making it an ideal candidate. In contrast to glucose, the use of fructose in these ORT solutions is contraindicated, as GLUT5 in the BBM is only expressed following weaning[49].

Glucose-galactose malabsorption (GGM) is a very rare autosomal recessive disease characterized by severe life-threatening diarrhea in the neonate, that resolves when the offending sugars (glucose, galactose and lactose) are removed from the diet[15,50]. Normal intestinal mucosal histology is observed, while phlorizin binding studies show reductions in SGLT1 protein in the BBM[51,52]. Electrophysiological studies and freeze fracture electron microscopy showed that this disease is due to a failure of the SGLT1 protein to traffic normally to the BBM[53]. Approximately 300 cases of GGM have been identified worldwide, affecting all racial and ethnic groups. The majority (70%) of patients are female, with two thirds coming from a consanguineous relationship[17]. Unlike genetic diseases like cystic fibrosis, in which a single

mutation accounts for most cases, in GGM each patient appears to have a unique mutation, ranging from missense mutations, to frame-shift mutations, to split-site-conservative mutations which produce truncated protein and mistrafficking of SGLT1 to the BBM[53-56]. This variety of mutations limits the usefulness of genetic testing for GGM, although prenatal diagnosis in a family at risk may be possible.

GGM is a difficult condition to diagnose. If GGM is suspected, the first step is the elimination of glucose, galactose and lactose from the infant's diet. Oral glucose tolerance tests in GGM patients produce a flat glucose response in the blood, as glucose is malabsorbed in the intestine. A hydrogen breath test performed following oral glucose produces abnormally high concentrations of H_2 in the breath (> 20 ppm) indicating glucose malabsorption, while oral fructose tolerance tests produce normal results. GGM is treated by using glucose-, galactose- and lactose-free formulas, and by eliminating the offending sugars from the diet[17]. Normal growth and neurological development are possible if infants receive fructose-based formula, and if dietary counselling is available[15,57].

GLUT5

GLUT5 is a 43 ku protein, with 12 transmembrane domains and intracellular N and C terminals. It was cloned by Burant and colleagues in 1992. GLUT5 was expressed in Xenopus oocytes, and its substrate specificity and kinetic properties were determined using radiolabelled substrates. Northern and Western blotting demonstrated the presence of GLUT5 in human small intestine and testis. Further work by Davidson *et al* (1992) focused on the developmental expression of GLUT5 in the human and fetal small intestine[58]. GLUT5 mRNA levels increase with age, and are highest in the adult small intestine. In adults, GLUT5 was localized to the BBM by Western blotting. Immunohistochemical techniques confirmed this finding, and further localized GLUT5 to only the mature enterocytes populating the upper half of the villus. This luminal localization provided further support for the notion that GLUT5 played a role in the intestinal uptake of dietary sugars.

Rand *et al*, (1993) characterized the expression of GLUT5 in rats[49]. GLUT5 mRNA was detected in the small intestine, kidney and brain by Northern blotting, and in the small intestine, testis, adipose and skeletal muscle using *in situ* hybridization. In the intestine, a proximal-distal gradient was observed, with GLUT5 mRNA levels being higher in the proximal small intestine when compared to the distal small intestine. A distinct pattern of expression was seen along the crypt-villous axis, with mRNA being highest in midvillus region.

The functional domain of GLUT5 was investigated by Buchs *et al*[59]. In order to ensure proper transport and insertion into the membrane, GLUT5-GLUT3 chimeras were created, and included various combinations of the GLUT3 and GLUT5 peptides. These chimeric GLUTs were expressed in Xenopus oocytes. This enabled the researchers to conclude that the regions necessary for fructose transport lie between the amino terminus and the third transmembrane domain, and between the 5th and 11th transmembrane domain.

The response of GLUT5 to dietary sugars was investigated by Miyamoto *et al*[23]. In this study, they fed sugar-enriched diets (55% D-glucose, D-galactose, 3-0-methylglucose, D-fructose, D-mannose or D-xylose) to male Sprague Dawley rats for 5 d. Northern blotting on intestinal samples showed that GLUT5 mRNA was increased only by dietary D-fructose, and was unaffected by the other sugars (Table 3). This was consistent with the suggestion that GLUT5 was a high affinity fructose transporter. Subsequent work by David *et al* (1995) showed that in 16 d old rats, feeding fructose but not glucose increased fructose uptake[60]. Furthermore, while both fructose and sucrose feeding enhanced absorption in older (21-60 d old) animals, glucose alone had no effect.

An interesting study by Castello *et al* (1995) demonstrated that GLUT5 mRNA in rats followed a circadean rhythm, with a 12-fold increase in mRNA at the end of the light cycle as compared to early in the light cycle[61]. BBM GLUT5 protein followed a similar pattern, which is also observed for other small intestinal genes such as BBM SI and lactase[62]. Although this pattern was thought to be a reflection of rodent feeding patterns, Corpe *et al* (1996) found that gene expression is hard-wired, because GLUT5 is up-regulated prior to the onset of feeding, even in the absence of dietary fructose[63]. Shu *et al* (1998) noted that this circadean rhythm was not developed at the time of weaning, possibly because the feeding patterns of suckling rats do not follow the same adult nocturnal patterns[64]. This diurnal variation in adult animals needs to be carefully considered when designing experiments in which levels of GLUT5 are measured, by performing studies in the morning in the early post-prandial period.

The regulation of GLUT5 was studied by Mahraoui *et al* (1994) using Caco-2 cells[65]. Treatment of the cells with forskolin, which stimulates adenylate cyclase and raises intracellular cAMP levels, increased fructose uptake 2-fold, and increased GLUT5 protein and mRNA 5-fold and 7-fold, respectively. Matosin-Matekalo *et al* (1999) used Caco2 cells transfected with a GLUT5 promoter inserted up-stream of the luciferase reporter gene[66]. They found that a region of the GLUT5 promoter binds the thyroid hormone receptor/retinoid X receptor heterodimers, and that both triiodothyronine (T3) and glucose increase GLUT5 mRNA.

Helliwell *et al*[67] looked at the regulation of GLUT5 by a number of signals that have well-established roles in the regulation of sugar transport. Isolated loops of rat jejunum were perfused with activators and inhibitors of the ERK, p38 and PI3K pathways. The findings suggest that the p38 pathway stimulates fructose transport, while the ERK and the PI3K pathways had little effect on fructose transport (Figure 3). Extensive cross-talk occurs between the pathways. For example, inhibiting the ERK pathway with PD98059 increased the sensitivity to anisomysin, which stimulates the p38 pathway. The authors concluded that the three pathways have the potential to regulate fructose transport during the digestion and absorption of a meal. They suggested that future work should focus on

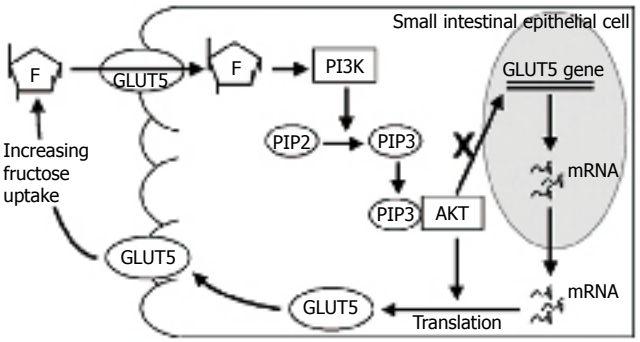

Figure 2 Proposed role of PI3K/Akt signalling pathway in the regulation of GLUT5 synthesis and trafficking (from Cui *et al*, 2005). Abbreviations: *F* = fructose, PIP3 = phosphatidylinositol-3, 4, 5-triphosphate, PIP2 = phosphatidylinositol-4, 5-biphosphate, PI3K = phosphatidylinositol 3-kinase.

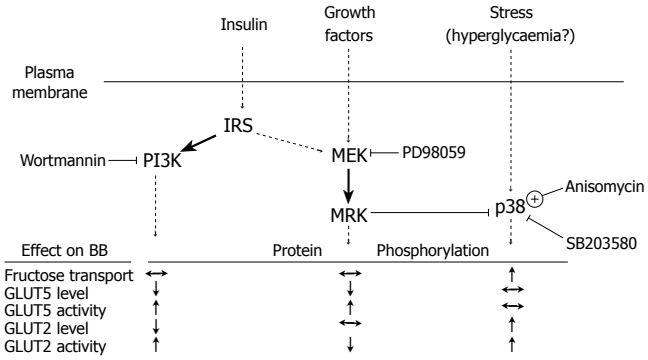

Figure 3 The regulation of BBM fructose transport by the PI3K, ERK and p38 MAPK signalling pathways (from Helliwell et al, 2000). Abbreviations: IRS=insulin regulatory subunit, ERK=extracellular regulated kinase, MEK=mitogen activated kinase kinases, PI3K=phosphatidylinositol 3-kinase, PD98059=ERK/MEK inhibitor, SB203580=p38 MAPK inhibitor, anisomycin=activator of p38 and jun kinase pathways.

determining the hormones that influence these pathways, and the molecular mechanisms that regulate the levels and activities of the sugar transporters.

Gouyon *et al*[68] used Caco-2 cells to investigate the mechanism by which fructose increases GLUT5 expression. Although both glucose and fructose increased the activity of the GLUT5 promoter, the effect of fructose was stronger and associated with higher cAMP concentrations. If cAMP signalling was blocked by a protein kinase A inhibitor, extensive GLUT5 mRNA degradation occurred, suggesting that the mRNA stability was influenced by PKA. A sugar response element was identified in the GLUT5 promoter. PABP-interacting protein 2, which represses translation[69,70], was identified as a component of GLUT5 3'-UTR RNA-protein complex, where it may act to destabilize transcripts. The differences between the effects of glucose and fructose on GLUT5 expression may be attributed to variations in their ability to increase cAMP levels, and to modulate the formation of protein complexes with GLUT5 3'-UTR.

Infection may also regulate fructose transport. Intravenous administration of Tumor necrosis factor-α (TNF-α) in rabbits significantly reduced jejunal fructose transport and GLUT5 protein[71]. This inhibition was related to the secretagogue effect of TNF-α, and both nitric oxide and prostaglandins were implicated in the inhibition of fructose uptake. Adaptive immunity also influences the expression of a number of developmentally regulated genes. In mice lacking in adaptive immunity (B cell deficient recombination-activating gene [RAG] mice), RNase protection assays demonstrated that GLUT5 was increased[72].

Recent advances have been made in understanding the signalling pathways involved in the regulation of GLUT5. Cui *et al*[73] have demonstrated that cAMP stimulates fructose transport in the neonatal rat intestine. Perfusing fructose (100mM) plus 8-bromo-cAMP in 22-d-old rats increased fructose uptake rates, while an inhibitor of adenylate cyclase abolished this effect. Despite the presence of two cAMP response elements in the human GLUT5 promoter region[65], GLUT5 mRNA was not affected by cAMP treatment. Interestingly, inhibitors of PKA did not prevent the fructose-associated increases in transport, suggesting that cAMP modulates fructose transport independent of PKA (Table 3).

Subsequent work by the same group has shown that fructose-induced increases in neonatal rat intestinal fructose uptake involve the PI3K/Akt signalling pathway[74]. In this study, PI3K inhibitors (wortmannin and LY94002) and an Akt inhibitor (SH-5) abolished the increase in fructose uptake, as well as the abundance of GLUT5 protein (but not mRNA) seen following fructose (100 mmol/L) perfusions in neonatal rats. Fructose perfusion increased phosphatidylinositol-3, 4, 5-triphosphate (PIP3), the product of PI3K, in the mid to upper regions of the villus, where most of the GLUT5 was located. The authors suggest that the PI3K/Akt pathway may be involved in the synthesis and/or recruitment of GLUT5 to the BBM in response to luminal fructose (Figure 2).

GLUT2

GLUT2 is a low affinity, high capacity facilitative transporter in the BLM that transports glucose, fructose, galactose and mannose[10,63,75-77]. It has 12 transmembrane domains, with intracellular N and C terminals. Using immunohistochemistry, Thorens *et al*[75] showed that GLUT2 expression increases as enterocytes migrate up from the crypt to the villous tip. Amino acid sequences in transmembrane segments 9-12 are primarily responsible for GLUT2's distinctive glucose affinity, whereas amino acid sequences in transmembrane segments 7-8 enable GLUT2 to transport fructose[78].

Luminal sugars[23,79] or vascular infusions of glucose or fructose[80,81] stimulate GLUT2 expression and activity. The response of GLUT2 to dietary sugars was investigated by Miyamoto *et al*[23]. In this study, they fed sugar-enriched diets to male Sprague Dawley rats for 5 d. GLUT2 mRNA was up-regulated by glucose, fructose and galactose. GLUT2 modulation required intracellular metabolism of the sugar, as it was unaffected by 3-O-methyglucose, a non-metabolized glucose analog.

In a study by Cui *et al*[82], the jejunum of 20-d-old anaesthetized rat pups was perfused with 100 mmol/L glucose or fructose. Increases in GLUT2 mRNA were

observed, and this effect was inhibited by actinomycin D, an inhibitor of transcription. Cycloheximide, an inhibitor of translation, did not block the enhanced expression of GLUT2 mRNA, suggesting that the synthesis of new proteins is not necessary for increases in GLUT2 mRNA. Because levels of GLUT2 mRNA and protein are tightly correlated, the regulation of GLUT2 may be transcriptional[83].

PASSIVE UPTAKE

For years there has been considered to be a "passive" component to sugar absorption. This traditional view has been challenged, with the suggestion that the kinetic characteristics of sugar uptake could also be described by a second high affinity, high capacity BBM transporter[83]. In order to better understand the new "GLUT2 trafficking model", we need first to consider the classic "passive permeation" model.

The fact that SGLT1 saturates at 30-50 mmol/L glucose was inconsistent with the observation that intestinal glucose absorption increases linearly with increases in luminal glucose concentrations up to several hundred millimolar[85]. This finding suggests the presence of two components: an active, phloridzin-sensitive component, and a phloridzin-insensitive, possibly passive component that does not appear to be saturable. Some studies have suggested that the "passive" component played a large role in glucose transport at high glucose concentrations, in some models contributing 3-5 times as much as the active component[86,87].

The passive component of glucose transport was characterized by Pappenheimer and Reiss (1987)[88]. The observation that high rates of water absorption accompany glucose absorption[89] provided a rationale for proposing that glucose in the intercellular spaces provided an osmotic force that resulted in bulk flow of nutrients. Pappenheimer and Reiss (1987) perfused isolated segments of hamster small intestine with 10-25 mmol/L glucose[88]. Structural studies using electron microscopy and freeze fracture analysis revealed large dilatations within junctions following glucose perfusion. They concluded that Na^+-coupled transport of solutes from the intestinal lumen to the cytosol of the enterocytes provides the driving force for the absorption of fluid and nutrients, and triggers the widening of intercellular junctions, thereby promoting the bulk absorption of nutrients by solvent drag. They calculated that the contribution of solvent drag exceeds that of active transport at luminal glucose concentrations greater than 250 mmol/L. Madara and Pappenheimer (1987) further demonstrated that the transport of glucose via SGLT1 caused dilatation of the tight junctions[90]. They concluded that passive glucose absorption is a result of paracellular solvent drag, and is indeed SGLT1 dependent. Therefore, like the more recent model suggested by Loo et al (2002)[39], these investigators suggest that the transport of water is SGLT1-dependent. However, this theory suggests the presence of a non-specific route, which could potentially allow passage of several solutes.

Ferraris and Diamond proposed an alternative theory, in which paracellular flow is negligible[91,92]. Based on the determination of up-dated kinetic constants for glucose absorption, and the determination of the usual free glucose concentrations in the intestinal lumen, they concluded that SGLT1 fully accounts for glucose absorption. Much of their work is based on studies examining long-term dietary adaptations, from which they concluded that BBM transporters are matched to dietary intake. Their model is supported by the findings of Lane *et al*[93], who demonstrated that paracellular flow in unanaesthetized dogs did not account for more than 2%-7% of total absorption.

Much of the controversy surrounding the role of the paracellular pathway stems from the discrepancies between the estimated concentrations of glucose in the intestinal lumen. Pappenheimer and Reiss[88] based their calculations on luminal glucose concentrations of 300 mmol/L, whereas Ferraris *et al*[92] did a detailed analysis of luminal glucose concentrations and concluded that physiological luminal values ranged from 0.2-48 mmol/L. Pappenheimer[94] used the rate of membrane hydrolysis of maltose to indirectly measure luminal glucose concentrations. They also point out that the techniques used by Ferraris *et al* (1990)[92], which involve glucose analysis of luminal contents, will underestimate the concentration found at the membrane following hydrolysis by disaccharidases. The actual physiological levels of glucose in the lumen remain a subject of debate.

The concept of more than one transport system for glucose was suggested by Malo[95]. Using human fetal and adult BBM vesicles, curvilinear Eadie-Hofstee plots and sodium activation curves were obtained when glucose concentrations were varied in the medium. These findings, coupled with determinations of phloretin-sensitive and -insensitive components, and the ability of the BBM vesicles to transport 3-O-methylglucose, suggested the presence of two transport systems: a high-affinity low-capacity system and a low-affinity high-capacity system[95,96]. This agrees with the observation that Na^+/glucose cotransport saturates at 30-50 mmol/L, yet absorption is linear from 50 mmol/L to several hundred mmol/L[85].

Although this concept was proposed many years ago, it was not until recently that interest in the area has re-emerged due to an alternative model of intestinal glucose transport proposed by George Kellett and his colleagues at the University of York, and by Edith Brot-Laroche and her colleagues at the University of Paris. Let us briefly explore this fascinating "voyage of discovery".

GLUT2 IN BBM

Several years ago, GLUT2 was detected in the BBM of enterocytes in diabetic animals, although at the time this was interpreted to be a pathological event[63]. More recently, Kellett and his colleagues proposed a model by which BBM SGLT1, in the presence of luminal glucose, promotes the rapid insertion of GLUT2 into the BBM via PKCβII and the MAP kinase-dependent signal transduction pathways[67,97,98]. PKCβII is located in the terminal web of mature enterocytes in the upper part of the intestinal villus[99]. Interestingly, these are the same cells that are responsible for glucose absorption.

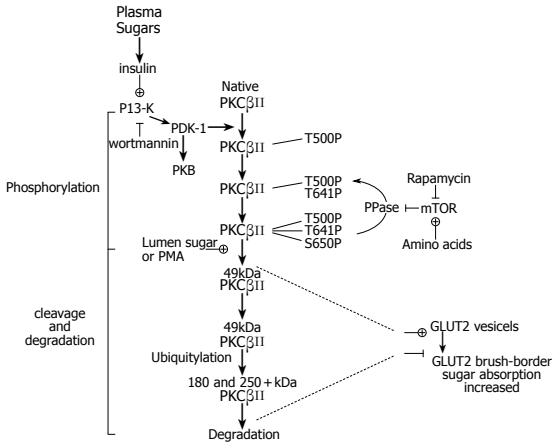

Figure 4 Potential signaling pathways for the regulation of GLUT2-mediated sugar absorption by insulin and amino acids through the control of PKC βII activity. (from Helliwell et al, 2003). Abbreviations: PMA= phorbol 12-myristate 13-acetate, PDK-1=protein-dependent kinase-1, PI3K=phosphatidylinositol-3-kinase, PKB=protein kinase B, mTOR=mammalian target of rapamycin, PKCβII=protein kinase C βII.

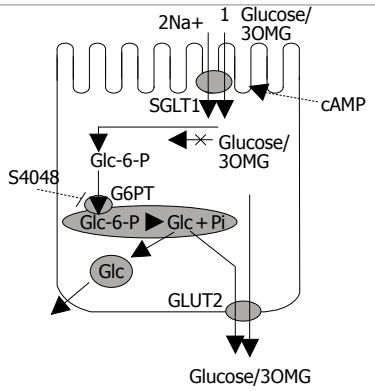

Figure 5 Proposed alternative mechanism for intestinal glucose transepithelial transport (from Stumpel et al, 2001). Abbreviations:Glc=glucose, Glc-6-P=glucose-6-phosphate, G6PT=glucose-6-phosphate translocase, 3-OMG=3-O-methylglucose, S4048=inhibitor of glucose-6-phosphate translocase, Pi=phosphate.

Using a luminal perfusion model, Kellett and coworkers measured the phloretin-insensitive (SGLT1) component and phloretin sensitive component (GLUT2) of glucose transport. They also showed using Western blotting that BBM GLUT2 increased 2.2 fold when the concentration of glucose in the perfusate increased from 0 to 100 mmol/L. Similarly, the BBM level of PKCβII increased with increasing glucose concentrations. This finding, coupled with the observation that PKCβII shows a saturation response and has a Km similar to that of SGLT1 (21-27 mmol/L), suggests that PKCβII is an important signal in the recruitment of GLUT2 to the BBM. PKCβII levels also correlate with levels of GLUT2 in the BBM, and this association offers further support for its role in the recruitment of GLUT2 to the BBM. The ability of the PKC inhibitor "chelerythrine" to block phorbol 12-myristate 13-acetate (PMA)-stimulated fructose transport and GLUT2 abundance in the BBM also supports this mode l[97].

Kellett's working hypothesis proposes that before a meal, when luminal concentrations of glucose are low, GLUT2 levels in the BBM are also low, which would minimize the escape of glucose from the cell (any glucose that did escape would be recycled by SGLT1, which can transport it against the glucose gradient). Once a meal is ingested and BBM enzymes hydrolyse disaccharides, luminal glucose concentrations increase. Glucose uptake via SGLT1 causes increases in enterocyte volume due to a rise in osmolarity (and the co-transport of water molecules by SGLT1), and may trigger the entry of Ca+, activating PKCβII and promoting the insertion of GLUT2 in the BBM. The involvement of SGLT1 in the recruitment of GLUT2 to the BBM agrees with observations that phloridzin (an SGLT1 inhibitor) fully blocks glucose uptake, and that patients with defective SGLT1 suffer from glucose-galactose malabsorption. Trafficking of GLUT2 is thought to be rapid, with a $t_{1/2}$ of less than 5 minutes. A rounding of the apical surface, due to a contraction of the peri-junctional actomyosin ring, allows luminal glucose to have increased access to the BBM enzymes and transporters. The authors recognize that there may also be an activation of the small amount of GLUT2 that is already present in the BBM.

Helliwell *et al*[67] investigated the role of several signalling pathways in intestinal fructose absorption. Using an *in vivo* perfusion model, they showed that fructose transport was mediated by both GLUT5 and GLUT2. Using PMA to stimulate a 4-fold stimulation of fructose transport, they saw a 4-fold increase in GLUT2 protein in the BBM that correlated with PKCβII activation. Only minor changes in GLUT5 levels were observed, suggesting that recruitment of GLUT2 to the BBM represents a mechanism by which absorptive capacity is matched to dietary intake.

Helliwell *et al*[100] also established a role for the PI-3K and the mTOR pathways in the phosphorylation, turnover and degradation of PKCβII. Using an *in vivo* perfusion model, they showed that inhibitors of these pathways (wortmannin and rapamycin, respectively) block GLUT2 trafficking to the BBM and inhibit sugar absorption. A role for insulin in the regulation of intestinal sugar absorption is suggested. In their model, they suggest that as sugar absorption increases, the plasma sugar concentration increases, stimulating the release of insulin, which activates PI 3-kinase, resulting in the phosphorylation of PKCβII (Figure 4).

They also proposed a model by which amino acids promote the formation of competent PKCβII by activating the mTOR pathway, which prevents dephosphorylation of PKCβII (Figure 4). Thus, the dynamic control of intestinal sugar absorption may be achieved by the rapid turnover and degradation of PKCβII.

Why haven't previous investigators been able to detect GLUT2 in the BBM? *In vivo* endogenous hormones and nutrients activate PKCβII. Kellett's group points out that the process of harvesting tissue for *in vitro* preparations causes the inactivation of PKCβII and the rapid trafficking of GLUT2 away from the BBM. This observation may help to explain why the passive component was more apparent in the *in vivo* studies, as compared to *in vitro* experiments. In order to minimize the loss of GLUT2 from the BBM, Kellett's group perform all stages of tissue harvesting and membrane vesicle preparations at 0-4℃

after perfusing the intestine with a sugar load.

Helliwell and Kellett [101] looked at perfusion conditions in order to determine if the so called passive component was SGLT1-dependent, as suggested by their work, or was SGLT1-independent, as suggested by earlier work by Debnam and Levin[102]. They concluded that the passive component is independent of the active component in high mechanical stress perfusions, suggesting that SGLT1-dependent recruitment of GLUT2 did not occur under these conditions. This may be related to the restrictions in blood flow and supply of endogenous nutrients and hormones caused by the high stress perfusions.

However, under conditions of low mechanical stress, inhibition of SGLT1 with phloridzin does decrease GLUT2 levels in the BBM. Clearly, the perfusion conditions affect the results of the experiment, and this may explain the discrepancies between various studies. Finally, Kellett and colleagues suggest that the term "facilitated" should be used rather than "passive" to more accurately describe the GLUT2 mediated component of sugar uptake.

The antibody used to detect GLUT2 is also critically important in being able to detect GLUT2 in the BBM. Currently, there are two commercially available antibodies that recognize GLUT2: one that recognizes the extracellular loop between transmembrane 1 and 2 (Biogenesis, Poole, England); and another that recognizes a portion of the C- terminus (Research Diagnostics, Flanders, NJ). The choice of antibody is important, as Au *et al* (2002) demonstrated that the biotinylation procedure they used to detect surface proteins interfered with the ability of the GLUT2 antibody to recognize the extracellular loop, forcing them to use the C-terminus antibody[103]. However, Thorens *et al*[103] were unable to detect GLUT2 in the BBM by immunohistochemistry using an antibody that recognizes the C-terminus[75]. In contrast, when using the antibody directed against the extracellular loop, Cheeseman's group was able to detect BBM GLUT2**.** In contrast, when using Western blotting on BBM vesicles, they were able to detect GLUT2 using either of the antibodies. Clearly, the choice of antibody depends on what method of detection is used.

Although earlier studies established a role for GLP-2 in modulating GLUT2 activity in the BLM[63,104], more recently Au *et al*[103] investigated the effect of GLP-2 on the transient expression of GLUT2 in the BBM. Using an *in vivo* perfusion model in rats, they showed that a one hour vascular infusion of GLP-2 (800 pM) doubled the rate of fructose absorption, and this enhanced absorption of fructose could be blocked by phloretin, an inhibitor of GLUT2. Immunohistochemistry localized GLUT2 to both the BBM and BLM, and identified a pool of transporter located just under the microvilli in the terminal web region. This raises the possibility that the cytoskeleton structure is involved in the insertion of GLUT2 into the BBM. Luminal glucose perfusion (50 mmol/L) or vascular GLP-2 infusion (800 pmol/L) increased GLUT2 in the BBM two-fold as determined by Western blotting of biotinylated surface proteins. Both Kellett and Cheeseman suggest that in addition to the insertion of GLUT2 in the BBM, the intrinsic activity of apical GLUT2 may also be regulated[103,105]. The concept of intrinsic activity and its regulation will be discussed in subsequent sections.

Gouyon *et al*[68] used confocal microscopy and immunofluorescence in mice to confirm the presence of GLUT2 in the BBM following five days of feeding a 65% sugar meal, or following an oral bolus of either fructose or glucose. Wild type and GLUT2 null mice were fed fructose, glucose or sucrose (65% glucose, fructose or sucrose)-rich diets for five days, or were fasted and then received a 40% fructose, glucose or sucrose bolus. The absence of GLUT2 did not significantly affect fructose absorption in animals fed a low-carbohydrate diet, suggesting that under these conditions GLUT5 is soley responsible for fructose uptake into the enterocyte.

In animals fed a high fructose diet, cytochalasin B (an inhibitor of GLUT2) inhibited fructose uptake 60% in wild type mice, whereas GLUT2 null mice were unaffected. A 40% reduction was observed in animals fed a high glucose diet. This suggests that under these conditions fructose enters the cell by both a cytochalasin B dependent process (GLUT2) and a cytochalasin B independent process (GLUT5). The trafficking of GLUT2 to the BBM may represent a mechanism by which sugar absorptive capacity is matched to dietary intake.

When GLUT2 null mice were challenged with oral fructose, transport was 60% lower than in wild type animals, indicating firstly that the absence of GLUT2 limited fructose uptake, and secondly that this could not be fully compensated for by GLUT5. Still, some compensatory changes were noted, as GLUT5 mRNA was found to be increased three-fold in the ileum, possibly indicating some fructose malabsorption in the GLUT2 null mice. Therefore, Gouyon's study demonstrates that while under control conditions GLUT5 is solely responsible for BBM fructose uptake, BBM GLUT2 may be responsible for 40%-60% of sugar uptake when the luminal sugar concentrations are high.

The role of AMP-activated kinase (AMPK), an intracellular energy sensor, in the regulation of intestinal sugar uptake has also been examined. Walker *et al*[106] demonstrated that the activation of AMPK resulted in the recruitment of GLUT2 to the BBM and a down-regulation of the energy-requiring SGLT1-mediated glucose uptake. The importance of this phenomenon, particularly in models of intestinal damage or stress, warrants further investigation.

The presence of GLUT2 in the BBM of humans has not been confirmed. Dyer *et al*[107] found GLUT2 expression was restricted to the BLM in humans, although critics of this work have speculated that this may be a result of the methods used to obtain and process biopsies, and the lack of feeding a high sugar diet or giving an oral sugar test prior to obtaining the biopsies[68].

In summary, the role of GLUT2 in the transport of sugars across the BBM remains controversial. The most recent data suggests that in specific situations, such as when luminal sugar concentrations are high, GLUT2 is recruited to the BBM and contributes to sugar uptake from the lumen. This may explain the discrepancies between studies and the inability of many researchers to detect GLUT2 in the BBM in their experimental models.

INTRINSIC ACTIVITY AND TRANSPORTER TRAFFICKING

A number of factors are involved in the regulation of intestinal sugar transport. These factors may modify sugar transport by altering the abundance of sugar transporters in the intestine. Alternatively, sugar transport may be regulated at an entirely different level. The intrinsic activity of the transporters (amount of substrate transported per unit of transporter protein) may be altered, in the absence of detectable changes in transporter abundance. Indeed, there has been a long history of reports of discrepancies between glucose uptake and the protein abundance of glucose transporters both in skeletal muscle[108], adipose[109] and in the intestine[67,103,110-114]. Changes in the intrinsic activity of glucose transporters have observed with hyperglycemia[110], diabetes[63], low luminal glucose concentrations[98] and following the activation of MAPK and PI3K[67]. The post-translational mechanism by which intrinsic activity is regulated is not known, but may involve phosphorylation or dephosphorylation of the transporter or the activation or inhibition of the transporter by a regulatory protein.

Kellett and his colleagues have shown that the PI3K pathway is involved in the modification of the intrinsic activity of GLUT2 and GLUT5[67]. Control of transport by the modulation of both the levels and activities of the transporters occurred as a result of extensive cross-talk between the extracellular signal-regulated kinase (ERK), p38, and phosphatidylinositol 3-kinase (PI 3-kinase) pathways. Activation of the p38 pathway stimulates fructose transport by increasing GLUT2 levels in the BBM, as well as increasing the intrinsic activity of GLUT2. In contrast, the ERK or PI 3-kinase pathways have regulatory effects on transporter trafficking and intrinsic activity, without having significant effects on fructose transport (Figure 3). However, these results are derived from independently modulating these pathways, when clearly there is extensive cross-talk. For example, when the ERK pathway is inhibited, fructose transport stimulated by the activation of the p38 pathway increases 50-fold, suggesting that the ERK pathway restrains the p38 pathway.

It is not known if PI3K/Akt modifies the intrinsic activity of SGLT1. However, a study by Alexander and Carey (2001) showed that orogastric IGF-1 treatment increased glucose uptake in piglets without increasing SGLT1 abundance, suggesting an effect on intrinsic activity of the transporter[115]. Inhibiting Akt blocked the increase in glucose uptake, possibly by modifying the activity of the transporter.

PI3K has also been implicated in the regulation of GLUT4 trafficking to the plasma membrane in adipocytes or muscle[108]. Despite this possibility, several studies have demonstrated that the trafficking of transporter protein to the BBM cannot fully explain changes in intestinal sugar uptake seen after IGF-1, GLP-2 or glucose administration [103,115,116]. Nevertheless, both alterations in trafficking and intrinsic activity may contribute to the changes seen in sugar uptake. Further work is required to further characterize the relative contributions of each of these mechanisms.

ALTERNATIVE THEORIES

The previously well-accepted role of GLUT2 as the sole BLM glucose transporter is also a subject of debate. The role of GLUT2 was originally based on it being immunolocalized to the BLM. However, this does not exclude the possibility of other basolateral transport pathways. Recently, GLUT2 null mice were developed, in which GLUT1 or GLUT2 was re-expressed in pancreatic β cells to enable survival. This was an important step in investigating the role of GLUT2 in sugar transport. In these animals, normal rates of glucose appearance in the tail vein blood were seen following an oral glucose load, suggesting that GLUT2 was not required for transepithelial glucose transport[117]. It is important to note that this paper has limitations, as the appearance of glucose in the tail vein is not a direct measure of intestinal sugar transport. Further work by Stumpel *et al*[118] using an isolated intestinal perfusion model demonstrated normal glucose transport kinetics despite a lack of GLUT2. This finding was noted under control conditions and following cAMP perfusion, which is known to increase glucose absorption via SGLT1[119]. Even with this accelerated apical uptake of glucose into the enterocyte, the basolateral transport of glucose did not appear to be rate-limiting.

Interestingly, sugar transport was dose-dependently inhibited by an agent that inhibits the glucose 6-phosphate translocase located in the endoplasmic reticulum (ER) membrane. Glucose 6-phosphate translocase transports glucose-6-phosphate from the cytosol into the lumen of the ER, where the active site of glucose-6-phosphatase is located. Furthermore, 3-O-methylglucose, which cannot be phosphorylated by the hexokinases, was not transported, despite the fact that it is a known substrate for both GLUT2 and SGLT1. Taken together, these findings suggest that a distinct pathway exists that involves glucose phosphorylation, transport to the ER, dephosphorylation, and release via a membrane-traffic based pathway (Figure 5). Interestingly, the expression of the glucose-6-phosphatase and the glucose-6-phosphate translocase, as determined by Northern blotting, were not increased in the GLUT2 null animals. This contrasts with the work of Gouyon *et al*[68], who used RT-PCR to demonstrate that GLUT2 null mice had increased mRNA expression of glucose-6-phosphatase.

Stumpel and colleagues[118] also noted that GLUT5 mRNA expression was increased in the GLUT2 null mice, while the expression of all other known GLUT transporters did not change. Human studies have demonstrated the presence of GLUT5 in the BLM of enterocytes[120]. The finding that fructose absorption was unaffected by GLUT2 status suggests that GLUT5 may have been present in the BLM, contributing to fructose release on the serosal surface of the enterocyte. However, the authors dismissed the possibility that fructose and glucose shared a common serosal transport system based on the observation that the release of glucose, but not fructose, was blocked by an inhibitor of the glucose 6-phosphate translocase.

Stumpel *et al*[118] also performed fructose perfusion experiments in GLUT2 null mice. The results showed

that intracellular fructose was not converted to glucose, further supporting the notion that this alternative pathway does not contribute to fructose efflux. The authors also discounted the possibility that the paracellular pathway significantly contributed to glucose absorption, as the SGLT1 inhibitor phloridzin greatly reduced glucose absorption. They concluded that a microsomal membrane traffic-based mechanism may be an important component of transepithelial glucose transport.

The investigators point out that the concept of a microsomal membrane-trafficking transport system is supported by the following observation: genes for glucose-6-phosphate translocase (G6PT1)[121] and glucose-6-phosphatase (G6PC)[122] are expressed in human intestinal cells, despite the fact that only minimal amounts of glycogen are found in jejunal biopsies[123]. Similarly, the high levels of hexokinase activity in intestinal cells [124] support the concept of an alternative transport system characterized by glucose phosphorylation and subsequent microsomal transport and trafficking.

Santer *et al*[125] re-evaluated the role of GLUT2 in intestinal sugar absorption in one patient with Fanconi Bickel syndrome (FBS). FBS is characterized by congenital GLUT2 deficiency. Oral glucose tolerance tests performed on this patient failed to demonstrate differences in breath hydrogen concentrations when compared to control subjects, indicating that sugar was not being malabsorbed, at least within the sensitivity limits of hydrogen breath testing. These findings also suggest that other mechanisms are in place to transport sugars across the basolateral membrane of enterocytes.

RECENT DISCOVERIES

The model of intestinal sugar transport is an ever-changing story. Recently, a new facilitative glucose transporter, GLUT7, has been cloned and characterized[126]. GLUT7 has a high affinity for glucose (Km = 0.3 mmol/L) and fructose (IC50 = 0.060 mmol/L), but not for galactose. GLUT7 mRNA is present in the human small intestine, colon, testis and prostate. GLUT7 protein was found in the intestine, mostly in the BBM. The transporter's high affinity led the researchers to speculate that it may be important in fructose absorption at the end of the meal, when concentrations of fructose in the intestinal lumen are low. The physiological relevance of GLUT7 is unknown, as it doesn't appear to compensate for the loss of SGLT1 in glucose-galactose malabsorption.

Tazawa *et al*[127] have also cloned SGLT4, a sodium-dependent sugar transporter found in the intestine, liver, and kidney. COS-7 cells expressing SGLT4 exhibited Na+-dependent α-methyl-D-glucopyranoside (AMG) transport activity (Km = 2.6 mmol/L), suggesting that SGLT4 is a low affinity transporter. Several sugars were able to inhibit AMG transport (D-mannose > D-glucose > D-fructose > D-galactose), suggesting that these sugars may also be substrates. However, only mannose was confirmed to be a substrate by studies demonstrating direct uptake of mannose into the cell. Because mannose is elevated in diabetes[128] and in the metabolic syndrome[128], the authors suggest that SGLT4 may be a potential therapeutic target for patients afflicted with these disorders. Further characterization of these novel intestinal transporters will add to understanding of intestinal sugar transport.

CONCLUSION

The process of intestinal sugar absorption remains a controversial topic. An increased understanding of this process will enable the development of better therapeutic strategies in conditions where the modulation of intestinal sugar transport could improve health. For example, reducing sugar absorption may be beneficial with regards to the treatment of diabetes or obesity. Conversely, stimulating sugar absorption may be desirable in patients with short bowel syndrome, or in malnourished elderly patients. Furthermore, the targeted delivery of drugs to tumour cells expressing glucose transporters is an exciting area of research that warrants further exploration.

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REVIEW

Helicobacter pylori eradication to prevent gastric cancer: Underlying molecular and cellular mechanisms

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Abstract

Numerous cellular and molecular events have been described in development of gastric cancer. In this article, we overviewed roles of *Helicobacter pylori (H pylori)* infection on some of the important events in gastric carcinogenesis and discussed whether these cellular and molecular events are reversible after cure of the infection. There are several bacterial components affecting gastric epithelial kinetics and promotion of gastric carcinogenesis. The bacterium also increases risks of genetic instability and mutations due to NO and other reactive oxygen species. Epigenetic silencing of tumor suppressor genes such as RUNX3 may alter the frequency of phenotype change of gastric glands to those with intestinal metaplasia. Host factors such as increased expression of growth factors, cytokines and COX-2 have been also reported in non-cancerous tissue in *H pylori*-positive subjects. It is noteworthy that most of the above phenomena are reversed after the cure of the infection. However, some of them including overexpression of COX-2 continue to exist and may increase risks for carcinogenesis in metaplastic or dysplastic mucosa even after successful *H pylori* eradication. Thus, *H pylori* eradication may not completely abolish the risk for gastric carcinogenesis. Efficiency of the cure of the infection in suppressing gastric cancer depends on the timing and the target population, and warrant further investigation.

Key words: Helicobacter; Cancer; Gastric acid; p53; Inflammation; Gastric atrophy; Intestinal metaplasia

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INTRODUCTION

Gastric cancer is one of the most common neoplasmas worldwide, accounting for over 870 000 new cases and over 650 000 deaths annually[1]. Mortality from gastric cancer is the second most in death from malignancies, following to lung cancer. With exceptions in countries that have developed screening programs for early diagnoses, most patients reach treatment with cancers already in advanced stages[2]. Since surgery can be palliative, and gastric cancers are largely resistant to chemotherapy and radiotherapy, advanced gastric cancer has a poor prognosis. Therefore gastric cancer still remains a major clinical challenge and a great public health concern.

Extensive epidemiologic studies have shown that *Helicobacter pylori (H pylori)* infection is a major risk factor for developing gastric cancer and its precursor lesions[3]. The bacterium affects more than 50% of the world popultion[4]. The risk of patients with *H pylori* infection developing gastric cancer is in the order of two- to six-fold according to most retrospective case-control and prospective epidemiologic studies[5]. Furthermore, some of the trials eradicating *H pylori* have shown that cure of the infection reduces development of gastric cancer in high risk populations[6-8]. Thus, a hope is emerging and growing that cure of the *H pylori* infection may reduce incidence of gastric cancer.

The goal of this review is to clarify whether eradication *H pylori* results in eradication of gastric cancer. To accomplish this, we will discuss what types of cellular and molecular events occur in the *H pylori*-infected gastric mucosa; what bacterial factors are involved in the process of gastric carcinogenesis; and what host factors are able or unable to be reversed after the cure of the infection.

CELLULAR BASIS OF *H PYLORI*-RELATED GASTRIC CARCINOGENESIS

Histopathologic alterations

Chronic infection with *H pylori* causes active inflammation of gastric mucosa in majority of the population, although it is mostly asymptomatic. The bacterium also alters physiologic and histological behaviors of gastric mucosa, including hypochlorhydria, hyperchlorhydria, and changes in mucosal population of gastric epithelial cells that are specifically differentiated. In the hypochlorhydric population, hypergastrinemia occurs, while parietal cells do not respond to gastrin to produce acid, probably due to corpus inflammation. Apoptotic loss of superficial epithelial cells in the process of differentiation and migration in gastric glands increases[9], while proliferation of neck cells also increases possibly by some sort of compensation and by transactivation of growth stimuli in gastric mucosa[10]. In corpus mucosa, parietal cell population is also diminished in a long term, which is associated with alteration in population of other types of cells in each gland. Together with lowered density of glands partly due to inflammatory and edematous changes in subepithelial tissues, the above changes are known as gastric mucosal atrophy or atrophic gastritis. In addition, epithelial clones with incomplete and complete intestinal phenotypes emerge in the long-term process.

Currently, origins of the epithelial clones for the intestinal metaplasia have not yet been clearly determined. It is likely that epithelial clones for incomplete and complete intestinal metaplasia are developed from gastric epithelial cells by an activation of a series of genes including cdx-1/cdx-2[11-16]. In addition, bone marrow-derived adult somatic stem cells are involved in the regeneration of gastric glands, and may be another source of epithelial population[17]. Although our own study suggest that bone marrow-derived epithelial cells do not harbor permanently in a gastric gland, gastric adenocarcinomas are recently reported to be bone marrow-derived[18]. Stem cells in gastric glands locate neck region, whereas those in intestinal glands reside bottom region, a location completely different from the neck. Transdifferentiation of gastric gland cells to metaplastic cells remains an important question in gastric carcinogenesis.

Bacterial and/or host factors affecting the histologic alterations

Several pathogenic factors of *H pylori* have been demonstrated to be involved in the above alterations in gastric mucosa and the following development of gastric diseases.

Ammonia (NH_3), produced by *H pylori* urease, has been shown to cause acute gastric injury[19] in rats *in vivo* and to accelerate gastric epithelial cell death *in vitro*[19-21]. Chronic administration of NH_3, whose concentration is comparable to that found in *H pylori*-infected patients, increases epithelial cell replication and epithelial cell turnover, associated with accelerated epithelial cell death, cell exfoliation, preferential loss of parietal cells and gastric mucosal atrophy[22, 23]. The damaging effects of NH_3 on gastric mucosa are pH-dependent, while sodium hydroxide at the same pH does not cause significant mucosal injury[19]. Ammonia dissolves readily in water where it forms, and is in equilibrium with, ammonium ions (NH_4^+). With decreases in pH, NH_4^+ predominates, but increases in pH may materially increase levels of non-ionized NH_3[19]. On the other hand, *per os* administration with ammonium chloride (NH_4Cl) results in intragastric NH_4^+, and does not induce significant mucosal atrophy. Rather, NH_4Cl is reported to stimulate antral mucosa to increase gastrin release[24], which possibly induces gastric mucosal hypertrophy[25]. Therefore, not only *H pylori* but also gastric acid secretion of the host is an important determinant of gastric cell kinetics.

NH_3 also increases incidence and size of gastric adenocarcinomas in rats pretreated with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)[26, 27]. Prior administration of NH_3 followed by MNNG does not increase incidence of gastric adenocarcinomas in rats, indicating that NH_3 may act as a promoter in the chemically-induced gastric carcinogenesis. Immunohistochemical analysis using bromo deoxy-uridine (BrdU) demonstrates that NH_3 increases cell replication in gastric tumors as well as non-cancerous tissues surrounding the tumors. Thus, NH_3 and the consequent host epithelial responses play important roles not only in increased cell proliferation in untransformed gastric mucosa but also in promotion of gastric cancer.

The other virulence factors including CagA and other cag pathogenicity island (PAI) proteins, VacA and adhesions have been considered to be involved in wide diversity of *H pylori*-related diseases. For an example, strains containing the cag PAI have been reported to trigger signaling cascades in gastric epithelial cells, resulting in NF-κB activation and other cellular responses. Furthermore, CagA, which can be injected into the host cells, is able to be phosphorylated in the host, and to alter epithelial morphology probably through signaling pathway similar to that of HGF/c-met[28-31]. Roles of phosphorylated CagA protein in gastric epithelium are under extensive investigation and reviewed elsewhere[32]. Since it is related to gastric inflammation, cag PAI may stimulate indirectly excessive production of reactive oxygen species, including nitric oxide, and lead to programmed cell death. Indeed, studies show conflicting results for an association between cag PAI and apoptosis[33, 34].

VacA reportedly induces gastric epithelial cell apoptosis[35, 36]. It is found that VacA also induces apoptosis of marophages and suppresses T-cell responses[37-40]. Shibayama *et al*[41] showed that γ-glutamyl transpeptidase induces apoptosis. Furthermore, several apoptotic mediators such as TNF-α, FAS-ligand, TRAILs and their receptors are reported to be upregulated[42-44]. Thus, proapoptotic factors from either the bacterium or the host appear to be involved in altered cell kinetics as well as disturbed immunologic surveillance in gastric mucosa. Once certain clones acquire the resistance from apoptotic or immunologic surveillance, they begin to grow to form clusters of neoplastic phenotypes.

MOLECULAR ALTERATIONS OF *H PYLORI*-RELATED GASTRIC CARCINOGENESIS

Events promoting gastric carcinogenesis

Gastric cancer is divided into two histologic entities: 'in-

testinal-type' and 'diffuse-type'. These two types differ in epidemiology and clinical outcome. Molecular profiles are also distinct between these phenotypes[45-47], and actually consist of wide variety of alterations including mutations, loss of heterozygosity (LOH), and epigenetic changes of expression of unmutated genes(Table 1). It is not surprising that numerous reviews have been published regarding this topic[45-52], considering the size of population with gastric cancers or with *H pylori* infection. In diffuse-type gastric adenocarcinomas, DNA-repair errors, p16 suppression and cyclin E amplification occur frequently in early stages. In early stages of intestinal-type gastric adenocarcinomas, inactivation of APC due to LOH or mutation and nonfunctioning p53 frequently occur. Events due to changes in tumor microenvironments, *i.e.,* overexpression or transactivation of growth factors such as EGF-family growth factors (TGFα, EGF, HB-EGF, etc), insulin-like growth factors (IGF-1 and IGF-2), transforming growth factor-β, cytokines, and gastrin, also play important roles in phenotypic change in gastric epithelial cells [53, 54]. For an example, elevated gastrin may transactivate HB-EGF and its receptors, resulting in upregulation of mitogen-inducible cyclooxygenase (COX-2) and its products (prostaglandin E_2, etc)[55, 56].

Recently, COX-2[57-71] attracts attention of many oncologists and gastroenterologists. In fact, some epidemiologic studies have shown that a long-term NSAID-use results in significant reduction of incidence and mortality of digestive cancers including not only colon but also stomach[72, 73]. We have shown that the COX-2 overexpression alters cell kinetics, suppresses programmed cell death, induces invasive phenotypes, supports tumor angiogenesis and influences cell adhesion to endothelial cells[54, 70, 71, 74-79]. *H pylori* infection induces gastric COX-2 upregulation[71, 80-86], and cure of the infection reduces the COX-2 expression[70]. However, in mucosa with intestinal metaplasia, COX-2 is overexpressed even after the cure of the infection (Figure 1)[70]. Procarcinogenic effects of COX-2 on stomach could be only partially reversed by successful *H pylori* eradication. Similar findings were also observed in the case of expression of nitrotyrosine, a product of nitric oxide (NO), in precancerous gastric mucosa. Expression of nitrotyrosine is elevated in gastric mucosa in patients with *H pylori* gastritis, which is reversible after successful *H pylori* eradication. However, in gastric mucosa with intestinal metaplasia, nitrotyrosine continue to be overexpressed even after the cure of the *H pylori* infection, suggesting that NO and other reactive nitrogen species is highly produced in metaplastic lesions[70].

Mismatch repair deficiency

Microsatellite instability (MSI) is defined as the presence of replication errors in simple repetitive microsatellite sequences due to mismatch repair (MMR) deficiency[48]. It is classified as high-frequency (MSI-H), low-frequency (MSI-L) or stable (MSS)[87]. MSI has been recognized as one of the earliest changes in carcinogenesis and results in genomic instability. MSI is detected not only in gastric cancer but also in intestinal metaplasia from subjects both with and without gastric cancer[88], suggesting that MSI can be an early event in gastric carcinogenesis[89-91]. Further-

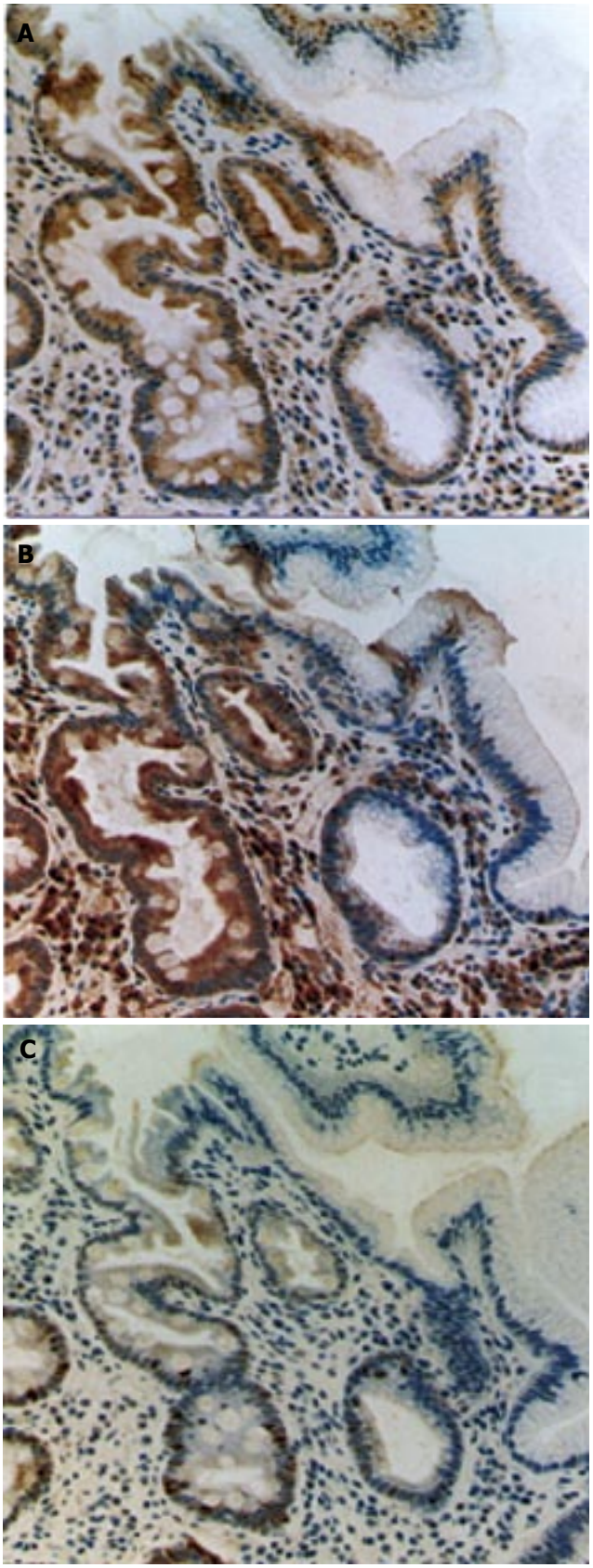

Figure 1 Expression of COX-2, nitrotyrosine and Ki-67 immunoreactivity in human gastric mucosa with intestinal metaplasia after cure of the H pylori infection. **A**: COX-2 immunostaining; **B**: nitrotyrosine immunostaining; **C**: Ki-67 immunostaining. The overexpression of COX-2 and nitrotyrosine, adduct of nitric oxide, are reported in gastric mucosa with *H pylori* infection[66, 68, 70, 71]. In these photographs, metaplastic gland with goblet cells (in the left side of each photograph) and non-metaplastic gastric glands (in the right side) are shown. COX-2 and nitrotyrosine immunoreactivities continue to exist in gastric mucosa with intestinal metaplasia after the successful *H pylori* eradication with PPI-triple therapy.

Table 1 Molecular alteration in the process of gastric carcinogenesis

Molecules	Major alterations	Comments	Category
p53	Mutation, LOH	Reported in diffuse-type and intestinal-type adenocarcinomas, as well as some precancerous lesions.	Tumor suppressor
APC	Mutation, LOH	Reported in diffuse-type and intestinal-type adenocarcinomas, as well as some precancerous lesions.	Tumor suppressor
DCC	LOH	Reported in intestinal-type adenocarcinomas. Related to cell adhesion	Tumor suppressor
CDH1	Mutation	Reported in diffuse-type adenocarcinomas.	Tumor suppressor
β-catenin	Mutation	Reported in intestinal-type adenocarcinomas.	Tumor suppressor
Fhit	LOH or deletion at chr. 3p14.2	Reported in diffuse-type and, in less frequency, intestinal-type adenocarcinomas, as well as some precancerous lesions.	Tumor suppressor
RUNX3	Hypermethylation	Related to TGF-β/SMAD signaling.	Tumor suppressor
K-ras	Mutation	Reported in intestinal-type adenocarcinomas. An element in signal transduction regulating cell proliferation, etc..	Oncogene
bcl-2	LOH	Reported in intestinal-type adenocarcinomas. Anti-apoptotic factor.	Oncogene
c-met	Amplification	Reported in diffuse-type and intestinal-type adenocarcinomas. The HGF receptor / tyrosine-kinase. Upregulation without mutation is also reported after mucosal injury.	Oncogene / Growth stimulus
c-erbB2	Amplification	Reported in intestinal-type adenocarcinomas. One of receptor-tyrosine kinases for EGF-family proteins.	Oncogene / Growth stimulus
Cyclin E	Amplification	Reported in diffuse-type and intestinal type adenocarciomas.	Cell cycle regulator
K-sam	Amplification	Reported in diffuse-type adenocarcinomas. One of bFGF receptor family proteins, FGFR2.	Oncogene
Mismatch repair (MMR) genes	Silencing due to hypermethylation	Reported in diffuse-type and intestinal-type adenocarcinomas, as well as some precancerous lesions. A possible source of mutations of other genes involving gastric carcinogenesis.	Determinants of microsatelite instability (MSI)
MMR genes	Mutation	Reported in diffuse-type and intestinal-type adenocarcinomas. There are conflicting data suggesting that mucosa with intestinal metaplasia is prone to and resistant to MSI.	Determinants of MSI
EGFR	Overexpression	Reported in diffuse-type and intestinal-type adenocarcinomas.	Growth stimulus
EGF	Overexpression	Reported in diffuse-type and intestinal-type adenocarcinomas.	Growth stimulus
TGF-α	Overexpression	Reported in diffuse-type and intestinal-type adenocarcinomas, as well as some precancerous lesions. Another EGF-family protein.	Growth stimulus
VEGF	Overexpression	Reported in diffuse-type and intestinal-type adenocarcinomas.	Angiogenic factor
iNOS	Overexpression	Reported in diffuse-type and intestinal-type adenocarcinomas, as well as some precancerous lesions and mucosa with H. pylori.	Enzyme
COX-2	Overexpression	Reported in diffuse-type and intestinal-type adenocarcinomas, as well as some precancerous lesions and mucosa with H. pylori. Cytokines and growth factors are possible inducer of COX-2.	Enzyme
ODC	Overexpression	Reported earlier in gastritis.	Enzyme
Telomerase	Activated	Enlongs telomere and prevents cell senescence.	Enzyme
CDXs	Overexpression	Reported in diffuse-type and intestinal-type adenocarcinomas, as well as precancerous lesions. Is involved in intestinal metaplasia.	Transcription factor
Ets1	Overexpression	A transcription factor involving angiogenesis.	Transcription factor
NF-κB	Overexpression	A transcription factor regulating expression of proinflammatory cytokines, chemokines, iNOS and COX-2.	Transcription factor
Sp-1	Overexpression	Reported in diffuse-type and intestinal-type adenocarcinomas.	Transcription factor
SC-1	Overexpression	Reported in diffuse-type adenocarcinomas.	Apoptosis receptor
Fas/CD95	Overexpression	Reported in diffuse-type adenocarcinomas.	Apoptosis receptor
E-cadherin	Mutation	Reported in diffuse-type and intestinal-type adenocarcinomas.	Cell adhesion
CD44	Splicing variant	Reported in diffuse-type and intestinal-type adenocarcinomas.	Cell adhesion
Gastrin	Elevation in serum	Elevation of amidated gastrin is reported. Transactivates EGF-family proteins.	Gut hormone

more, hypermethylation of CpG islands in the promoter region of the hMLH1 gene is associated with decreased hMLH1 protein, and often occurs in gastric cancer cases with MSI-H, indicating that epigenetic inactivation of hMLH1 may underlie MSI[92]. MSI in gastric cancer is associated with antral tumors, intestinal-type differentiation, and a better prognosis. Cancer cases with MSI exhibit mutations in BAX, hMSH3, hMSH6, E2F-4, TGF-β receptor II, and IGF-R II, which have simple tandem repeat sequences within their coding regions[93-99]. *H pylori* infection and following gastric mucosal alteration are closely related to MSI[100-102]. In particular, Park *et al*[100] recently reported an immunohistochemical study demonstrating that DNA MMR protein expression (hMLH1 and hMSH2) decreases in patients with *H pylori* infection. Cure of the infection resulted in significant increases in the percentage of hMLH1 (76.60 ± 20.27, 84.82 ± 12.73, $P = 0.01$) and hMSH2 (82.36 ± 12.86, 88.11 ± 9.27, $P < 0.05$) positive epithelial cells[100], suggesting that the effects of *H pylori* on MSI are reversible at least in a part. On the other hand, MSI results in frame-shift mutations of hMSH3 and hMSH6, and loss of hMSH1 and hMSH2 functions, which may lead gastric

Table 2 "p53" mutation in gastric cancers of early stages and precancerous gastric lesions. In gastric cancers of early stages and precancer gastric lesions, LOH and splicing are merely reported. Abbreviations for mutation: Del: deletion; Ins: insertion; F/S: frame shift. Abbreviati for lesion: EGC: early gastric cancer; AD: adenoma, CA/AD: carcinoma in adenoma; D: dysplasia; IM: intestinal metaplasia; N: muc without dysplasia, IM or carcinoma. Data are collected from references 104, 105, 113-118. (Modified from Tsuji *et al*[119, 120])

First author	Year	Case	G:C→A:T	G:C→T:A	A:T→G:C	A:T→C:G	A:T→T:A	G or C	Del	Ins	F/S	Lesions
Yokozaki	1992	1			2	1	1	1				EGC
Tohdo	1993	5			3						1	AD or CA/AD
Uchino	1993	12	10			2		1				EGC
Correa	1994	8	4		3				1			D, IM, or N
Hongyo	1995	9	10					1	1	2		Cancer at stage I
Sakurai	1995	7	4									AD, CA/AD or EGC
Tamura	1995	1	1									AD
Tamura	1995	4	3	2								EGC
Ranzani	1995	18	13	1			1	1	1	2		EGC
Summary			45	3	8	3	2	3	4	4	1	
(%)			62	4	11	4	3	4	5	5	1	

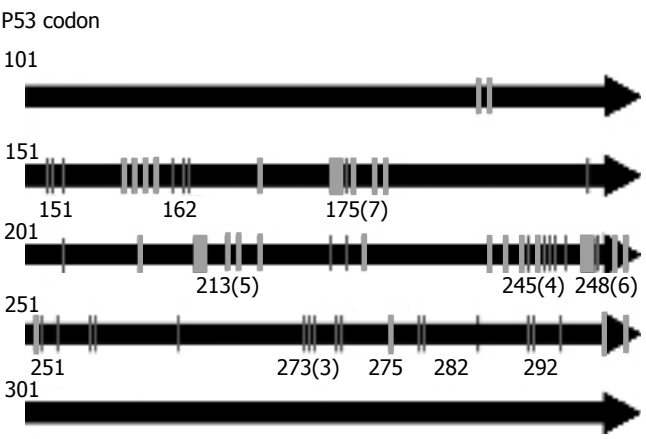

Figure 2 Location of point mutations of p53 in gastric cancers and premalignant lesions of the stomach. Horizontal lines mean codons of p53 gene. Thick and thin vertical lines respectively mean 5 and 1 mutations of the corresponding codon. Gray numbers indicate location of mutated codon followed by number of mutated cases in parentheses. As shown in this figure, codons 175, 213, 245, and 248 are preferably mutated in early stages of gastric cancer. Data are collected from references 104, 105, 113-118.

epithelial cells to further genetic instability that cannot be reverted by *H pylori* eradication. Therefore, the precise mechanism for *H pylori*-induced suppression on MMR protein has not yet been clarified and one of the important topics in *H pylori*-related gastric carcinogenesis.

Oncogenes

Certain EGF-like growth factors and their receptors are activated by membrane-type proteases called ADAMs (a disintegrin and metalloproteinase) following the stimulation including gastrin[56], endothelin and IL-8 that have G-protein coupled receptors[103]. IL-1β is also known to transactivate EGF-receptor via pathways dependent and independent of IL-8[103].

In addition, certain growth factors, their receptors and components of intracellular signaling have mutations or amplifications activating cell growth, inhibiting programmed cell death, and altering cell phenotypes. These oncogenes include HGF receptor (c-met), c-erbB2 (HER-2/neu), c-erbB3, K-sam, ras, c-myc and others, and have been reported to be mutated, amplified, or overexpressed in the process of gastric carcinogenesis[47, 52]. Once these oncogenes are mutated, it would be hardly possible for *H pylori* eradication to suppress oncogenes.

Tumor suppressor genes

Various tumor suppressor genes have been reported to be inactivated and involved in gastric carcinogenesis. For example, inactivation of p53 and p16 has been shown in both diffuse- and intestinal-type gastric cancers[52,104,105]. On the other hand, mutation of adenomatous polyposis coli (APC) gene occurs more often in intestinal-type gastric cancer. Another important tumor suppressor gene in intestinal-type gastric cancer is runt-related gene 3 (RUNX3) coding a subunit of polyomavirus enhancer binding protein 2[106-110], since expression of RUNX3 is greatly reduced in intestinal metaplasias in human stomachs[111] and Runx3-/- mouse gastric epithelial cells have a potential to differentiate into Cdx-2 positive intestinal type cells[112]. The product of the gene appears to interact with smad 2/3, which mediates TGF-β signaling pathway, and induces $p21^{WAF1/Cip1}$ expression.

Inactivation of these tumor suppressor genes includes, inactivating mutations, LOH, and epigenetic silencing. For example, hot spot mutations on CpG islands in p53 have been reported not only in gastric cancers at early stages but also in non-cancerous tissues with intestinal metaplasia [104, 105, 113-118]. In stomach, mutated p53 proteins are largely non-functioning and accumulate in the cells. Interestingly, p53 mutation frequently include G:C→A:T transition (Table 2, Figure 1)[119, 120], and NO is an important mutagen causing this type of mutation[120-122]. On the other hand, silencing of RUNX3 by promoter hypermethylation is frequently found in gastric cancers and in intestinal metaplasia. Although the silencing of tumor suppressor genes due to mutation may not be reversed, the epigenetic silencing may be reversed in methylation and demethylation processes. At present, there is no evidence indicating *H pylori per se* increases aberrant hypermethylation of tumor suppressor genes[123]: rather, Epstein-Barr virus-related gastric cancer is associated with a high frequency of DNA

hypermethylation, suggesting that viral oncogenesis might involve DNA hypermethylation with inactivation of tumor suppressor genes[124]. However, male gender, intestinal metaplasia and chronic inflammation with monocytic infiltration are strongly associated with increased methylation in non-cancerous gastric mucosa[123], and *H pylori* infection is one of the major causes of gastric inflammation. Thus, it remains an important question whether cure of the infection reduces the epigenetic alterations in tumor suppressor genes in non-transformed gastric epithelia.

Telomere and/or telomerase

Activation of telomerase that prevents shortening of telomeres during cell division may play an important role in immortalizing cells[47, 125-127]. In brief, telomeres cover the ends of chromosomes and are important in maintaining chromosomal integrity. In intestinal metaplasia, shortening of telomeres[52] as well as telomerase activation[127, 128] are observed, suggesting an important role in development of gastric cancer with intestinal type. Interestingly, it has been reported that *H pylori* infection reactivates telomerase[129, 130], and that cure of the infection appears to reduce telomerase activity[130]. Since clinical studies using human subjects may suffer from sampling errors, it remains an open question whether *H pylori* eradication reverses telomerase activation.

HOST GENETICS OF *H PYLORI*-RELATED GASTRIC CARCINOGENESIS

Genetic predisposition affecting inflammation and acidity of stomach

Genetic predisposition plays an important role in developing gastric cancer. The most widely reported are IL1B and NAT1 polymorphisms[131-138]. The association of IL1B polymorphism and gastric carcinogenesis was hypothetically explained by El-Omar *et al*[134] to be a strong acid-inhibiting and proinflammatory capacity of the gene product. Indeed, gastric acid secretion is known to be suppressed by IL-1β, which is mediated by nitric oxide[139]. These genetic factors may have strong association with *H pylori* infection, since the bacterium induces production of interleukins, inflammation, and elevates intragastric pH, which may result in increase of xenobiotic products. On the other hand, IL-1β and IL-8 were recently reported to transactivate EGF-receptor *via* ADAM-10 activation[103]. IL-1β is also known to up-regulate COX-2 in gastric epithelium[140]. Therefore, the reason for the association of IL1B polymorphisms and the risk for gastric cancer remains an open question and may require further investigation.

Genetic predisposition possibly independent of acidity of stomach

Another example of the genetic predisposition is families of hereditary nonpolyposis colorectal cancer (HNPCC) kindred of which have an excess of gastric carcinoma; complete intestinal metaplasia and chronic atrophic gastritis restricted to the antrum[141-143]. Interestingly, HNPCC patients frequently have a mutation in one of two DNA mismatch repair genes, hMSH2 or hMLH1, and demonstrate MSI-H. As mentioned earlier, *H pylori* has an ability to decrease MMR activity. Several genetic predispositions in MSI may share the same mutations to those found in *H pylori*-induced carcinogenesis. In these cases, the bacterial infection has a potent impact on gastric carcinogenesis, since it could lower the MMR activity more than the hereditary predisposition alone.

Hereditary gastric cancer due to germline mutation of the E-cadherin has been reported[144], which is a risk factor possibly independent of *H pylori* infection.

DOES *H PYLORI* ERADICATION ERADICATE GASTRIC CANCER?

Unlike the typical adenoma-carcinoma sequence of colon, development of gastric cancer appears to be a complex process. Due to the complexity of molecular events of gastric carcinogenesis, factors discussed here do not cover every aspect of gastric carcinogenesis. Rather, we tried to overview some of the possible factors initiating, promoting and supporting the development of gastric cancer. By doing so, we discussed what types of risks exists in *H pylori* positive subjects and what extent of these risks could be withdrawn after the cure of the infection.

Certain bacterial factors affect gastric epithelial cells directly to support establishment and development of metaplastic or dysplastic clones. Successful *H pylori* eradication withdraws these bacterial factors and therefore lowers the promotional effects on tumor development. The bacterium also increases genetic instability and risks of mutation. Some host factors such as NO and other reactive oxygen species are induced by *H pylori* and increase risks of mutation. Although cure of the infection may reduce these risks leading to epithelial mutagenesis, it does not abolish the risk completely. Particularly, in gastric mucosa with intestinal metaplasia and other phenotypically altered tissues, increases in MSI and NO synthesis, as well as COX-2 overexpression are unaltered after the cure of the *H pylori* infection. Thus, *H pylori* eradication is an effective strategy in reducing the risk of gastric cancer; however, it is not efficient enough to eradicate gastric cancer. Prevention of the infection, *H pylori* immunization, *H pylori* eradication in the youth, selection of the high risk population, and alternative chemopreventive measures may be essential for optimal management of malignancy of the stomach.

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REVIEW

Interleukin-10 and chronic liver disease

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Abstract

Interleukin (IL)-10 is an important immunoregulatory cytokine produced by many cell populations. Numerous investigations suggest that IL-10 plays a major role in chronic liver diseases. IL-10 gene polymorphisms are possibly associated with liver disease susceptibility or severity. Recombinant human IL-10 has been produced and is currently tested in clinical trials. These trials may give new insights into the immunobiology of IL-10 and suggest that the IL-10/IL-10 receptor system may become a new therapeutic target.

Key words: Interleukin (IL)-10; Cytokine; Chronic liver diseases; Polymorphisms

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BIOLOGICAL CHARACTERISTICS AND REGULATION OF INTERLEUKIN-10

Interleukin-10 (IL-10) first described as a cytokine synthesis inhibitory factor for T lymphocytes produced by T helper 2 (Th_2) cell clones, can inhibit interferon (IFN)-γ synthesis in Th_1 cell clones[1]. The human IL-10 gene, a homodimer with a molecular mass of 37 ku, is located on chromosome 1 and encodes for 5 exons. Each monomer consists of 160 amino acids. X-ray crystal-structure-analysis showed the two identical intertwining polypeptide chains of 160 animo acids are rotated by 180° to each other, forming two domains in a V- shape structure, each containing six helices[2-4]. Murine and human IL-10 exhibits a homology of about 80%. Various cell populations produce IL-10 in the body, including T cell subsets (Th_2, Tc_2, Tr_1, *etc*), monocytes, and macrophages. IL-10 is produced also by various cell types in other organs, including the liver[5-6]. Also, the stress axis plays a significant role in regulating IL-10 expression *in vivo*. Inflammation of the central nervous system or indirect activation of the stress axis by endotoxemia/bacteremia triggers the release of catecholamines that up-regulate IL-10 production in macrophages, particularly in the liver[7-8]. Within the liver, production of IL-10 has been documented within hepatocytes, sinusoidal endothelial cells, Kupffer cells, hepatic stellate cells and liver-associated lymphocytes[9]. These cells are stimulated to produce IL-10 through the cAMP/protein kinase A/CREB-1/ATF-1 signaling by several endogenous and exogenous factors such as stress, exdotoxin, tumor necrosis factor-α, catecholamines, and cAMP-elevating drugs. Recent data suggest that the p38 mitogen-activated kinase pathway also regulates the human IL-10 promoter via the activation of sp1 transcription factor[10]. IL-10 activity is mediated by its specific cell surface receptor-IL-10 receptor, which is expressed on a variety of cells, especially in immune cells[11]. Only a few copies of IL-10R are expressed on the surface of cells[12-13]. The expression is variable, but so far only a few regulating factors are known. IL-10R is composed of two different chains[14]. The interaction of hIL-10R with hIL-10 has been characterized recently and seems to be highly complex[15-16]. The IL-10Rβchain is essential for IL-10-mediated effects and CRFB4-deficient mice display the same phenotype as IL-10 deficient mice[17]. Only in cells expressing both IL-10R α and βchains, can the characteristic pattern of IL-10 signaling be observed[18]. The IL-10/IL-10R interaction activating the tyrosine kinases Jak1 and Tyk2, inhibiting the activity of NF-κB, results in transcriptional activation of several hundred genes[19]. The effects of IL-10 have been confirmed by experimental research in animals including IL-10 knockout mice[20] as well as by the effects of IL-10 observed in several inflammatory, autoimmune, and tumor models. IL-10 inhibits the ability of monocytes and macrophages to produce antigens to T cells[21-22] and monocytic production of IL-12. Inhibits proliferation and cytokine synthesis of $CD4^+$ T cells by exerting some direct effects of T cells, but does not exert potent direct inhibitory effects on $CD8^+$ T cells. IL-10 has various but weak stimulatory effects on B cells. IL-10 prevents apoptosis and enhances proliferation and differentiation of plasma cells as well as IgM synthesis, and inhibits the release of various chemokines by neutrophils. One of the most important properties of IL-10 is its anti-inflammatory action[23], which restrains the

immune response under various stimuli. Evidence of *in vivo* function of IL-10 indicates that inflammatory bowel disease is exacerated in the absence of IL-10.

EFFECTS OF IL-10 ON CHRONIC LIVER DISEASE AND LIVER FIBROSIS

Experimental data from animal models and clinical data from patients suggest that inflammation-associated cytokines including pro-inflammatory cytokines such as TNF-αand TGF-β, and anti-inflammatory cytokines such as IL-10 , are involved in the development of liver injury[24]. The effects of IL-10 have been observed in viral or autoimmune hepatitis, alcoholic liver disease, and animal models. Patients with a strong Th_1 response during acute HCV infection can clear the virus, while patients presenting with a Th_2 response (high levels of IL-10) evolve into chronicity[25]. In Con A-induced liver injury model[26-28], using a blocking IL-10 monoclonal antibody could lead to severe hepatic necrosis. On the other hand, administration of recombinant IL-10 in mice challenged with Con A could dramatically reduce secretion of pro-inflammatory cytokines, apoptosis of hepatocytes, hepatic neutrophil infiltrate and delay hepatic necrosis. In the model of liver injury induced by lipopolysaccharide (LPS) or staphylococcal enterotoxin B (SEB) in D-galactosamine (GalN)-sensitized mice[29-31], treatment with IL-10 could markedly reduce serum transaminase activities in a dose-dependent manner and hemorrhagic liver damage in sensitized mice exposed to toxins. IL-10 also inhibits increases in serum TNF-alpha and IFN-gamma concentrations with the toxins. Treatment with IL-10 could significantly reduce TNF-alpha mRNA and IFN-gamma mRNA expression in the liver and spleen after administration of the toxins to sensitized mice. These findings suggest that IL-10 is capable of regulating hepatic injury *in vivo* mediated by T cells macrophages. Injury of the liver requires the participation of proinflammatory cytokines and chemokines, many of which are regulated by the transcription factor, nuclear factor κB (NFκB). Other data suggest that IL-10 protects against hepatic ischemia/reperfusion injury by suppressing NFκB activation and subsequent expression of proinflammatory mediators[32]. IL-10 has been shown to be beneficial in the setting of liver transplantation[33], treatment with IL-10 can increase allograft survival. Current studies demonstrate that IL-10 may protect against surgery-or trauma related organ injuries secondary to hepatic ischemia-reperfusion. In human alcoholic liver disease or in rats fed with alcohol, defective production of IL-10 might result in chronic liver disease, suggesting that IL-10 might be of therapeutic value for alcoholic hepatitis by decreasing hepatocyte death[34]. In the model of CCl_4-induced chronic liver injury, IL-10 deficient animals had a persistently increased inflammatory infiltrate, and developed a more extensive fibrosis than the animals able to produce IL-10, indicating that IL-10 is involved in the control of fibrogenesis[35-37]. Several studies indicate that IL-10 might play an important role in antifibrogenesis during CCl_4-induced hepatic fibrogenesis[38-39]. Hepatic stellate cells (HSCs) are involved in liver fibrogenesis since, *in vitro* experiments have shown that HSCs express IL-10 receptor and produce IL-10[40-42]. In highly purified preparations of rat HSCs, messenger RNA (mRNA) for IL-10 can be detected by reverse-transcription polymerase chain reaction (RT-PCR). Long-term incubation of unstimulated mouse HSCs secrete IL-10 protein as detected by immunoblotting and specific enzyme-linked immunosorbent assay (ELISA). IL-10 protein is undetectable by immunohistochemistry in mouse HSCs during the first 3 d of culture. The percentage of IL-10-positive cells increases to 45% on d 7 and 100% on d 14, and IL-10 continues its expression in long-term culture of up to 120 d. These data indicate that IL-10 plays an important role in liver fibrosis by suppressing the function of HSC and promoting apoptosis of HSC[43-45]. IL-10 has a direct effect on the production of collagen and collagenases, modulates remodeling of the extracellular matrix[46-47], and indirectly limits the fibrogeneic response by controlling $TGF\beta_1$ secretion.

IL-10 GENE POLYMORPHISMS IN CHRONIC LIVER DISEASE

Genetic markers in cytokine genes are widely used in studies of immune-mediated diseases to determine disease susceptibility and severity[48]. In recent years, increasing attention has been paid to the role of cytokine levels in inflammatory and immune response., which may account for some of the heterogeneity observed in the outcome of chronic liver diseases, such as HBV and HCV infection, alcoholic and autoimmune hepatic disease. Possible linkage of IL-10 promoter haplotypes to disease susceptibility or severity has been reported[49]. The IL-10 promoter is highly polymorphie with two informative microsatellites, IL-10G and IL-10R. Single nucleotide polymorphisms (SNPs) in the promoter form SNP combinations (ATA, ACC, GCC) associated with differential IL-10 expression[50]. There are several lines of evidence that ATA haplotype in the IL-10 gene promoter is relevant to a genetically low capacity for IL-10 production, whereas GCC haplotype is identified as a high IL-10-producing phenotype, suggesting that the difference in disease progression of patients results from the inheritance of the IL-10 gene promoter polymorphisms. The influence of cytokine genotypes either on different clinical features of liver disease or in the response to antiviral therapy has been evaluated in several studies. Since inadequate expression of IL-10 seems to be of pathophysiological relevance in several diseases and the expression levels seem to have a genetic background. Increased serum levels of IL-10 are often observed in chronic HCV infection and inheritance of the interleukin-10 -1082 G/G may be associated with susceptibility to chronic hepatitis C infection and resistance to combined antiviral therapy[51-53], suggesting that chronic HCV infection patients with the haplotype conferring a high production of IL-10 have a lower rate of response to interferon therapy. IL-10 promoter allelic frequencies of T and A at positions -819 and -592, as well as the frequencies of ATA haplotype at positions -1082/-819/-592, are significantly higher in asymptomatic carriers than in patients with progressive chronic liver disease, suggesting that patients with haplotype

conferring a high production of IL-10 develop chronic progressive liver disease, while patients with a lower production of IL-10 tend to be asymptomatic carriers[54, 55]. Possession of the A allele at position -627 in the IL-10 promoter (low IL-10 expression) is associated with an increased risk of advanced liver disease in heavy drinkers[56, 57].

Genetic association analysis has revealed that one of the IL-10 haplotypes, IL10-ht2 (-1082A/-819T/-592C/+117T) is strongly associated with hepatocellular carcinoma (HCC) occurrence in a dose-dependent manner[58]. The frequency of susceptible IL10-ht2 is much higher in HCC patients and significantly increased in order of susceptibility to HBV progression from chronic to cirrhosis and HCC. In addition, the onset age of HCC is also accelerated among chronic hepatitis B patients who carry IL10-ht2. Increased IL-10 production mediated by IL10-ht2 suggests that up-regulation of IL-10 accelerates progression of chronic HBV infection, to HCC.

APPLICATION OF IL-10 AS A THERAPEUTIC AGENT

The promising results from IL-10 applied to several inflammatory diseases in experimental models induce the interest in clinical application of IL-10. So far human recombinant IL-10 has been tested in healthy volunteers, patients with Crohn's disease, rheumatoid arthritis, psoriasis, hepatitis C and HIV infection[59]. In phase I clinical trials, safety, tolerance, pharmacokinetics, pharmacodynamics, immunological and hematological effects of single or multiple doses of IL-10 administered by intravenous (i.v.) or subcutaneous (s.c.) route have been investigated in healthy volunteers[60]. IL-10 is well tolerated without serious side effects at the dose of 25 μg/kg and mild to moderate flu-like symptoms are observed in a fraction of recipients at the doses of 100 μg/kg.

Single i.v. or s.c. of IL-10 results in transient dose-dependent changes in white blood cell population, including increase of total white blood cells and neutrophils, lymphocytopenia and monocytosis as well as decrease in platelet counts are observed[61]. Following i.v. administration, IL-10 serum levels initially decline rapidly but yields a less steep terminal phase. IL-10 is cleared mainly through the kidneys as indicated by the increased $t_{1/2}$ and AUC of IL-10 in patients with moderate to severe renal insufficiency. Taken together, IL-10 application induces a number of immunological changes and is well tolerated[62]. IL-10 treatment does not result in significantly higher remission rate or clinical improvement for Crohn's disease compared with placebo treatment[63]. IL-10 can prevent postoperative recurrence of Chorine's disease but the clinical results are unsatisfactory[64]. The data from rheumatoid arthritis patients are rather discouraging, showing only marginal activity of the drug[65]. For psoriasis, IL-10 is likely to have antipsoriatic activity[66].

IL-10 is able to express antifibrotic properties in experimental models of liver cirrhosis[59]. It has been postulated that *in vivo* administration of IL-10 to patients with HCV infection may shift the intrahepatic immunologic balance away from Th1 cytokine predominance, thus exerting its anti-inflammatory and subsequent antifibrotic effect[67]. It was reported that long-term therapy with interleukin-10 decreases hepatic inflammatory activity and fibrosis, but leads to increased HCV viral levels[68].

IL-10 increases the susceptibility to infections due to its immunosuppressive activity and inhibition of bactericidal activity[69]. In the future, it may be used to target the delivery of IL-10 to avoid systemic side effects and low biodisponibility. IL-10 could be delivered locally with an adenovirus in the liver[70], suggesting that anti-inflammatory cytokines may have a future in the treatment of liver injury and the prevention of its complications.

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ESOPHAGEAL CANCER

Association of smoking, alcohol drinking and dietary factors with esophageal cancer in high- and low-risk areas of Jiangsu Province, China

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Abstract

AIM: To study the main environmental and lifestyle factors that account for the regional differences in esophageal cancer (EC) risk in low- and high-risk areas of Jiangsu Province, China.

METHODS: Since 2003, a population-based case-control study has been conducted simultaneously in low-risk (Ganyu County) and high-risk (Dafeng County) areas of Jiangsu Province, China. Using identical protocols and pre-tested standardized questionnaire, following written informed consent, eligible subjects were inquired about their detail information on potential determinants of EC, including demographic information, socio-economic status, living conditions, disease history, family cancer history, smoking, alcohol drinking, dietary habits, frequency, amount of food intake, etc. Conditional logistic regression with maximum likelihood estimation was used to obtain Odds ratio (OR) and 95 % confidence interval (95% CI), after adjustment for potential confounders.

RESULTS: In the preliminary analysis of the ongoing study, we recruited 291 pairs of cases and controls in Dafeng and 240 pairs of cases and controls in Ganyu, respectively. In both low-risk and high-risk areas, EC was inversely associated with socio-economic status, such as level of education, past economic status and body mass index. However, this disease was more frequent among those who had a family history of cancer or encountered misfortune in the past 10 years. EC was also more frequent among smokers, alcohol drinkers and fast eaters. Furthermore, there was a geographic variation of the associations between smoking, alcohol drinking and EC risk despite the similar prevalence of these risk factors in both low-risk and high-risk areas. The dose-response relationship of smoking and smoking related variables, such as age of the first smoking, duration and amount were apparent only in high-risk areas. On the contrary, a dose-response relationship on the effect of alcohol drinking on EC was observed only in low-risk areas.

CONCLUSION: The environmental risk factors, together with genetic factors and gene-environmental interactions might be the main reason for this high-risk gradient in Jiangsu Province, China.

Key words: Esophageal cacner; Case-control study; Smoking; Alcohol drinking; Dietary factors

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INTRODUCTION

Esophageal cancer (EC) is the sixth most common cause of cancer mortality worldwide. The incidence of this disease shows a striking geographic variation in the world; a 20-fold variation is observed between high-risk China and low-risk western Africa[1]. Jiangsu Province, southeastern China is one of the highest EC incidence areas with a mortality rate of 30.0/100 000 between 1990-1992, which was significantly higher than the national average of 17.0/100 000[2,3]. EC has been the third leading cause of cancer mortality in Jiangsu Province since the 1970s[3]. Although the mortality rates of EC are high in most counties

of Jiangsu, national surveys conducted in the 1970s and 1990s have shown that rates differ considerably between different counties within the province, despite their similar geographic characteristics and socioeconomic status[3,4].

Comprehensive studies on the etiology and carcinogenesis of EC in high-risk areas have been carried out during the past decades. Epidemiological evidence suggests that the independent risk factors, tobacco smoking and alcohol drinking, are strongly associated with EC risk and have approximately multiplicative joint effects[5-7]. Dietary factors were also found to play an important role in the development of EC. Increased risk of EC was found to be associated with low intake of raw vegetables and fresh fruits, a deficiency in vitamins or protective antioxidants (e.g. Vitamin C and E, β-carotene, and selenium), high intake of carcinogens (frequent consumption of pickled vegetables and fungi toxins) and thermal injuries (fast eating speed for hot drinks and soups)[8-10]. While the contributory factors of EC are the high consumption of tobacco and alcoholic beverages in Western countries, the causative factors of EC in high-risk areas of China are nutrition deficiency, N-nitrosamines, fungi toxins and genetic factors[11].

Although numerous epidemiological studies have been conducted to explore the associations between environmental, lifestyle, dietary factors and the risk of esophageal cancer, few studies have been conducted to compare the association between risk factors and EC in apparently similar areas with a high risk gradient. Thus, a population-based case-control study has been conducted since 2003 in both low and high-risk areas of Jiangsu Province, China to study main environmental and lifestyle factors that account for regional differences in EC risk.

This paper reports the preliminary results on the independent and joint effects of smoking, alcohol drinking and dietary factors on EC risk and compares their associations with EC in both high-risk and low-risk areas.

MATERIALS AND METHODS

Study area

This study has been conducted in Dafeng and Ganyu counties since late 2003. Both counties are less developed coastal areas in northern Jiangsu Province, China, with 0.7 million and 1.1 million inhabitants, respectively. Dafeng is a high cancer incidence area and has a much higher mortality rate of EC than Ganyu. From 1996 to 2002, the yearly average age-adjusted mortality rate of EC in Dafeng was 36/100 000, whereas Ganyu had a considerably lower age-adjusted EC mortality rate of 24/100 000 during the same period ($P < 0.01$)[12].

Selection of cases and controls

Cases Newly diagnosed patients with primary esophageal cancer were recruited using data from regional cancer registry agencies. The cancer registry agencies in both counties were established in the late 1990s and are connected to the local Center for Disease Control and Prevention (CDC). All cases were coded according to the International Classification of Diseases, tenth revision (ICD-10, code C15.0 to C15.9). Secondary and recurrent cancers were excluded. All cases were restricted to local inhabitants of the two counties who have lived in either area for at least 5 years. In 2004, 45% and 72% of all newly registered EC cases were recruited and interviewed in Dafeng and Ganyu, respectively. The comparatively low response rate in Dafeng was partly due to the low involvement of local hospitals during the beginning of the study. A small number of cases were also unwilling to participate. Presently, the response rate in Dafeng is much higher. A system of rapid case recognition was used in the study. All regional hospitals were required by the local health authorities to report new EC patients shortly after diagnosis. As the cancer registry agencies are connected to the local CDC, the field investigators from the local CDC were able to identify and interview most patients within one month after their diagnosis. Of all the EC cases in Dafeng, 46% were histologically confirmed, 40% and 13% were diagnosed by endoscopy and radiology, respectively. In Ganyu, 30% of EC cases were histologically confirmed, 50% and 16% were diagnosed by endoscopy and radiology, respectively.

Controls Cases and controls were individually matched and derived from the same county. The criteria for the eligibility of controls were established as: controls had to be the same gender and within 5 years of age as the case, had to have lived in the area for at least 5 years, and had to have had good physical and mental health to answer questions reliably. Controls were randomly selected by a computer from the demographic database of the general population in the county police station. Local interviewers were responsible for locating and interviewing controls. If a selected control refused to participate, a replacement was found using the same recruitment criteria. The response rate of controls in both Dafeng and Ganyu was around 70%. Till March 2005, more than 400 EC cases were recruited in each county. However, the control recruitment rate is slower than the rate of case identification; thus only 291 and 240 pairs of cases and their matched controls were used in this analysis.

Data collection

Identical protocols and pre-tested standardized questionnaires were used. Data collection included a written informed consent, a face-to-face interview, a physical examination, and a 5 ml blood sample taken by professional interviewers from the local CDC in both counties. The questionnaire elicited detailed information on potential determinants of EC, including demographic information, socio-economic status, living conditions, disease history, family cancer history (any malignant neoplasm in first-degree relatives), smoking, alcohol drinking, dietary habits, and frequency and amount of food intake.

In our study, never-smokers were defined as having smoked fewer than 100 cigarettes in their lifetime. Current smokers and drinkers were defined as those who had the habit during the time of interview or those who stopped the habit because of health problems within one year of the date of interview. The dietary questionnaire used in this study included 90 food items. For each food item, the amount and frequency of consumption over the past year

were inquired. For cases, the amount and frequency of consumption referred to the year prior to the onset of the disease. In the final analysis, foods were categorized into several major groups: staple foods, preserved foods, meat, fish, eggs, soybean, and fruits and vegetables.

An anthropometric measurement and physical examination also took place at the time of interview to evaluate the subject's health status. Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared (i.e., kg/m^2). BMI was grouped into quartiles according to the Chinese national standard (underweight: <18.5, normal: 18.5-23.9, overweight: 24~27.9, obesity: ⩾28)[13].

Statistical analysis

Data were double entered using Epidata 2.1b and cleaned and analyzed using SAS v8.2. Chi-square and Student *t*-tests were used to compare the distribution of relevant factors among the control groups between the two counties. Conditional logistic regression with maximum likelihood estimation of parameters was applied for both univariate and multivariate analyses. This was done by transforming each matched pair into a single observation, where the explanatory variable value was the difference between the corresponding values for the case-control pair [14]. Continuous variables such as income level and amount of food intake were divided into quartiles based on the frequency distribution among control groups.

The strength of the association was quantified as odds ratio (OR) obtained from conditional logistic regression. Statistical significance was set at 0.05, and accordingly, 95% confidence interval (CI) around the OR was used to address precision.

RESULTS

Subject characteristics

There were 291 pairs of cases and controls (200 male and 91 female pairs) in Dafeng and 240 pairs (181 male and 59 female) in Ganyu, respectively. By design, cases and controls had similar distributions in terms of gender and age in both two counties (Table 1). The differences in the distribution of the above-mentioned variables between the two counties were examined by comparing the two control groups. The proportion of patients who were older than 70 years of age and the proportion of illiteracy in Ganyu were higher than that of Dafeng ($P=0.002$ and $P<0.001$, respectively). There was also a geographic variation between the two counties between the proportion of BMI ($P=0.014$), occurrences of misfortunes such as fire disasters, lost of family members, divorces, etc in the past 10 years ($P=0.008$) and family history of cancer ($P<0.001$). On the other hand, there was no significant difference in past economic status, ever-smoking and ever-alcohol drinking habits between the two counties.

Socio-economic status

EC occurred less in higher socio-economic groups characterized by high levels of education and high economic status in both counties (Table 2). On the contrary, low levels of education, low economic status, family history of cancer in first-degree relatives (Dafeng OR: 1.53, Ganyu OR: 2.07), and occurrences of misfortune in the past 10 years (Dafeng OR: 1.26, Ganyu OR: 1.64) increased the risk of developing EC in both areas. In Dafeng, when compared to the lowest quartile (underweight people) of BMI, the second quartile (normal weight people, OR = 0.45) and the third quartile (overweight people, OR = 0.26) significantly showed a reduced risk of EC; whereas the OR increased in the highest quartile (obese people, OR = 0.49). A similar association between BMI and EC risk was also found in Ganyu, although the trend was not significant.

Tobacco smoking and alcohol drinking

Consistent smoking elevated the risk of developing EC in both counties (Table 3). In Dafeng, former smokers and current smokers have a 1.93- and 2.42- fold higher risk of developing EC than never-smokers. In Ganyu former smoking and current smoking also increased the risk of developing EC (OR = 1.28 and 2.36 respectively). We found in Dafeng that smoking at an earlier age (for trend $P=0.016$), long durations of smoking (for trend $P=0.006$), and large amounts of cigarettes per day (for trend $P=0.029$) were significantly associated with increased EC risk, with an apparent dose-response relationship. However, these associations were not significant in Ganyu.

In Ganyu, subjects who had drunk alcohol tended to have a higher risk of EC (OR = 1.71, 95% CI: 1.02-2.88). Moreover, drinking at an early age (for trend $P=0.012$) and long durations of drinking (for trend $P=0.061$) showed an increasing association with EC (Table 4). A high consumption of pure ethanol per week, 10 years ago, slightly elevated the risk of EC, but no significant dose-response relationship was found. We did not find any significant association between alcohol drinking and EC in Dafeng, despite its similar alcohol drinking prevalence as Ganyu. The joint effects by smoking and alcohol drinking were also explored in both counties, but no significant interaction was observed either in Dafeng ($P=0.900$) or in Ganyu ($P=0.870$).

Dietary factors

After adjusting for potential confounders in both Dafeng and Ganyu, subjects with fast eating speeds showed an increased risk of developing EC (Dafeng: OR=4.01; Ganyu: OR=3.09). On the other hand, high food temperatures, the possibility of being exposed to grain fungi pollution, and frequent intake of fresh garlic did not influence EC risk significantly (Table 5).

With regard to the consumption of major food groups, high consumptions of fish and seafood products significantly elevated the risk of developing EC in Dafeng (for trend $P=0.024$). Staple foods, preserved foods, fruits and vegetables, and soybean, however, were not apparently associated with EC risk in either county.

DISCUSSION

This population-based case-control study, conducted in high and low-risk areas of Jiangsu Province, China demonstrated an association between tobacco smoking, alcohol drinking, dietary factors and EC. These

Table 1 Characteristics among EC cases and controls in Dafeng and Ganyu[1] *n* (%)

Characteristics	Dafeng (high-risk)		Ganyu (low-risk)		*P* value[2]
	Case (*n* = 291)	Control (*n* = 291)	Control (*n* = 240)	Case (*n* = 240)	
Gender: Male	200 (68.7)	200 (68.7)	181 (75.4)	181 (75.4)	
Female	91 (31.3)	91 (31.3)	59 (24.6)	59 (24.6)	0.088
Age (yr) Mean±SD	64.8 ± 8.6	64.6 ± 8.9	65.4 ± 10.3	65.6 ± 10.4	
<50	14 (4.8)	17 (5.8)	19 (7.9)	17 (7.1)	
50-59	61 (30.0)	59 (20.3)	48 (20.0)	51 (21.3)	
60-69	137(47.1)	137 (47.1)	78 (32.5)	76 (31.7)	0.002
70-79	71 (24.4)	69 (23.7)	77 (32.1)	78 (32.5)	
⩾80	8 (2.8)	9 (3.1)	18 (7.5)	18 (7.5)	
Level of education					
Illiterate	156 (53.6)	130 (44.7)	138 (57.7)	164 (68.6)	
Primary school	95 (32.7)	119 (40.9)	63 (26.4)	54 (22.6)	<0.001
Secondary school & above	40 (13.7)	42 (14.4)	38 (15.9)	21 (8.8)	
Past economic status (By separate cut-off points)					
Median (CNY/yr)	1250	1500	1000	775	
1 (lowest)	97 (33.5)	55 (19.0)	38 (16.2)	47 (20.3)	
2	68 (23.5)	64 (22.1)	71 (30.2)	87 (37.5)	
3	73 (25.2)	96 (33.1)	71 (30.2)	59 (25.4)	0.235
4 (highest)	52 (17.9)	75 (25.9)	55 (23.4)	39 (16.8)	
Smoking status[3]					
Neversmoker	92 (31.6)	122 (41.9)	95 (39.6)	82 (34.2)	
Former-smoker	71 (24.4)	64 (22.0)	19 (7.9)	17 (7.1)	0.067
Current smoker	128 (44.0)	105 (36.1)	126 (52.5)	141 (58.7)	
Alcohol drinking status[4]					
Never drinker	175 (60.1)	181 (62.2)	143 (59.6)	131 (54.6)	
Former drinker	5 (1.7)	7 (2.4)	7 (2.9)	7 (2.9)	0.076
Current drinker	111 (38.1)	103 (35.4)	90 (37.5)	102 (42.5)	
Encountered misfortune in past10 yr:					
No					
Yes	54 (18.6)	41 (14.2)	33 (14.0)	54 (23.3)	
	237 (81.4)	248 (85.8)	203 (86.0)	178 (76.7)	0.008
History of family cancer					
No	112 (38.5)	86 (29.6)	16 (6.7)	29 (12.1)	
Yes	179 (61.5)	205 (70.5)	224 (93.3)	211 (87.9)	<0.001
Body mass index					
<18.5	60 (20.8)	27 (9.3)	18 (7.6)	31 (13.4)	
18.5-23.9	182 (63.0)	192 (66.2)	161 (67.9)	155 (67.1)	0.014
23.9-27.9	34 (11.8)	60 (20.7)	46 (19.4)	28 (12.1)	
⩾28	13 (4.5)	11 (3.8)	12 (5.1)	17 (7.4)	

[1] Some strata do not match the total because of missing values; [2] The *P*-value for comparing the distribution of factors between the two counties; [3] Never-smokers and ever-smokers were used for comparing the smoking habits between the two counties; [4] Because of the few numbers of former drinkers in both two counties, alcohol drinking status was categorized to never-drinkers and ever-drinkers for the comparison between the two counties and following analyses.

associations were compared separately in the two regions which had similar socioeconomic status and geographic characteristics. From our awareness, this is the first comparative population-based case-control study conducted in low-risk and high-risk areas simultaneously to compare the different associations of risk factors and EC in similar areas with high-risk gradients. Like other epidemiological researches, our study showed that EC was inversely associated with socioeconomic status, such as level of education and income. However this disease was more frequent among subjects with smoking and alcohol drinking habits and unhealthy dietary factors. Furthermore, a geographic variation of some associations was observed between the low-risk and high-risk areas. Smoking elevated the risk of EC in both areas concordantly, but the dose-response relationship of smoking and smoking related variables (age of first smoking, duration and amount) was apparent only in the high-risk area. On the contrary, the effect of alcohol drinking on EC and a dose-response relationship was only observed in the low-risk area.

Supporting previous studies, the risk of EC was inversely associated with socioeconomic status in the present study[15,16]. People with higher levels of education and better financial situations tend to have a lower risk of developing EC due to good living conditions and better health care access. Increased risk was found in people who had encountered misfortune in the past 10 years (Dafeng OR: 1.26, Ganyu OR: 1.64), or had a history of family cancer in first-degree relatives (Dafeng OR: 1.53, Ganyu OR: 1.57). These results were consistent in both high-risk and low-risk areas.

Associations between body mass index (BMI) and EC have been explored in several studies. Chow *et al*[17] reported a tendency towards a decreasing risk of esophageal squamous cell carcinoma with increasing BMI. Engeland found that low BMI increased the risk

Table 2 OR[1] and 95% CI of socioeconomic status in EC of Dafeng and Ganyu

Socioeconomic status	Dafeng (high-risk)	Ganyu (low-risk)
Level of education		
Illiterate	1.00 (Referent)	1.00 (Referent)
Primary school	0.54 (0.35-0.84)	0.58 (0.33-1.03)
Secondary school & above	0.74 (0.39-1.41)	0.42 (0.21-0.83)
P value for trend	0.08	0.008
Past economic status		
1 (lowest)	1.00 (Referent)	1.00 (Referent)
2	0.67 (0.43-1.06)	1.03 (0.61-1.75)
3	0.44 (0.28-0.68)	0.76 (0.44-1.31)
4 (highest)	0.39 (0.23-0.65)	0.73 (0.41-1.28)
P value for trend	<0.001	0.024
Encountered misfortune in past 10 yr		
No	1.00 (Referent)	1.00 (Referent)
Yes	1.26 (0.80-1.98)	1.64 (0.98-2.73)
History of family cancer		
No	1.00 (Referent)	1.00 (Referent)
Yes	1.53 (1.06-2.19)	2.07 (1.03-4.17)
Body mass index		
<18.5	1.00 (Referent)	1.00 (Referent)
18.5-23.9	0.45 (0.26-0.76)	0.50 (0.28-0.90)
23.9-27.9	0.26 (0.13-0.50)	0.36 (0.17-0.75)
⩾28	0.49 (0.18-1.33)	0.80 (0.33-1.98)
P value for trend	0.002	0.376

[1] Matched by age and gender, further adjusted for education level and past economic status (quartile).

Table 3 OR[1] and 95% CI of tobacco smoking in EC of Dafeng and Ganyu

Tobacco smoking	Dafeng (high-risk)	Ganyu (low-risk)
Smoking status[2]		
Former smoker	1.93 (0.91-4.08)	1.28 (0.28-5.83)
Current smoker	2.42 (1.28-4.56)	2.36 (0.89-6.26)
P value for trend	0.005	0.07
Age at first smoke[2]		
<20	2.02 (0.93-4.38)	1.60 (0.31-7.90)
20-34	2.32 (1.15-4.67)	2.25 (0.80-6.35)
⩾35	1.80 (0.62-5.24)	0.98 (0.29-3.24)
P value for trend	0.016	0.249
Duration of smoking (yr)[2]		
1-29	1.61 (0.67-3.86)	1.44 (0.46-4.42)
30-49	2.65 (1.28-5.49)	2.04 (0.60-6.92)
⩾50	2.04 (0.78-5.35)	1.98 (0.43-9.11)
P value for trend	0.009	0.194
Amount of smoking (Cig/d)[2]		
1-9	1.36 (0.50-3.74)	1.12 (0.27-4.68)
10-19	2.21 (1.01-4.80)	1.56 (0.42-5.78)
⩾20	2.04 (1.00-4.18)	0.91 (0.32-2.61)
P value for trend	0.015	0.915
Total consumption of cigarettes[2]		
1 (lowest)	1.40 (0.61-3.21)	0.96 (032-2.82)
2	2.55 (1.06-6.14)	3.50 (0.37-32.8)
3	1.88 (0.79-4.49)	1.94 (0.25-14.7)
4 (highest)	1.81 (0.57-5.74)	0.74 (0.19-2.81)
P value for trend	0.029	0.959

[1] Matched by age and gender, further adjusted for level of education, past economic status (group) and alcohol drinking; [2] Never-smokers were used as the reference group.

of esophageal squamous cell carcinoma, while high BMI increased the risk of esophageal adenocarcinoma. In general, lowest BMI had the highest risk of EC[18]. Our study found similar results in both low-risk and high-risk areas. The risk of developing EC was significantly lower in normal and overweight groups when compared to the underweight group. However, the OR was high in the obese group. An increased risk of esophageal adenocarcinoma among obese persons has been explained by a dose-dependent association between increasing BMI and the risk of gastro-esophageal reflux symptoms, as observed by Nilsson *et al*[19].

Table 4 OR[1] and 95% CI of alcohol drinking in EC of Dafeng and Ganyu

Alcohol drinking	Dafeng (high-risk)	Ganyu (low-risk)
Alcohol drinking		
Never	1.00 (Referent)	1.00 (Referent)
Ever	1.01 (0.70-1.46)	1.71 (1.02-2.88)
P value	0.964	0.043
Age of first drink[2]		
<20	0.83 (0.44-1.58)	2.59 (1.03-6.50)
20-34	1.23 (0.79-1.91)	1.95 (1.08-3.53)
⩾35	0.81 (0.48-1.35)	1.18 (0.56-2.47)
P value for trend	0.815	0.012
Duration of drinking (yr)[2]		
1-24	0.96 (0.56-1.59)	1.28 (0.58-2.79)
25-34	0.89 (0.48-1.64)	1.48 (0.75-2.94)
35-44	1.57 (0.92-2.70)	1.47 (0.71-3.01)
⩾45	0.77 (0.43-1.40)	1.88 (0.95-3.75)
P value for trend	0.834	0.061
Alcohol consumption 10 years ago[2] (pure ethanol mL/wk)		
1-249	0.87 (0.49-1.54)	0.79 (0.36-1.74)
250-499	1.06 (0.60-1.89)	0.61 (0.30-1.25)
500-749	0.97 (0.52-1.79)	1.63 (0.77-3.43)
⩾750	1.10 (0.63-1.93)	1.27 (0.71-2.28)
P value for trend	0.74	0.223

[1] Matched by age and gender, further adjusted for level of education, past economic status (group) and tobacco smoking; [2] Never-drinkers were used as the reference group.

In conformity with other epidemiological studies shown in Western countries and some areas of Asia and Africa[20-22], increased risks of EC among former smoking and current smoking subjects were observed in both areas of our study. Tobacco smoke contains over 3000 constituents including 30 carcinogens, such as polycyclic aromatic hydrocarbons (PAHs), aromatic amines, and N-nitrosamines. The metabolites of these carcinogens may lead to gene mutation and cancer[23]. Age of first smoke, duration and dosage of tobacco use were also strongly associated with an elevated risk of developing EC in Dafeng, with an apparent dose-response relationship. Although Ganyu had a similar smoking prevalence, these time and dosage dependent results were not statistically significant in this area.

Several studies have reported a strong correlation between EC and alcohol abuse[24,25]. Alcoholic beverages also contain carcinogens and other compounds and may facilitate the absorption of esophageal mucosal cells and make them more susceptible to chemical carcinogens[26]. On the contrary of tobacco smoking, the positive association between alcohol drinking and EC was only found in Ganyu in our study (OR=1.71). Several studies

Table 5 OR and 95% CI of dietary habits, food consumption in EC of Dafeng and Ganyu

Dietary factor	County	Category				*P* value for trend
		1 (lowest)	2	3	4 (highest)	
Food temperature[1]	Dafeng	1	0.51 (0.24-1.09)	-	-	0.08
(1-Normal; 2- Hot)	Ganyu	1	1.14 (0.55-2.41)	-	-	0.714
Eating speed[1]	Dafeng	1	4.01 (1.87-8.62)	-	-	<0.001
(1-Normal; 2-Fast)	Ganyu	1	3.09 (1.24-7.70)	-	-	0.015
Self reported grain fungi pollution[1]	Dafeng	1	2.27 (0.79-6.54)	-	-	0.131
(1-Likely; 2-Not likely)	Ganyu	1	1.18 (0.45-3.11)	-	-	0.741
Fresh garlic/wk[2,3]	Dafeng	1	0.64 (0.26-1.60)	-	-	0.337
	Ganyu	1	1.17 (0.57-2.41)	-	-	0.664
Staple foods[1]	Dafeng	1	0.45 (0.19-1.10)	0.54 (0.21-1.38)	0.73 (0.26-2.04)	0.474
	Ganyu	1	0.98 (0.10-9.77)	0.45 (0.05-4.38)	0.54 (0.05-4.34)	0.324
Meat[4]	Dafeng	1	0.73 (0.29-1.85)	1.66 (0.68-4.10)	1.93 (0.64-5.77)	0.16
	Ganyu	1	0.54 (0.12-2.42)	1.17 (0.28-4.92)	0.65 (0.11-3.67)	0.305
Fish and seafood products[4]	Dafeng	1	1.14 (0.64-2.03)	2.11 (1.12-3.96)	1.91 (1.00-3.64)	0.023
	Ganyu	1	0.98 (0.42-2.28)	0.64 (0.28-1.44)	1.04 (0.46-2.33)	0.794
Eggs[4]	Dafeng	1	0.53 (0.20-1.44)	1.23 (0.54-2.80)	1.99 (0.72-5.49)	0.146
	Ganyu	1	0.69 (0.28-1.73)	0.30 (0.10-1.10)	0.95 (0.41-2.22)	0.936
Soybean[4,5]	Dafeng	1	1.81 (0.88-3.74)	-	-	0.11
	Ganyu	1	1.31 (0.37-4.59)	-	-	0.677
Preserved foods[4]	Dafeng	1	0.26(0.09-0.75)	0.49 (0.16-1.46)	0.94 (0.37-2.36)	0.635
	Ganyu	1	1.05 (0.37-2.97)	0.56 (0.21-1.48)	1.21 (0.46-3.20)	0.932
Vegetables[4]	Dafeng	1	1.26 (0.50-3.16)	0.94 (0.39-2.30)	1.37 (0.49-3.83)	0.72
	Ganyu	1	0.34 (0.08-1.54)	0.80 (0.20-3.18)	0.76 (0.15-3.72)	0.889
Fruits[2]	Dafeng	1	1.02 (0.42-2.47)	0.42 (0.16-1.12)	1.23 (0.51-2. 98)	0.802
	Ganyu	1	1.61 (0.68-3.80)	1.13 (0.43-2.95)	1.17 (0.41-3.37)	0.746

[1] Matched by age and gender, further adjusted for level of education, past economic status (group), smoking, alcohol drinking, BMI group, cancer family history, eating speed, food temperature and self-reported grain fungi pollution; [2] Matched by age and gender, further adjusted for education level, past economic status (group), smoking, alcohol drinking, BMI group, eating speed and family history of cancer; [3] Less than 3 times per week=1, 3 times per week and above=2; [4] Matched by age and gender, further adjusted for level of education, past economic status (group), smoking, alcohol drinking, BMI group, cancer family history, eating speed and food temperature; [5] Categorized by median among controls.

have reported a linear relationship between an overall daily ethanol consumption and EC risk[27,28]. However, in our study only the age of initial drinking and years of alcohol drinking were found to be associated with EC risk in Ganyu. No clear relationship between daily alcohol consumption and EC was found.

The interaction between tobacco smoking and alcohol drinking has been studied in many researches. It has been suggested that alcohol and tobacco interact in a multiplicative way[29,30]. In a large scale study, Castellsagué reported that the risk of EC in the highest joint level of alcohol and cigarette smoking increased 50.85-fold and 35.34-fold among men and women[7]. However the joint effect of smoking and alcohol was not found to be statistically significant in the high-risk area and the low-risk area in our study. The link between smoking, alcohol and EC in China are not as apparent as in Western countries. Several previous studies conducted in other high-risk areas of Jiangsu, China either did not find any relation or found only a weak association between smoking, alcohol drinking and EC[31,32].

Dietary factors are thought to play an important role in the pathogenesis of EC. Some epidemiological studies have suggested that the risk of EC is inversely associated with a higher intake of fruits and vegetables[10,33], while a detrimental effect was observed among high intake of certain types of meat, butter and saturated fatty acids[34]. Increased risk was related to N-nitrosamine compounds (mainly from preserved foods), foods contamination by fungus and the presence of toxins. Some unhealthy dietary habits such as fast eating speeds, consumption of hot foods and soups can cause the injury of esophageal mucosa and render the mucosa more susceptible to carcinogens.

An increased OR was found among fast eating subjects in both areas of this study (Dafeng OR: 4.01, Ganyu OR: 3.09). However, the associations between high food temperatures, the possibility of fungi pollution of grain, frequent intake of fresh garlic and EC was not statistically significant. In the food group analysis, after adjusting for potential confounders, we did not find any significant association between major food consumption and EC risk in either area. A positive association of fish and seafood product intake in Dafeng was found (for trend $P=0.024$). Fish is a rich dietary source of n-3 fatty acids. It has been reported that this long chain of fatty acid can suppress mutation, inhibit cell growth, and enhance cell apoptosis, thus reducing the risk of developing cancer[35]. The contradicting results found in our study of the increased risk found in the association between fish consumption and EC in Dafeng was probably due to water contamination or other unidentified confounders. However, this hypothesis needs to be further clarified and studied. Moreover, it may be more reasonable to study food composition and micronutrients in our future analysis rather than to use individual foods or food groups[36].

Ganyu has a high proportion of ageing and illiterate residents. The economic status of residents in Ganyu is also lower than Dafeng, although the difference is not significant (Table 1). As disease is more prevalent

among ageing populations and the level of education and economic situation are inversely associated with the risk of developing EC, it can be expected that the two counties would have a far higher risk gradient if they had a similar distribution of age and socioeconomic related factors.

As mentioned above, a heterogeneous association between smoking, alcohol drinking and EC was observed in the low- and high-risk areas in our study, despite their similar geographic characteristics and general socioeconomic statuses. A malignant tumor is the result of a series of DNA alterations in a single cell, which leads to a loss of normal functioning. A large number of gene coding for enzymes and receptors are involved in xenobiotic metabolism, with many of them showing polymorphisms. Many molecular epidemiological studies have proved that polymorphisms in activation and detoxification enzymes can interact with environmental carcinogens. It has been reported that GSTM1 null carriers may be especially susceptible to the action of tobacco with regards to EC[32], while inactive the ALDH2 genotype increases the risk of EC in alcoholics[37]. Genetic polymorphisms can interact with dietary factors. For example, cruciferous vegetables can inhibit the metabolic activation of phase I enzymes and induce the detoxification of carcinogens via phase II enzymes[38]. The polymorphism of one gene may also have an effect on other genes. Gene-gene interactions between GSTM1 0/0 and CYP1A1 and CYP1A2 enzyme induction have been observed in smokers[39]. Another example is that individuals with CYP1A1 Ile/Val alleles have greater CYP1A2 activity than those with wild type CYP1A1[40]. Furthermore, it has been suggested that genes can influence individual behaviours such as smoking, alcohol drinking and excess calorie intake, thereby having the potential to affect cancer risk[41].

Both environmental factors and human genes can show considerable regional variability. The variation in these factors together with their separate and joint effects ultimately determine the risk of cancer in different regions and may be the main reason for the large EC risk gradient between the counties in Jiangsu Province. Unfortunately scientific evidence on genetic polymorphisms, gene-environmental and gene-gene interactions remains inconsistent and inconclusive because of low statistical power and few candidate genes in previous studies. Moreover, no study has ever been conducted to compare the association between gene-environmental interaction and EC risk in apparently similar areas with a high risk gradient. Therefore, our future study will focus on genetic polymorphisms and their interactions with different environmental, lifestyle and dietary factors in the etiology of EC in high and low-risk areas, with a sufficient sample size and candidate genes.

Our present population-based case-control study has some limitations. Differences in the etiological factors between esophageal adenocarcinoma and squamous cell carcinoma may exist. Because of the low histological examination rate in China, it is difficult to differentiate between the subtypes of EC in a population-based study. Additionally, most risk factors in our study are based on self-reported data and may be subject to recall bias. Moreover, the relationship between BMI and EC was examined by using height and weight measurements obtained at the time of interview. Some cases might have begun to lose weight at an earlier time because of the disease. This factor could also have caused bias in our study.

In summary, the present study demonstrated the association between smoking, alcohol drinking, dietary factors and EC risk in the low-risk and high-risk areas of Jiangsu Province, China. Heterogeneous effects of smoking and alcohol drinking were found between the two areas, despite their similar geographic characteristics and general socioeconomic status. The variation in environmental risk factors, together with gene-environment and gene-gene interactions may be the main reason for these heterogeneous associations and may contribute to the large risk gradient of EC mortality in Jiangsu Province, China.

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GASTRIC CANCER

Effects of tachyplesin and n-sodium butyrate on proliferation and gene expression of human gastric adenocarcinoma cell line BGC-823

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Abstract

AIM: To investigate the effects of tachyplesin and n-sodium butyrate on proliferation and gene expression of human gastric adenocarcinoma cell line BGC-823.

METHODS: Effects of tachyplesin and n-sodium butyrate on proliferation of BGC-823 cells were determined with trypan blue dye exclusion test and HE staining. Effects of tachyplesin and n-sodium butyrate on cell cycle were detected by flow cytometry. Protein levels of c-erbB-2, c-myc, p53 and p16 were examined by immunocytochemistry.

RESULTS: The inhibiting effects were similar after 2.0 mg/L tachyplesin and 2.0 mmol/L n-sodium butyrate treatment, the inhibitory rate of cellular growth was 62.66% and 60.19% respectively, and the respective maximum mitotic index was decreased by 49.35% and 51.69% respectively. Tachyplesin and n-sodium butyrate treatment could markedly increase the proportion of cells at G_0/G_1 phase and decrease the proportion at S phase. The expression levels of oncogene c-erbB-2, c-myc, and mtp53 proteins were down-regulated while the expression level of tumor suppressor gene p16 protein was up-regulated after the treatment with tachyplesin or n-sodium butyrate. The effects of 1.0 mg/L tachyplesin in combination with 1.0 mmol/L n-sodium butyrate were obviously superior to their individual treatment in changing cell cycle distribution and expression of c-erbB-2, c-myc, mtp53 and p16 protein. The inhibitory rate of cellular growth of BGC-823 cells after combination treatment was 62.29% and the maximum mitotic index was decreased by 51.95%.

CONCLUSION: Tachyplesin as a differentiation inducer of tumor cells has similar effects as n-sodium butyrate on proliferation of tumor cells, expression of correlative oncogene and tumor suppressor gene. It also has a synergistic effect on differentiation of tumor cells.

Key words: Tachyplesin; n-sodium butyrate; Gastric adenocarcinoma cell; Cell differentiation

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INTRODUCTION

Tachyplesin, isolated from acid extracts of hemocytes of Chinese horseshoe crab (*Tachypleus tridentatus*), a sort of marine arthropod known as "live fossil", is a low-molecular-weight polypeptide [1]. This bioactive component has antitumor effect and induces tumor cell differentiation [2]. We used the small molecule polar compound n-sodium butyrate[3] as a parallel control to compare the effects on proliferation, cell cycle and expression of correlative oncogene and tumor suppressor gene of BGC-823 cells treated with tachyplesin, n-sodium butyrate and their combination. This research could provide information for further identification of antitumor low-molecular-weight crude bioactive peptides such as tachyplesin that can modulate the proliferation and differentiation of tumor cells.

MATERIALS AND METHODS

Tachyplesin isolation and extraction

Tachyplesin was isolated from acid extracts of Chinese horseshoe crab *(Tachypleus tridentatus)* hemocytes as previously described[4]. The crude extract was separated by SephadexG-50, CM-sepharose CL-6B column chromatography.

Cell culture

BGC-823 cells were cultured in RPMI-1640 medium supplemented with 20% heat-inactivated fetal calf serum,100 units/mL penicillin,100 mg/L streptomycin and 50 mg/L kanamycin at 37℃ in atmosphere containing 50 mL/L CO_2. BGC-823 cells were treated with culture medium containing inducers after seeded for 24 h.

Inducing treatment

The powder of tachyplesin obtained from separation, purification and lyophilization was dissolved in D-Hank's solution to prepare 100 mg/L concentrated solution. The mother liquor was prepared for solution of a given concentration with culture medium. N-sodium butyrate purchased from Sigma Co., was dissolved in appropriate concentration of D-Hank's solution to prepare 200 mmol/L concentrated solution. The concentrations of the three treatment solutions were as follows: 2.0 mg/L tachyplesin-treatment (Ta), 2.0 mmol/L n-sodium butyrate-treatment (Tb), and 1.0 mg/L tachyplesin + 1.0 mmol/L n-sodium butyrate for the combination treatment (Ta+Tb). The experimental groups were treated with the three reagents after medium was changed, while the control group was cultured continuously with fresh culture medium for future use.

Determination of cell growth curve

BGC-823 cells were collected in logarithmic phase, then suspension of BGC-823 cells was made in 5.0×10^4 cells/mL. The cells were seeded into 15 mL culture flasks with 2 mL per flask. After seeded for 24 h, the experimental groups were treated with the reagents containing different kinds of differentiation-induced gradients while the control group was cultured continuously in fresh culture medium. During the first seven days, untreated or treated cells were harvested from three flasks everyday, and the viable cells were counted by the trypan blue dye exclusion test to get average value. The similar results were found in triplicate experiments, the growth curve was derived from one of the results.

Determination of cell mitotic index

BGC-823 cells (5.0×10^4 mL) were seeded in to bottles containing little penicillin with cover slips. Treatments were performed after the cells were seeded for 24 h. During the first seven days, the cover slips were removed from two bottles of the untreated or treated cells everyday, fixed in Bouin-Hollande fixative, and stained with Hematoxylin-Eosin (HE). The mitotic cells in 1000 cells on each cover slip were counted, and the mitotic index curve was drawn.

Determination of cell cycle

BGC-823 cells were collected respectively from the treated groups and the control group after digested and centrifuged at 1000 r/min for 5 min. All the cells collected were rinsed three times with D-Hank's solution. The cells grown on cover slips were fixed in 75% pre-cooled ethanol at 4 ℃ overnight, centrifuged and resuspended in 100 mg/L RNase at 37℃ for 30 min. Then 50 mg/L propidium iodide was added into the suspended cells at 4 ℃ in dark for 30 min. The cell cycle was analyzed by flow cytometry (Bacton-Dickson Co.) and the data were analyzed by Cell FIT cell cycle analysis software（Version2.01.2）.

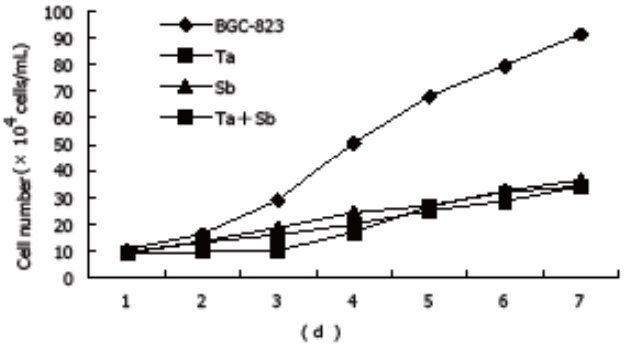

Figure 1 Cell growth inhibition in BGC-823 cells after tachyplesin, n-sodium butyrate and their combination treatment (×10^4 cells/mL).

Immunohistochemical analysis

BGC-823 cells (5.0×10^4/mL) and cells treated with different inducers were seeded into bottles containing little penicillin with cover slips for 36 h respectively. The cells grown on cover slips were rinsed with D-Hank's solution at 37℃. The changes in c-erbB-2, c-myc , p53 and p16 expression of treated and untreated BGC-823 cells were determined by SABC immunohistochemical assay. The reagent kit (Wuhan Boster Bioengineering Corporation) was used to determine the expression of these genes. PBS was used to take the place of primary antibody as the negative controls and positive specimens were used as positive controls.

RESULTS

Effects of tachyplesin and n-sodium butyrate on proliferation of BGC-823 cells

The cell growth curve determination showed that the proliferation of untreated BGC-823 cells was very fast. The cell number increased to 91.29×10^4/mL on the seventh day which was 18.26 times of that of the original 5.0×10^4/mL on the first day, with a doubling time of 45.82 h. However, after treated with tachyplesin or n-sodium butyrate, the growth rate of BGC-823 cells was inhibited. After treatment with 2.0 mg/L tachyplesin, the number of cells was 34.09×10^4/mL which was 6.8 times of that of the original number on the 7th day, with the doubling time prolonged to 75.7 h and the growth inhibitory rate increased to 62.77%. After treatment with 2.0 μg/mL n-sodium butyrate, the number of cells was 36.34×10^4/mL which was 7.27 times of that of the original number on the 7th day, the doubling time was 67.10 h and the growth inhibitory rate was 60.19%. After treatment with 1.0 mg/L tachyplesin + 1.0 mmol/L n-sodium butyrate, the number of cells was 33.87×10^4/mL on the 7th day, the doubling time was 69.75 h and the growth inhibitory rate was 62.29% (Figure 1).

The cell mitotic index determination showed that BGC-823 cells had vigorous proliferation capability, which reached to the divided peak on the fourth day and the maximum mitotic index was 38.5‰. However the mitotic

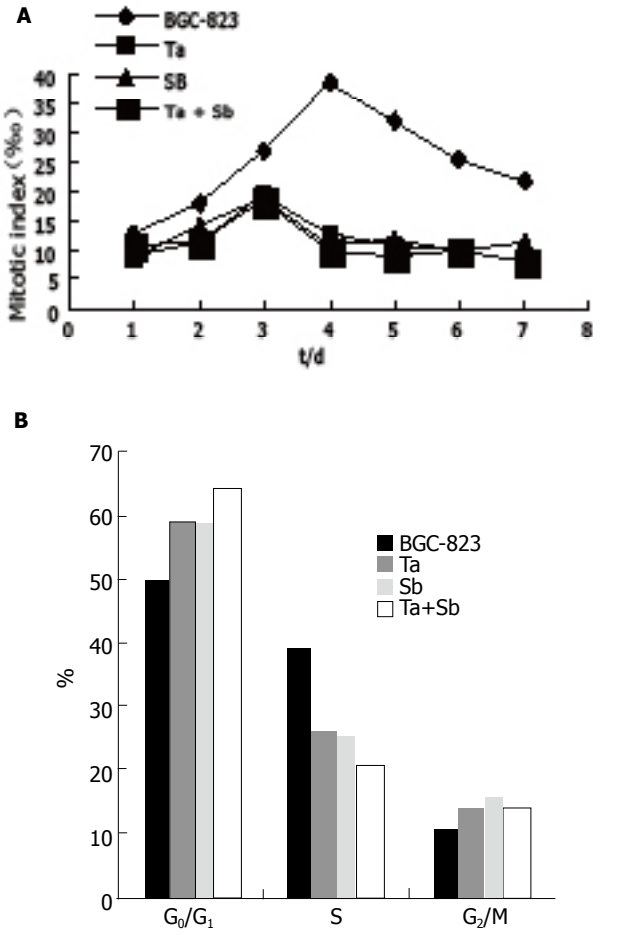

Figure 2 Influence of tachyplesin, n-sodium butyrate and their combination on mitotic index (**A**) and cell cycle (**B**) of BGC-823 cells.

index of cells treated with tachyplesin or n-sodium butyrate or their combination was only 19.5‰, 18.6‰ and 18.5‰ respectively at the divided peak, and declined by 49.35%, 51.69% and 51.95% respectively. The divided peak occurred on the third day after inducing treatment (Figure 2A).

Effects of tachyplesin and n-sodium butyrate on cell cycle of BGC-823 cells

Cell cycle of BGC-823 cells was analyzed by flow cytometry. The results showed that the cell cycle distribution of BGC-823 cells changed obviously when the cells were treated with tachyplesin or n-sodium butyrate. The proportion of untreated cells was 50.7% in G_0/G_1 phase, 39.1% in S phase and 10.2% in G_2+M phase. However, the proportion of cells at G_0/G_1 phase was 59.5%, 26.3% at S phase and 14.2% at G_2+M phase after treated with 2.0 mg/L tachyplesin. Similar changes occured in cells treated with 2.0 mmol/L n-sodium butyrate r the proportion of cells in G_0/G_1 phase was 59.2%, 25.2% in S phase and 15.6% in G_2+M phase. Meanwhile, the proportion of BGC-823 cells increased from 50.7% to 64.5% in G_0/G_1 phase and decreased from 39.1% to 20.9% in S phase(Table 1). The proportion of cells in G2+M phase increased from 10.2% to 14.6%(Figure 2B).

Table 1 Influence of tachyplesin, n-sodium butyrate and their combination on cell cycle of BGC-823 cells (%)

Group	G_0/G_1	S	G_2/M
BGC-823	50.7	39.1	10.2
Ta	59.5	26.3	14.2
Sb	59.2	25.2	15.6
Ta+Sb	64.5	20.9	14.6

Effects of tachyplesin and n-sodium butyrate on expression of oncogene and tumor suppressor gene of BGC-823 cells

Immunocytochemistry showed that c-erbB-2 protein level in the control group was high. The dark brown-yellow granules were mainly detected in cytoplasm and membrane of BGC-823 cells. But in nuclei, the protein level was low, while a few unevenly-distributed buff granules could be detected (Figure 3A). After treated with tachyplesin, the positive rate of c-erbB-2 expression in BGC-823 cells decreased. The brown-yellow particles were mainly detected in cytoplasm around nuclear membrane but a few in nuclei (Figure 3B). After treated with n-sodium butyrate, relatively bigger brown-yellow granules were mainly detected in nuclei and cytoplasm around the nuclei but a few around the cell membrane(Figure 3C). After treated with the combination of the two inducers, the immunocytochemistry signal became weaker than after treated with n-sodium butyrate alone. The smooth and evenly-distributed brown-yellow granules were mainly detected in nucleoli and nuclear membrane of the cells (Figure 3D).

Immunocytochemistry showed that the c-MYC protein level in the control group was high, the brown granules were mainly detected in nuclei and cytoplasm around the nuclei of BGC-823 cells (Figure 3E). After treated with tachyplesin, the level of c-MYC protein in BGC-823 cells decreased. The light brown-yellow granules were mainly detected in karyoplasms around the nuclear membrane, while in nuclei, the protein level was very low (Figure 3F). After treated with n-sodium butyrate, the brown particles were mainly detected in nucleoli and cytoplasm, while in nuclei of the cells, the signal was almost negative (Figure 3G). After treated with combination of the two inducers, the signal became weaker than after treated with n-sodium butyrate alone. The evenly-distributed brown-yellow granules were mainly detected in nucleoli and cytoplasm while in nuclei of the cells, the signal could be scarcely detected (Figure 3H).

Immunocytochemistry showed that mtP53 protein level in the control group was high. The dark brown-yellow granules were mainly detected in nuclei, while a few granules were detected in cytoplasm of BGC-823 cells. Their distributing was irregular(Figure 3I). After treated with tachyplesin, the rate of positive expression of mtP53 in BGC-823 cells decreased. The light brown-yellow granules were mainly detected in cytoplasm, while in nuclei of the cells the expression was negative (Figure 3J). After treated with n-sodium butyrate, the light brownish-red granules

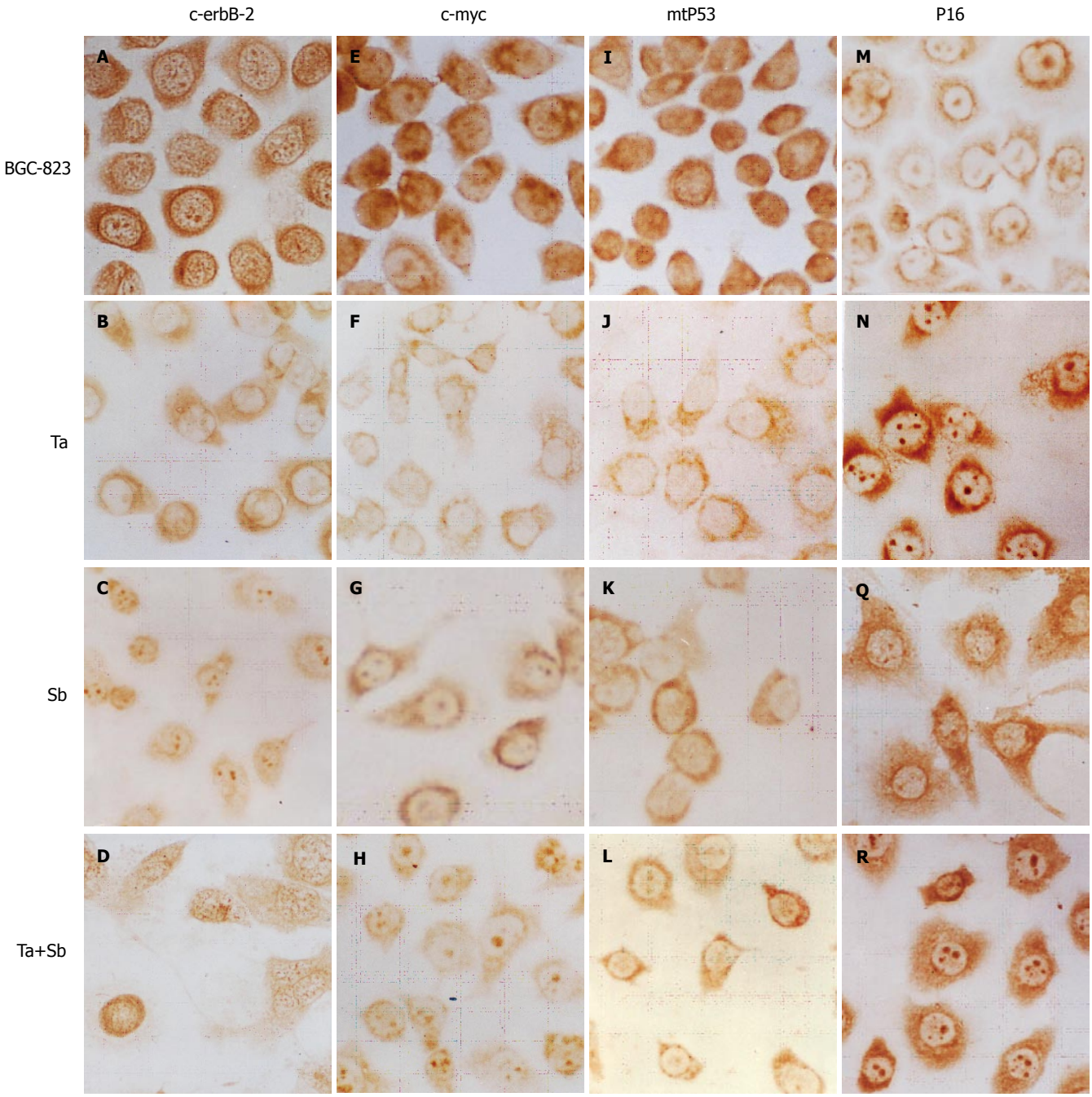

Figure 3 Expression of c-erbB-2 (A-D), c-myc (E-H), p53 (I-L), and p16 (M-R) in BGC-823 cells, tachyplesin-treated cells, n-sodium butyrate-treated cells, and tachyplesin + n-sodium butyrate-treated cells. ×536

were mainly detected in cytoplasm, while in nuclei of the cells, the mtP53 expression decreased greatly, and the signal became almost negative(Figure 3K). After treated with combination of the two inducers, the immunocytochemistry signal as a whole became weaker than after treated with n-sodium butyrate alone. The light brown granules were mainly detected in cytoplasm and could be hardly detected in nuclei of the cells (Figure 3L).

Immunocytochemistry showed that P16 protein level in the control group was low. The brown-yellow granules were detected in nucleoli and cytoplasm around the nuclear membrane of BGC-823 cells. The signal in cytoplasm and cell membrane was very weak (Figure 3M). However, after treated with tachyplesin, the P16 protein level in BGC-823 cells became very high. The evenly-distributed brown granules were detected in cytoplasm (Figure 3N). After treated with n-sodium butyrate, the P16 protein level became high. The brown granules were mainly detected in cytoplasm in a spare and dispersed manner. Some smaller granules were detected in marginal area of cytoplasm and protuberance of cells (Figure 3Q). After treated with combination of the two inducers, the P16 protein level became very high. The evenly-distributed brown granules were mainly detected in nucleoli and cytoplasm. The protein level in the combination treatment group was higher than in the group treated with n-sodium butyrate alone and the signal in nucleoli

became very strong (Figure 3R).

DISCUSSION

Sine continual division and constant proliferation are important characteristics of tumor cells, the proliferation of tumor cells is one of the significant indexes in identifying exogenous inducers of differentiation [5]. In our study, the cell growth curve, mitotic index and cell cycle indicated that BGC-823 cells had vigorous proliferation capability, the doubling time was 45.82 h, the maximum mitotic index was 38.5%, and the proportion of cells was 50.7% in G_0/G_1 phase and 39.1% in S phase. However the doubling time of cells treated with tachyplesin or n-sodium butyrate was 75.7 h and 67.1 h respectively, the rate of cell growth inhibition was 62.66% and 60.19% respectively, the maximum mitotic index decreased to 12.6% and 10.9% respectively, the proportion of cells in G_0/G_1 phase was 59.5% and 59.2% respectively, while the proportion of BGC-823 cells in S phase decreased to 26.3% and 25.2% respectively. The results demonstrated that the inhibitory effects of tachyplesin and n-sodium butyrate on proliferation of BGC-823 cells were significant. n-sodium butyrate, a small molecule polar compound, has been widely used in inducing differentiation of tumor cells [3, 6, 7]. The effects of n-sodium butyrate on cell growth and cell cycle are coincident with the reports about the anti-proliferative effects of n-sodium butyrate on the cell cycle arrest of human gastric, colonic and endometrial carcinoma cell lines [6-9]. Our experiment showed that the effects of tachyplesin and n-sodium butyrate on cell growth and cell cycle of BGC-823 were similar, indicating that tachyplesin has identical effects on inhibiting proliferation as chemical inducers of cancer cells.

The changes in expression of oncogenes and tumor suppressor genes play a role in cell carcinogenesis and reversal movement, amplification and inactivation of some associated genes such as c-erbB-2, c-myc, p53, p16 [10-11] are a main index in differentiation of human gastric carcinoma cells [11]. In our study, the levels of c-erbB2, c-myc, mtp53 proteins were high but p16 expression was low in untreated BGC-823 cells. However, after treated with tachyplesin or n-sodium butyrate, the level of c-erbB-2, c-myc, mtp53 proteins decreased and p16 protein expression increased significantly in BGC-823 cells, demonstrating that both tachyplesin and n-sodium butyrate can influence the expression of oncogenes and tumor suppressor genes. Previous studies showed that n-sodium butyrate could down-regulate the expression of oncogenes (c-erbB-2, c-myc, p53) and up-regulate the expression of tumor suppressor genes (p16) in endometrial and colonic carcinoma and melanoma[7,8,12,13], confirming that n-sodium butyrate has obvious effects on differentiation of BGC-823 cells. Our results also showed that tachyplesin had the same effect on up-regulating the expression of tumor suppressor genes and down-regulating the expression of oncogenes as n-sodium butyrate in BGC-823 cells.

The combination of different inducers may have a synergistic effect on differentiation and reduce toxicity and side effects by cutting down the dosage. It is not only an important subject in the field of differentiation, but also has a positive impact on the clinical application of different therapies for cancer [14]. Our study showed that at the concentration of 1.0 g tachyplesin + 1.0 mmol/L n-sodium butyrate, the growth inhibitory rate was 62.29%, the mitotic index decreased to 18.5‰ at the divided peak and the proportion of cells in G_0/G_1 phase increased to 64.5% and decreased to 20.9% in S phase. Immunocytochemistry also showed that the levels of c-erbB-2, c-myc, p53 proteins in BGC-823 cells treated with the combination were lower and the level of p16 protein was higher.

In conclusion, tachyplesin has synergistic effects with n-sodium butyrate and can be used in treatment of cancer.

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Assessment of metastatic liver disease in patients with primary extrahepatic tumors by contrast-enhanced sonography versus CT and MRI

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Abstract

AIM: To evaluate contrast-enhanced ultrasonography (CEUS) using SonoVue® in the detection of liver metastases in patients with known extrahepatic primary tumors versus the combined gold standard comprising CT, MRI and clinical/histological data.

METHODS: It is an international multicenter study, and there were 12 centres and 125 patients (64 males, 61 females, aged 59 ± 11 years) involved, with 102 patients per protocol. Primary tumors were colorectal in 35%, breast in 27%, pancreatic in 17% and others in 21%. CEUS using SonoVue® was employed with a low-mechanical-index technique and contrast-specific software using Siemens Elegra, Philips HDI 5000 and Acuson Sequoia; continuous scanning for at least five minutes.

RESULTS: CEUS with SonoVue® increased significantly the number of focal liver lesions detected versus unenhanced sonography. In 31.4% of the patients, more lesions were found after contrast enhancement. The total numbers of lesions detected were comparable with CEUS (55), triple-phase spiral CT (61) and MRI with a liver-specific contrast agent (53). Accuracy of detection of metastatic disease (i.e. at least one metastatic lesion) was significantly higher for CEUS (91.2%) than for unenhanced sonography (81.4%) and was similar to that of triple-phase spiral CT (89.2%). In 53 patients whose CEUS examination was negative, a follow-up examination 3-6 mo later confirmed the absence of metastatic lesions in 50 patients (94.4%).

CONCLUSION: CEUS is proved to be reliable in the detection of liver metastases in patients with known extrahepatic primary tumors and suspected liver lesions.

Key words: CT; MRI; Metastasis; Detection; SonoVue®

Dietrich CF, Kratzer W, Strobel D, Danse E, Fessl R, Bunk A, Vossas U, Hauenstein K, Koch W, Blank W, Oudkerk M, Hahn D, Greis C. Assessment of metastatic liver disease in patients with primary extrahepatic tumors by contrast-enhanced sonography versus CT and MRI. *World J Gastroenterol* 2006; 12(11): 1699-1705

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INTRODUCTION

The continuous routine follow-up of cancer patients requires an easily available, reliable and cost-effective diagnostic method for the detection of liver metastases. Sonography is a widely used method for the detection of liver lesions, but is generally regarded as inferior to contrast-enhanced computed tomography (CT) and magnetic resonance imaging (MRI). The detection of liver

lesions with acoustic properties similar to those of the surrounding normal liver parenchyma has always been the significant limitation of grey scale (B-mode) imaging. To improve the detection of focal liver lesions, ultrasound imaging must also provide information on vascularity, exploiting the differences in blood flow between normal and pathological tissue. The concept of contrast-enhancing agents is not new, being derived from (bolus) dynamic computed tomography (CT) and magnetic resonance imaging (MRI).

Recent advances in contrast-enhanced techniques using high-mechanical-index imaging with Levovist® have improved the detection rate to a level similar to that attained using CT and MRI. This is clear from several, mostly single-centre, studies[1-5]. Owing to the high spatial resolution of sonography, CEUS may also detect subcentimetre metastases.

But there are still some important limitations since contrast-enhanced high-mechanical-index techniques must be performed intermittently and the examination technique is therefore difficult. It is expected that real-time scanning over the whole enhancement period of approximately three to five minutes using more stable contrast agents such as SonoVue® will facilitate routine use, making the examination easier and more reliable[6].

The purpose of this prospective international multicenter study using a real-time technique was to assess the ability of low-mechanical-index contrast-enhanced ultrasound (CEUS) to detect liver metastases in the presence of a known primary tumor versus a combined gold standard.

MATERIALS AND METHODS

The rationale of this study was to evaluate the diagnostic value of dynamic contrast sonography for the assessment of liver metastases versus established reference methods (CT and MRI) in combination with all clinical data except ultrasound techniques as a combined gold standard.

Study design

The study was a prospective, multicenter, open-label, intraindividual comparison. The new diagnostic procedure, dynamic contrast-enhanced ultrasound (CEUS), was compared with conventional sonography (without assessment of vascularity using contrast enhancement) and contrast-enhanced triple-phase CT, the method most commonly used in routine diagnosis. Furthermore, contrast-enhanced MRI was performed and the results from other diagnostic tests (biopsy, clinical data, etc.) were collected where available, to define the final diagnosis using the combined gold standard.

Patients

Between August 2001 and June 2002, 131 patients were enrolled at 12 European centres (see list of contributing centres). Included were male and female patients with known extrahepatic primary tumors and an indication for diagnostic assessment of possible liver metastases. Exclusion criteria were age < 18 years, pregnant or lactating women, known allergies to ingredients of the contrast agent, unstable medical conditions impairing the diagnostic procedure or contraindications to such a procedure, insufficient sonographic window for liver examination and participation in another investigational study.

Signed informed consent was obtained from all study patients before enrollment. The study protocol was approved by the ethics committee of the Landesärztekammer Baden-Württemberg and the local ethics committee of each centre. The study complied with Good Clinical Practice and the Declaration of Helsinki.

Methods and procedures

Ultrasonography

Ultrasound examinations were performed using a high-end sonographic scanner [Siemens Elegra (Ensemble Contrast Imaging), Acuson Sequoia (Coherent Contrast Imaging), Philips ATL HDI 5000 (Pulse Inversion Harmonic Contrast Imaging] with contrast-enhanced imaging software as indicated in square brackets. The contrast examinations were performed with low insonation power (low-MI imaging, mean MI = 0.22), to avoid destruction of microbubbles. Optimized pre-settings were provided for each type of machine, adjusting the imaging parameters to the contrast agent used.

BR1 (SonoVue®, Bracco International) was used as ultrasound contrast agent. BR 1 contains microbubbles of sulphur hexafluoride gas surrounded by a flexible phospholipid shell, allowing contrast-specific imaging at low insonation power. Owing to the size of the SonoVue® microbubbles (mean 2.5 μm), this contrast agent remains (as a so-called blood pool agent) within the vascular system, unlike current X-ray and MRI contrast agents which spread into the interstitial fluid. Thus contrast wash-in and wash-out can be assessed continuously during the whole enhancement phase.

Using a 20-gauge needle, BR1 was injected as an intravenous bolus of 2.4 mL (1 mL/second) into the cubital vein, followed by a 3-10 mL saline bolus for flushing. Additional injections of 4.8 mL (up to 3 contrast injections) were given, if required, to allow optimization of the procedure. There was an interval of at least 6 min between each injection of SonoVue®. To allow contrast clearance of the previous contrast injection the bubbles were destroyed by using high insonation power.

Prior to injection of the contrast agent (native) and throughout contrast enhancement (arterial and portal-venous phase), the entire liver tissue was examined by conventional B-mode ultrasonography as recently described[2,7]. Unenhanced and contrast-enhanced examinations (including the native and arterial and portal-venous enhancement phases) were evaluated separately.

All examinations were documented on S-VHS videotapes and some examinations were additionally digitally stored on magnetic-optical discs (MOD).

Computed tomography (CT)

Standard triple-phase spiral CT examinations were performed and evaluated in the radiology departments or associated radiology units of each centre. The CT examinations include native, arterial and portal-venous phase scans, using bolus injections of 123 mL (mean,

Table 1 Demographic data of the study population

Parameter	Mean	Range	
Age (yr)	59 ± 11	22-82	
Weight (kg)	76 ± 13	45-115	
Height (cm)	170 ± 8.9	152-192	
Sex			51.2 % male, 48.8 % female
Race			99.2 % white, 0.08 % Asian

Table 2 Nature of primary tumor

Parameter	Mean	Range
Primary tumor	*n*	%
Colorectal tumor	44	35.2
Breast tumor	27	21.6
Pancreas tumor	17	13.6
Bronchial tumor	7	5.6
Gastric tumor	6	4.8
Renal tumor	3	2.4
Endocrine gastrointestinal tumor	2	1.6
Melanoma	2	1.6
Others	23	18.4
In 6 patients, several primary tumors were present		

range 100-370 mL, in one patient 60 mL) iodinated contrast agent (300-350 mg/mL). In most cases a multislice scanner was used. Examinations were performed with a slice thickness ⩽ 5 mm (in two patients 8 mm).

Magnetic resonance imaging (MRI)

Standard MRI examinations were performed and evaluated in the radiology departments or associated radiology units of each centre. The MRI examinations included native and liver-specific late-phase scans with T1 and T2 weighted images obtained by SE, TSE and/or GE sequences, using a liver-specific contrast agent (in 18 patients only dynamic phase with a Gd agent). Slice thickness was ⩽ 6 mm (in 10 patients up to 10 mm).

Final reference diagnosis

The final reference diagnosis was defined by combining all available information from imaging (CT and MRI examinations) plus additional information from histology (17), surgery (8) and other clinical examinations (4). Thus the final reference diagnosis includes all information available at the end of the diagnostic evaluation, with the exception of the results from the ultrasound examination (being the test method).

Follow-up examination

Patients with negative findings at the initial examination (i.e. no metastatic lesions detected in the liver) were asked to come back for a follow-up examination 3-6 mo after the initial examination, either US, CT or MRI. This follow-up examination was used as an additional reference standard for patients with negative initial contrast-enhanced sonography, to assess the predictive clinical value of contrast-enhanced sonography.

Safety and tolerability of the ultrasound contrast agent

All adverse events occurring during the examination and a 2 h post-examination observation period were collected and listed, irrespective of a causal relationship. Adverse events were assessed with regard to severity and causal relationship.

Statistical analysis

Unenhanced and contrast-enhanced sonographies were compared by calculating the percentage difference with the two-sided 95% confidence interval. A difference of 10% between methods was defined a priori as clinically significant. The assessment of contrast-enhanced sonography invariably included native (representing tissue) as well as contrast-enhanced sequences (representing vascularity), in parallel to the assessment of CT and MRI. This reflects clinical reality, where vascularity information is always assessed in combination with tissue information. Contrast sequences are performed as a supplement to native baseline sequences, not as an alternative.

For all methods sensitivity, specificity, accuracy, and negative and positive predictive values were calculated together with the respective 95% confidence intervals, using the combined final reference diagnosis as gold standard. Thus CT, MRI, histology, clinical data, etc, but not ultrasound were part of the gold standard. This could introduce a bias in favour of CT and MRI (for example, if sonography showed a small metastasis but all other CT + MRI did not, sonography was assessed as false positive), especially in cases where invasive confirmation was impossible for ethical reasons (6 lesions in 3 patients).

For the assessment of lesion numbers only patients having at most 8 lesions were considered, since in cases with a very high number of lesions the result is more indicative of the counting efforts and moreover there is no real clinical relevance. For the assessment of the presence of metastatic disease a patient was rated as positive if at least one lesion classifiable (on the basis of characteristic features mainly of the perfusion pattern, e.g. lack of portal-venous enhancement) as metastasis could be identified. Owing to the inclusion criteria, all patients had a current or previous primary nonhepatic tumor. The comparison of the methods included all patients for whom valid results from both methods were available, irrespective of the number of lesions. The assessment of follow-up data included all patients having negative metastatic disease at the initial examination (no metastases found with CEUS) and having follow-up data available.

As statistical tests the Wilcoxon signed rank test (comparison of lesion numbers) and the McNemar test (assessment of metastatic disease) were used. For the comparison of CEUS *vs* CT the test was performed as a two-sided test (testing equivalence) and as a one-sided test (testing non-inferiority of CEUS *vs* CT).

RESULTS

Study population

Epidemiological data are summarized in Table 1. All 131 patients enrolled had a primary extrahepatic tumor (Table 2). 125 of the 131 patients received an adequate dose of

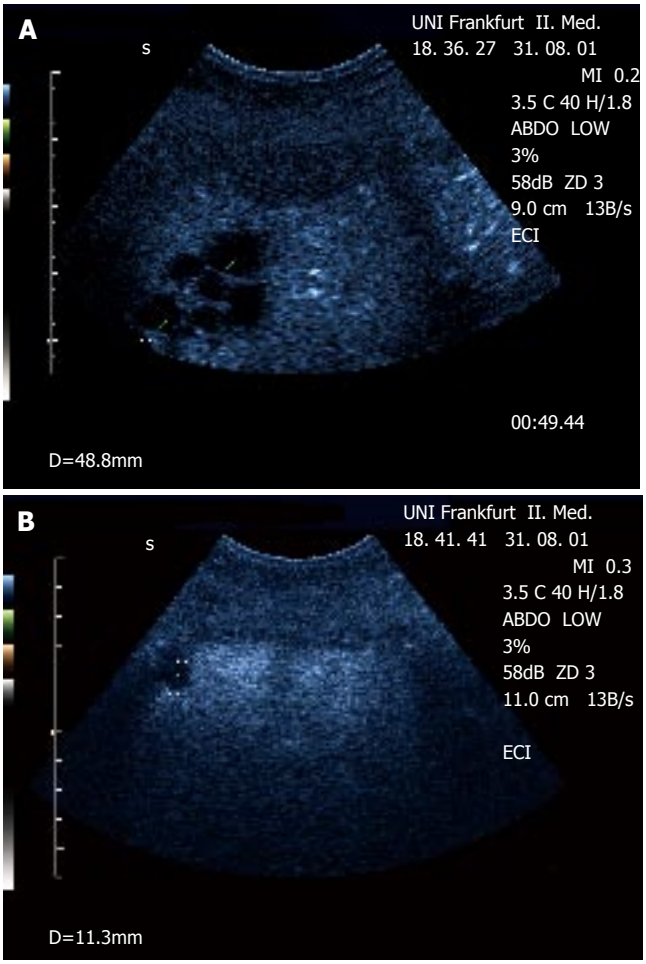

Figure 1 Demonstration of a focal (multicystic) lesion in a patient with cervix carcinoma using contrast-enhanced ultrasound (CEUS). **A**: The lesions can be delineated in the portal-venous phase as 'black spots' lacking portal-venous enhancement within normally enhanced liver tissue. **B**: An additional small lesion next to the diaphragm (not visible in native B-mode) was detected by CEUS but not with CT. Biopsy confirmed metastatic disease.

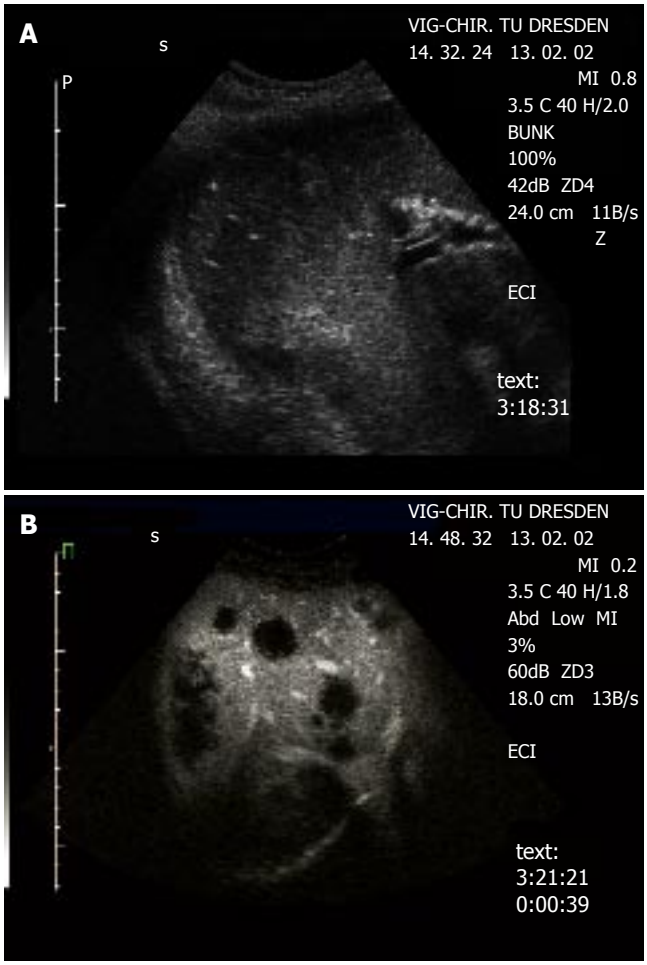

Figure 2 Detection of metastases in a patient with colorectal carcinoma **A**: Native B-mode sonography revealed 3 metastases (segment 6/7) in agreement with CT, MRI revealed 4 metastases (segment 6/7 and 4). **B**: Contrast-enhanced sonography identified diffuse metastatic disease in both liver lobes. The metastatic lesions are clearly delineated in the portal-venous phase as 'black spots', due to the lack of portal-venous blood supply.

the study medication (ultrasound contrast agent) and were considered eligible. 102 patients had no relevant protocol violations (per protocol population) and were used for the efficacy analysis. The reasons for exclusion from the primary efficacy analysis were (multiple instances possible): reference examination outside the stipulated time window of +/- 14 d (11 patients), reference examination missing or incomplete (8 patients), and inadequate visualization of the entire liver (5 patients).

The examination of the liver was performed as part of the initial staging in 63 patients (50.4%), as follow-up examination in 52 patients (41.6%), for presurgical diagnosis in 11 patients (8.8%) and for other purposes (abdominal pain, recurrent tumor staging, postsurgical assessment) in 3 patients (2.4%) (multiple reasons possible, percentage related to $n = 125$).

62 patients had negative contrast-enhanced sonography, i.e. no metastatic lesions were found at the initial examination. In 53 of these 62 patients, a follow-up examination was performed 3-6 mo after the initial examination and the absence of metastatic lesions was assessed additionally versus the information from follow-up.

Number of metastatic lesions

The metastatic lesions could be identified most clearly in the portal-venous phase, as lesions lacking portal-venous enhancement surrounded by normal liver tissue (Figure 1 and 2). Lack of portal-venous enhancement during CEUS occurred in 171 of 186 metastatic lesions (91.9%). In 60 of 186 lesions (32.3%) peripheral arterial enhancement could be detected (Figure 3). The number of lesions was assessable in 74 patients having fewer than 8 focal liver lesions. In 36 of these patients no metastatic lesion could be detected with any of the 3 imaging modalities (CEUS, CT, MRI). In the remaining 38 patients the number of metastatic lesions detected was 55 (CEUS), 61 (CT) and 53 (MRI). A comparison of the number of lesions per patient revealed that in 50 patients (67.6%) the same number of metastases was found with CEUS and CT. In 16 patients (21.6%) CT found more metastases, whereas in 8 patients (10.8%) CEUS found more metastases. However, since the histological gold standard was not available for every lesion, the rates of true and false positive lesions cannot be reliably defined. The differences between the numbers of metastases detected were not statistically significant (Wil-

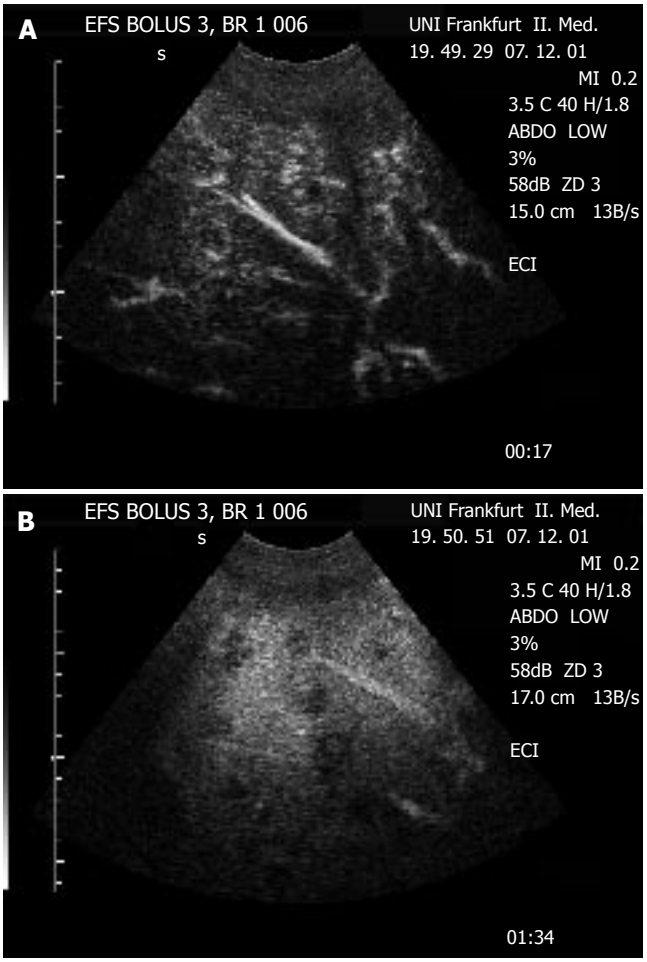

Figure 3 Detection of multiple metastatic lesions in the liver of a patient with sarcoma. **A**: In contrast-enhanced sonography, the lesions show a periperal enhancement in the arterial phase ("rim sign"), indicating the arterial blood supply to the peripheral proliferation zone of this metastatic lesions from a hypervascular tumor. **B**: During portal-venous phase, the lesions show lack of portal-venous enhancement ('black spot'), indication the absence of portal-venous blood flow typical for malignant liver lesions.

coxon signed rank test: $P = 0.28$ and $P = 0.95$ for CEUS versus CT and CEUS versus MRT, respectively). Thus with all 3 imaging modalities a comparable number of metastatic lesions could be found.

Assessment of metastatic disease

Contrast-enhanced ultrasound (CEUS) versus conventional (unenhanced) ultrasound On a patient basis, the detection of metastatic disease (i.e. of liver metastases irrespective of the number) was assessed and compared with the combined gold standard. With the use of contrast enhancement, the number of correctly classified patients rose from 83 out of 102 (native US) to 93 out of 102 (CEUS), showing clear superiority of contrast-enhanced sonography over unenhanced sono-graphy (Table 3). The superiority of CEUS was statistically significant (McNemar test: $P < 0.01$).

Contrast-enhanced ultrasound (CEUS) versus CT The correctly diagnosed number of patients with metastatic disease was 91 out of 102 patients in the case of triple-phase CT, compared with the 93 out of 102 patients who were correctly diagnosed using CEUS (Table 4). Thus contrast-enhanced ultrasound and spiral CT showed comparable accuracy for the detection of metastatic liver disease. The slight difference in favour of contrast-enhanced ultrasound was not statistically significant. The McNemar test revealed a two-sided 95% confidence interval [-6.2%; +10.1%],not clearly exceeding the stipulated 10% equivalence range.In comparison to the final reference diagnosis the sensitivi-ties were 84.6%, 88.5% and 92.3% and the respective specificities were 78.0%, 94.0% and 89.2% (unenhanced sonography, contrast-enhanced sonography and CT). Thus the accuracy for the detection of metastatic liver disease rose from 81.4% to 91.2% on use of contrast-enhancement for sonography, compared with 89.2% for spiral CT. Of the three methods, contrast-enhanced ultrasound showed the best specificity and accuracy for the detection/exclusion of metastatic liver disease.

Table 3 Patients with correct diagnosis (existence of metastatic disease) with unenhanced sonography and contrast-enhanced sonography (CEUS)

		CEUS Correct diagnosis		
		Yes	No	
Unenhanced sonography correct diagnosis	Yes	82	1	83
	No	11	8	19
		93	9	102

Table 4 Patients with correct diagnosis (existence of metastatic disease) with contrast-enhanced sonography (CEUS) and triple-phase spiral CT

		CEUS Correct diagnosis		
		Yes	No	
CT correct diagnosis	Yes	83	8	91
	No	10	1	11
		93	9	102

Contrast-enhanced ultrasound (CEUS) versus follow-up examination Of the 62 patients with a negative diagnosis (metastatic disease) on contrast-enhanced sonography, 53 had a follow-up examination 3-6 months after the initial examination. In 47 patients (88.7%) no additional lesions could be found at the follow-up examination. In 3 patients (5.7%) new lesions were found, which turned out not to be metastatic. Only 3 patients (5.7%) showed new metastatic lesions at the follow-up examination which were not diagnosed at the initial CEUS examination. One of these patients also showed no lesion on initial CT and MRI, so that this metastasis initially could not be detected by any of the 3 imaging modalities. In the other 2 patients, CEUS initially detected 5 lesions and 1 lesion respectively, but these lesions were not classified as metastatic and so, based on CEUS alone, the initial diagnosis was false negative. In the first patient (with 5 lesions), CT identified 1 of these lesions as metastasis

with peripheral portal-venous enhancement and MRI with SPIO particles identified 2 lesions as metastases. At follow-up, 3 lesions proved to be metastatic lesions. In the other patient (with 1 lesion), all methods (CT, MRI and CEUS) initially classified the lesion as nonmetastatic, and only at follow-up were 3 metastatic lesions identified. However, even versus the 3-6 mo follow-up, CEUS showed 94.4% correct assessment for nonexistence of metastatic liver disease, demonstrating the suitability of contrast-enhanced ultrasound for follow-up examinations of patients with primary extrahepatic tumors.

Safety and tolerability of the ultrasound contrast agent

The patients received 1-3 bolus injections of 2.4 or 4.8 mL SonoVue®, with a total dose of 2.4 mL (11.2%), 4.8 mL (12.8%), 7.2 mL (41.6%), 9.6 mL (5.6%) or 12.0 mL (28.8%) per patient. Only one adverse event - dry mouth of mild intensity - was reported. Thus the overall adverse event rate per patient in this study was 0.8%.

DISCUSSION

Brightness(B)-mode ultrasonography is highly sensitive and specific in characterizing cysts and calcifications, leading to a definitive diagnosis, but shows several limitations in patients with primary and secondary liver tumors. In addition, some focal lesions have the same echogenicity of normal liver parenchyma leading to false negative findings. To improve the detection of focal liver lesions, ultrasound imaging must also provide information on vascularity, exploiting the differences in blood flow between normal and pathological tissue.

It was recently shown that CEUS using contrast-specific nonlinear high-mechanical-index imaging techniques improves the detection rate of liver metastases in comparison with B-mode ultrasound, achieving a detection rate similar to that reported for computed tomography and magnetic resonance imaging techniques[1,2,4,5,8-9] In these recently published detection studies mainly using Levovist® in the portal venous and liver-specific late phase in patients with known malignancies, additional lesions could be found in 30-55% of patients. Additionally, it was shown that examination techniques employing Levovist® allow differentiation of histologically proven benign and malignant liver lesions. In 79 patients with histologically proven malignant liver lesions and in 95 patients with benign liver lesions it was shown that hypoechoic contrast enhancement in the portal venous or late phase as a predictive sign of malignancy had 100% sensitivity in patients mostly without underlying liver disease. Homogeneous Levovist® enhancement in the portal venous and late phase had 93% specificity as an indicator of benign disease[7]. It should be noted that there were no false negative findings in patients without underlying parenchymal liver disease. Furthermore, a lower interobserver variability was found in contrast-enhanced sonography than in baseline ultrasonography[10]. But there are still some important limitations since contrast-enhanced high-mechanical-index techniques must be performed intermittently and the examination technique is therefore difficult.

The present study employed a new-generation contrast medium (SonoVue®) allowing real-time imaging, providing similar information to the more difficult intermittent imaging technique using Levovist®. This method proved useful in routine application and is easy to learn. Contrast-enhanced sonography using low-mechanical-index techniques with SonoVue® has also proved useful intraoperatively[11] and in conjunction with 3D techniques[12] and additionally may differentiate between adenoma and focal nodular hyperplasia[13]. It was shown that these techniques gave a statistically significant improvement in the accuracy of detection of metastatic disease versus unenhanced sonography. In comparison with baseline ultrasound the number of metastatic lesions increased with a sensitivity comparable to contrast-enhanced triple-phase CT. In two thirds of patients the same numbers of metastatic lesions were found with contrast-enhanced sonography and CT. In the remaining patients, sometimes CT and sometimes sonography found more lesions, with no significant superiority of one method. Since contrast-enhanced sonography was not part of the gold standard this may introduce a possible bias in the calculation of accuracy. There were two patients in whom CEUS found a lesion but CT and MRI did not. One patient (female, 54 years, with breast cancer) had a 29 mm lesion and the other (female, 67 years, with pancreatic cancer) had an 11 mm lesion, which were both non-enhancing in the portal-venous phase. However, owing to the definition of the gold standard both were rated as false positive for CEUS, since no biopsy was obtained for clarification. This illustrates the limitation of such studies comparing imaging methods, since there is no absolute non-invasive gold standard. Additionally, contrast-enhanced sonography has the best specificity and accuracy for detection or exclusion of metastatic disease when compared with unenhanced sonography and triple-phase spiral CT. In 11% of the patients the existence of lesions could be ruled out, indicating the high specificity of contrast-enhanced sonography.

Monitoring of adverse events was mandatory and performed in all patients and demonstrated no relevant reaction, leading to an excellent tolerability of the ultrasound contrast agent.

In conclusion, contrast-enhanced ultrasound in the portal venous and late phase following injection of SonoVue® considerably improves the detection of liver tumors compared with conventional B-mode sonography and is therefore a suitable method for the follow-up of patients with primary extrahepatic cancer.

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VIRAL HEPATITIS

Protocol liver biopsies in long-term management of patients transplanted for hepatitis B-related liver disease

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Abstract

AIM: To evaluate the long-term histological outcome of patients transplanted for HBV-related liver disease and given HBIg prophylaxis indefinitely after LT.

METHODS: Forty-two consecutive patients transplanted for hepatitis B were prospectively studied. HBsAg, HBV-DNA and liver function tests were evaluated in the serum 3, 6 and 12 mo after LT and then yearly. LB was obtained 6 and 12 mo after LT and yearly thereafter. Chronic hepatitis (CH) B after LT was classified as minimal, mild, moderate or severe.

RESULTS: HBV recurred in 7/42 (16.6%) patients after 6-96 mo of follow-up. A hundred and eighty-seven LB were evaluated. Four of 7 patients with graft reinfection, all with unknown HBV DNA status before LT, developed cirrhosis at 12-36 mo of follow-up. Of the 122 LB obtained from 28 HBsAg+/HCV- recipients with no HBV recurrence after LT, all biopsies were completely normal in only 2 patients (7.1%), minimal/non-specific changes were observed in 18 (64.2%), and at least 1 biopsy showed CH in the remaining 8 (28.5%). Twenty-nine LB obtained from 7 patients transplanted for HBV-HCV cirrhosis and remaining HBsAg- after LT revealed recurrent CH-C. Actuarial survival was similar in patients with HBsAg+ or HBsAg- liver diseases.

CONCLUSION: Though protocol biopsies may enable the detection of graft dysfunction at an early stage, the risk of progression and the clinical significance of these findings remains to be determined.

Key words: Liver transplantation; Hepatitis B virus; Liver biopsy; Anti-HBs Immunoglobulins

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INTRODUCTION

Hepatitis B virus (HBV)-related liver disease is a common indication for liver transplantation (LT)[1,2]. Before the advent of effective prophylaxis, the rate of HBV recurrence after LT was reportedly more than 90% at one year in viremic patients and around 30% in those who were negative for serum HBV-DNA at the time of LT[3]. The majority of patients with recurrent infection develop progressive liver damage resulting in graft and patient survival rates being reduced to around 50% at 2 years after LT[4,5]. The prevention of HBV recurrence is based on long-term treatment with hepatitis B immunoglobulin (HBIg), with or without lamivudine[1,6-9,10-15]. The major drawback of using HBIg is its high cost, while the efficacy of lamivudine is limited by the onset of drug resistance[13,14,16-18].

The most reliable way to diagnose and establish the severity of any liver disease recurrence is by histological evaluation of the graft. Serial liver biopsies (LB) may identify subclinical histological changes in LT recipients with normal biochemical data and are useful in assessing disease progression during long-term follow-up[19]. At most centers, liver biopsies are performed in patients with graft dysfunction due to HBV reinfection. There is limited experience of protocol biopsies in patients after LT for HBV. The aim of the present study was to evaluate the usefulness of protocol liver biopsies in assessing long-term histological outcome in patients on indefinite HBIg

Table 1 Pre-transplant HBV-DNA in the study population and outcome after liver transplantation

Indication	*n*	HBV DNA		Total recurrence
		Negative	Unknown	
Cirrhosis	38	34	4	7
HBV	19	16	3	5
HBV-HDV	12	11	1	2
HBV-HCV	7	7		
Fulminant hepatitis	4		4	

Subgroups of HBV patients (with HDV/HCV coinfection and fulminant hepatitis) showing pre-transplant HBV-DNA status and disease recurrence.

prophylaxis following LT for hepatitis B.

MATERIALS AND METHODS

Study population

From November 1990 to December 2000, 246 adults underwent 268 LT at our center, and 55 (22.3%) of them were hepatitis B surface antigen (HBsAg)-positive. Among the patients with HBV infection, 45 (81.8%) had cirrhosis and 10 (18.2%) had fulminant hepatic failure (FHF). Eight patients with cirrhosis also had hepatocellular carcinoma (HCC), 6 were diagnosed pre-LT and 2 were incidental findings. HBV DNA detection methods became available at our center in August 1991. Since then, patients with HBV-related cirrhosis have only been listed for LT if their serum HBV DNA was negative. HBV DNA was unavailable for 5 cirrhotic patients transplanted before August 1991 and for all patients with FHF. Hepatitis delta virus (HDV) antibodies (anti-HDV IgG) were positive in 14 patients (25.4%), hepatitis C virus (HCV) antibodies in 7 (12.7%). Three patients (5.4%) had a history of alcohol abuse but had all abstained for at least 6 mo before LT. Eleven patients who died within 6 mo of LT (5 with cirrhosis and 6 with FHF) and 2 patients with a follow-up of <6 mo (both with cirrhosis) were excluded from the analysis.

Liver tests and serological/virological tests for HBV, HCV and HDV were obtained for all patients 6 and 12 mo after LT and yearly thereafter. Enzyme immunoassays were used to detect HBsAg (ELISA II Abbott Diagnostics, North Chicago, IL), anti-HDV (ELISA II Abbott Diagnostics) and anti-HCV (ELISA II-III, Ortho Diagnostics Raritan, NJ). Samples positive for anti-HCV were confirmed by recombinant immunoblotting assays (RIBA II, Ortho Diagnostics Raritan, NJ). Serum HCV RNA was investigated by RT-PCR and HBV DNA by chemiluminescence (Digene Hybrid Capture System) in serum samples and by PCR in liver tissue using primers from the conserved region of the surface gene of HBV[20].

Histological analysis

Liver biopsies were performed using a modified Menghini needle (16-17 gauge). Informed consent for liver biopsy was obtained from all patients. Hematoxylin and eosin, periodic acid-Schiff (PAS), van Gieson, reticulin and iron stains were available for all biopsy samples. Immunohistological staining was done for HBsAg and HBcAg.

Chronic viral hepatitis was defined as minimal, mild, moderate or severe according to Scheuer and Desmet[21-23]. Recurrent hepatitis C was graded according to Ishak's classification[24]. Acute and chronic rejection was classified according to Snover's classification[25]. Minimal and not-otherwise-specified changes were defined according to Pappo's classification[26].

HBIg prophylaxis

Patients received 10 000 IU of iv HBIg (VENBIG, Hardis) during the anhepatic phase of LT, daily from days 1 to 7 and weekly for 4 wk following LT. Long-term immunoprophylaxis consisted of weekly doses of 1600 IU of im HBIg (IMMUNOHBS, Hardis) indefinitely. Serum anti-HBs concentrations were investigated weekly before hospital discharge, at 3, 6 and 12 mo after LT and yearly thereafter. Target anti-HBs titers were >400 IU/L throughout the follow-up. HBIg therapy was discontinued in patients who became HBsAg-positive following LT.

Immunosuppression

Immunosuppressive therapy was a combination of cyclosporine or tacrolimus with corticosteroids. Azathioprine or mycophenolate mofetil were added in patients with serum creatinine >200 mol/L. No anti-lymphocyte antibody induction therapy was used. Hist-ologically-proven acute cellular rejection was treated with one or two courses of 1 g/d of iv methylprednisolone for 3 consecutive days.

Antiviral therapy

Lamivudine therapy (100 mg/d) was indicated in patients with detectable serum HBV DNA following LT, with or without reappearance of HBsAg.

Recurrent HBV infection and related liver disease

Recurrent HBV infection was defined as the detection of HBsAg in the serum at any time after LT.

Statistical analysis

The Kaplan-Meier method was used to calculate actuarial survivals and the log-rank test for comparisons between groups.

RESULTS

The study population included 42 patients transplanted for hepatitis B with a mean follow-up of 96 mo.

The overall rate of HBV recurrence was 16.6% (7/42 patients): 18.4% in patients with cirrhosis (7/38) and 0% in cases of FHF (0/4) (Table 1). Among the 38 patients with cirrhosis, the HBV recurrence rates varied according to etiology, pre-LT HBV DNA status and presence of HCC. Graft reinfection occurred in 5/19 patients with pure HBV-cirrhosis (26%), 2/12 patients with HBV/HDV cirrhosis (16.6%) and none of 7 with HBV/HCV-cirrhosis (Table 1). HBV DNA before LT was unavailable in 4 patients, who all developed recurrent infection - as opposed

to only 3/34 (8.8%) with negative HBV DNA ($P=0.013$). Finally, HBV recurred in 2/7 patients (28.5%) with HCC (1 with unknown pre-LT HBV DNA) and 5/31 (16.1%) without HCC: among the 4 patients with unknown pre-LT HBV DNA status, 2 died of recurrent HBV 17 and 44 mo after LT, 1 developed graft cirrhosis after 2 years and 1 had moderate chronic hepatitis with a stable clinical course; the remaining 3 patients with HBV recurrence had undetectable HBV DNA prior to LT and became HBsAg-positive 12, 12 and 14 mo after LT. In the first of these 3 patients, HBV recurrence at 12 mo coincided with de novo HCV infection. Although histology revealed mild chronic hepatitis, it was difficult to differentiate the role of HBV and HCV infection in allograft injury. Protocol liver biopsies showed cirrhosis 3, 4 and 5 years after LT. Now, 9 years after LT, without lamivudine treat-ment (which was not indicated because transaminases always remained within the normal range), the patient has compensated cirrhosis.

The second patient, though non-replicating at the time of LT, had intermittently positive HBV-DNA while on the waiting list. At the time of HBV recurrence, 12 mo after LT, liver biopsy showed mild chronic hepatitis. Lamivudine led to serum HBV DNA clearance after 2 mo of treatment. Repeated liver biopsies 2 and 3 years after LT showed stable, mild chronic hepatitis with focal cytoplasmic HBsAg on immunohistochemistry.

The third patient, who had HDV coinfection, developed mild chronic hepatitis 14 mo after LT and was treated with lamivudine.

Six of 7 patients with HBV-HCV coinfection developed histologically-proven recurrent HCV 12 to 48 mo after LT.

Liver HBV DNA, obtained from the first 11 transplanted patients, was positive in 1 patient who had recurrent positive serum HBsAg.

HBIg administration was well tolerated in all patients and no episodes of immunocomplex syndrome were observed. Mean anti-HBs titers 5 years after LT were 713±314 mIU/mL.

Only 2 of the 7 patients with recurrent HBV received lamivudine treatment because it was unavailable in 1991 and 1992 when the other 5 developed graft reinfection. Both patients were cleared of serum HBV-DNA after 6 mo of lamivudine therapy; they are both still taking the treatment with no evidence of any development of the escape mutants.

Actuarial survival was similar among patients with HBsAg-positive or HBsAg-negative liver disease (Figure 1), while it was significantly lower ($P=0.009$) in patients who had LT for fulminant HBV hepatitis than in those transplanted for HBV cirrhosis, HBV/HDV cirrhosis or HBV/HCV cirrhosis. Moreover, it was not significantly different in patients transplanted for HBV cirrhosis with vs without HCC, despite a decreasing trend in survival 100 mo after LT in patients with HCC (70% *vs* 88%).

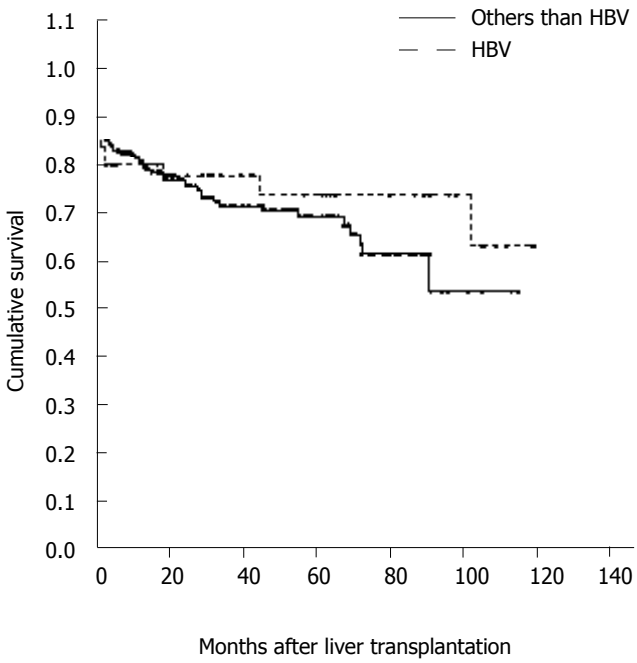

Figure 1 The figure shows the rate of cumulative survival in patients transplanted for HBV-related liver disease (cirrhosis plus fulminant hepatic failure) compared to the survival of patients transplanted for liver diseases of different etiologies. Outlined bar: survival in patients after liver transplantation fot HBV-related liver disease. Plain bar: survival in patients after liver transplantation for non-HBV-related liver disease. The difference is not statistically significant (Kaplan Meier Survival Analysis).

Histopathological features

The results of the 187 protocol biopsies performed were analyzed depending on the patients' HBV and HCV status after LT. A hundred and fifty-eight biopsies were obtained from the 35 HCV-negative recipients (mean 4.5 biopsies per patient; range 1-10) during the 6-96 months of follow-up: 36 from 7 patients with recurrent HBV (5.1 per patient; range 2-10) and 122 from 28 patients with no HBV recurrence (4.3 per patient; range 1-11).

Among the 6 HBsAg+/anti-HCV- patients (there were originally 7, but 1 became anti-HCV+ a year after LT and was consequently included in the HBsAg+/anti-HCV+ group), two had signs of mild chronic hepatitis in all biopsies (follow-up 6-24 mo), and one had at least 1 biopsy showing moderate chronic hepatitis (follow-up 6-96 mo). The other 3 patients, all with HBV-DNA unavailable before LT, developed cirrhosis (two at 12 mo and one at 24) (Figure 2).

As for the 28 HBsAg-/anti-HCV- patients (follow-up for 6-96 mo), biopsies were normal in 2 (7.1%), intermittent, mild inflammatory changes (follow-up for 6-96 mo) were observed in 18 (64.2%), at least 1 biopsy showed mild chronic hepatitis (follow-up for 6-84 mo) in 6 (21.4%) and at least 1 biopsy revealed moderate chronic hepatitis (follow-up for 6-84 mo) in 2 (7.1%). None of the patients in this group had severe chronic hepatitis or cirrhosis. Only 3 patients were HBeAg+ and due to this small number, no biochemical or histological correlations between HBeAg+ and anti-HBeAg+ patients were performed.

The histological features of the 28 patients without HBV recurrence are shown in Table 2. Twenty-nine biopsies in all were obtained from the 7 HCV+/HBsAg- patients during 6-60 mo of follow-up (4.1 biopsies per patient; range 2-6). All 7 patients developed chronic hepatitis with

Table 2 Histological features in anti-HCV negative patients, transplanted for HBV-related liver cirrhosis with no HBV recurrence

	6 mo	1 yr	2 yr	3 yr	4 yr	5 yr	6 yr	7 yr	8 yr
n	28	26	19	16	12	11	8	8	4
No. of biopsies/total biopsies (%)									
Negative	5/26 (19.2%)	9/23 (39.1%)	4/19 (21.0%)	6/14 (42.8%)	6/12 (50.0%)	3/10 (30.0%)	4/8 (50.0%)	4/8 (50.0%)	1/2 (50.0%)
Minimal, not otherwise specified changes	19/26 (73.0%)	8/23 (39.7%)	11/19 (57.8%)	6/14 (42.8%)	5/12 (41.6%)	6/10 (60.0%)	2/83/81/2 (25.0%)	(37.5%)	(50.0%)
Mild chronic hepatitis	2/26 (7.6%)	6/23 (26.0%)	4/19 (21.0%)	2/14 (14.2%)	- -	- -	1/8 (12.5%)		
Moderate chronic hepatitis	-	-	-	-	1/12 (8.3%)	1/10 (10.0%)	1/8 (12.5%)	1/8 (12.5%)	
Severe chronic hepatitis	-	-	-	-	-	-	-	-	-
Cirrhosis	-	-	-	-	-	-	-	-	

Histological features in 122 liver biopsies performed in 28 anti-HCV negative patients transplanted for HBV-related liver disease with no HBV recurrence. The number of patients considered declined year by year and the total number of liver biopsies performed each year is less than the number of patients per year.

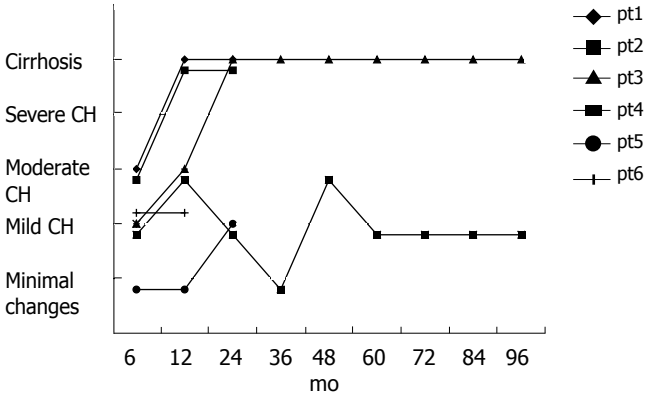

Figure 2 The figure shows the histological damage progression in the 6 anti-HCV negative patients with HBV recurrence.

Table 3 Staging and grading of liver damage in patients transplanted for HBV- and HCV-related liver cirrhosis

Pts	Stage	Grade	Months after transplantation
1	1	4	36
2	2	4	48
3	1	4	24
4	2	10	12
5	0	2	36
6	2	5	12
7	1	2	12

The worst grades and stages were in the 7 HBV/HCV coinfected patients. Staging and grading are based on Ishak's classification [24].

fibrosis 12 to 48 mo after LT (Table 3). The patient with recurrent HBsAg+, who also became anti-HCV positive 1 year after LT, developed mild chronic hepatitis a year after transplantation and cirrhosis 3 years after LT.

Immunohistochemistry was performed on 106/187 (56.7%) liver biopsies. Focal HBcAg positivity was seen in 4/106 (3.7%) biopsies, obtained from 4 HBsAg negative patients, all of them HBV-DNA negative at the time of the biopsy and offering no clue as to the clinical relevance of this finding. Focal HBsAg positivity was seen in only 1 patient with recurrent HBV.

Acute cellular rejection was histologically confirmed in 11/42 patients (26.1%), with a total of 14 episodes (0.33 episodes/patient), 1 to 6 mo after LT; 8 patients had one episode of rejection and 3 patients had two. None of the patients developed steroid-resistant or chronic rejection.

DISCUSSION

HBV-related liver disease is now a common indication for liver transplantation[1,27,28], since graft and patient survival rates are comparable with those of patients transplanted for other conditions[29]. Although perioperative mortality was high in this study, it was unrelated to recurrent HBV infection. Previous studies have shown that the outcome of LT is worse in patients with fulminant HBV hepatitis[30,31] and fulminant non-HBV liver failure[32], as confirmed by our findings. The survival rate was similar, however, between HBsAg positive and negative patients transplanted at our center.

The overall HBV recurrence rate in this series (16.6%) was low and similar to the rate reported in other studies using long-term HBIg monotherapy[14,33-36]. When the analysis was restricted to the cirrhotic cases with negative pre-transplant HBV-DNA, the recurrence rate was even lower (8.8%). These good results confirm that patients without HBV replication before LT and given long-term immunoglobulin prophylaxis are at low risk of recurrent infection, as amply reported elsewhere[5,37-41]. Our study also shows that iv infusions of HBIg are not required beyond the perioperative period; putatively protective levels of anti-HBs (>400 IU) can be obtained using the im route, as reported elsewhere[42-45].

In our series, the rate of HBV recurrence was similar in HBsAg-positive patients with and without HDV coinfection, unlike the situation observed in previous publications[28,46-49], where HDV coinfection was associated with

a lower rate of HBV recurrence and improved survival. Only 2 of our patients with HBV/HDV-cirrhosis developed recurrence, however, and one of them had unknown (but probably positive) pretransplant HBV-DNA.

Similarly, although HBV recurred more frequently in cirrhotic patients with HCC, as in previous publications [50-52], the difference was not statistically significant. In addition, one of the 2 HCC patients with HBV recurrence was intermittently positive for HBV-DNA, while the other had unknown (but probably positive) HBV-DNA pre-LT.

The strength of this study lies mainly in the performance of long-term per-protocol serial liver biopsies, the clinical utility of which is still not clear (though the value of protocol biopsies has recently been confirmed[19,53-55]). The International Liver Transplantation Society Expert Panel Consensus Conference on liver transplantation for hepatitis C considered per protocol biopsies essential: all anti-HCV+ liver transplant recipients should undergo annual liver biopsy to determine histological progression and provide additional data on the natural history of the disease[56].

Among the 28 patients treated with HBIg who remained HBsAg and anti-HCV-negative after LT, only 7% had a completely normal histology up to 3 years after LT. Although the majority of abnormal biopsies showed only minimal inflammatory changes, almost 30% fulfilled the criteria for the diagnosis of chronic hepatitis, as reported elsewhere[26,55]. The features of mild or moderate chronic hepatitis were seen in nearly 1/3 of our cases.

Protocol biopsies obtained from the 7 HBsAg-negative recipients with post-LT HCV re-infection showed recurrent hepatitis C with variable degrees of inflammation and fibrosis. It is worth noting, however, that none of the patients progressed beyond stage 2 fibrosis after a mean follow-up of 36 mo, suggesting that the HBIg preparation used may contain HCV neutralizing antibodies that may attenuate the severity of recurrence, as reported by Feray *et al*[57], but this is a controversial issue because no other papers have confirmed these data since 1990. HBcAg expression was found in the liver tissue of a minority of biopsies from HBsAg-negative recipients, but it was focal and transient. In addition, the finding of HBV-DNA in liver tissue correlated with serum HBV-DNA positivity. Despite the limited number of cases studied, we feel confident in saying that serum assay is clinically useful, whereas tissue HBV DNA should not be investigated because it is expensive and time-consuming. We did not assess HBV genotypes, but it has been recently reported that genotype C seems to be associated with a higher risk of reactivation of hepatitis B and progression to cirrhosis than genotype B[58]. As previously reported, we observed a low rate of acute rejection among patients transplanted for hepatitis B[59-60]. This may be an indirect consequence, at least in part, of the long-term administration of human polyclonal immunoglobulins, which have immunosuppressive properties[59].

In short, this study shows that 25% of patients transplanted for hepatitis B and remaining HBsAg-negative with HBIg prophylaxis develop histological features of mild or moderate chronic hepatitis despite having normal liver test results and negative virological markers[26,55]. These cases will require additional follow-up to estimate the risk of disease progression and further clarify the cause of liver damage. Our findings confirm that normal liver function test results cannot guarantee a healthy graft. The performance of protocol liver biopsies may enable the identification of early histological anomalies potentially capable of progressing to significant liver damage[19,53], but this needs further confirmation.

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Inter-observer variability in histopathological assessment of liver biopsies taken in a pediatric open label therapeutic program for chronic HBV infection treatment

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Abstract

AIM: To our knowledge, the inter-observer variability of the liver biopsy findings in HBV-infected children have not been studied as yet. Hence, we aimed to compare different pathologist's assessment of grading and staging in liver biopsies obtained from children prior to interferon treatment.

METHODS: We collected 920 biopsies from 11 medical centers. The biopsies were independently reviewed by 6 pathologists from academic centers who assessed Batts-Ludwig score for grading and staging. Satisfactory agreement among observers was defined as at least 60% of observers having the same opinion. Satisfactory dispersion between maximal and minimal score for the same biopsy specimen was defined as a maximum 1 point.

RESULTS: Satisfactory inter-observer agreement for grading was obtained in 51.6% and for staging in 75.7% of biopsies. Satisfactory dispersion for grading scores was observed in 44.5% and for staging in 72.7% of cases.

CONCLUSION: Our study demonstrates that: (1) pathologists differ in their assessment of grading and staging of liver biopsies; (2) inter-observer variability for staging is lower than that for grading; and (3) regardless of the inter-observer variability of assessments, the majority of children with chronic HBV infection have mild to moderate inflammation and mild to moderate fibrosis.

Key words: Grading; Staging; Type B Hepatitis; Children

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INTRODUCTION

Many authors believe that a liver biopsy is a gold standard in hepatology[1]. However, there is considerable confusion in terminology and the methods of liver biopsy evaluation are still under discussion. These are the reasons why many different scoring systems for liver histological evaluation were proposed. The first scoring system developed by Knodell *et al*[2] has been used in clinical trials since 1981. This classification differentiated necroinflammatory changes and fibrotic liver damage. However, both features were combined in one histological activity index. This was the reason why the Knodell classification was replaced by other systems with clear differentiation between grading of inflammation and staging of fibrosis[3-6]. Nowadays, the scoring systems are widely used by pathologists and clinicians. It often happens that the grading and staging scores replace the descriptive evaluation of biopsy slides. In this situation, one can raise the question: which grading/staging classification is a true gold standard for a liver biopsy assessment? The other issues that can be discussed are results reproducibility and inter-observer variability of the grading and staging scores for the same biopsy. These problems are vital for the clinicians who receive the biopsy reports. However, they are not frequently addressed in the literature.

Table1 The inter-observers agreement definitions

Agreement rate Number of equal assessment/number of observations	Description	Percentage of agreement (%)
6/6, 5/5, 4/4	All observers gave the same score	100
3/4, 5/6, 4/5,	One observer scored differently than others	75-85
4/6, 3/5	Two observers scored differently than others	60-66
3/6. 2/4	Agreement of half of observers	50
2/6, 2/5, 1/4	Agreement of less than half of observers	25-40

We met the problem of liver biopsy interpretation when we analyzed the results of a nationwide program for interferon treatment of chronic HBV infection in children, conducted in Poland between 1993 and 1999. Twenty-six centers from all over the country participated in this program and 3700 children received treatment there. All centers used their local laboratory facilities, but the entry criteria and treatment regime (interferon 3 MU TIW for 20 wk) were the same. The data collected in the local centers was entered into CRF and sent for central analysis. The results published for 1688 children showed 51.5% of HBe clearance at one year after interferon discontinuation. The response was better in younger children and there was a positive correlation with ALT activity[7]. This observation clearly illustrates the problems with liver biopsy result interpretation that are often met by physicians who review the biopsy reports provided by different pathologists.

The liver biopsy specimens could be easily stored in paraffin blocks and as microscopical slides and used for microscopic evaluation many times. These gave us the opportunity to return to the biopsy slides taken from children with chronic HBV infection, treated with interferon in a Polish therapeutic program, and to re-evaluate them according to selected grading and staging classification. Therefore, we asked all participating centers to provide liver biopsy slides for central review. We wanted to see whether the liver biopsies obtained as part of the routine procedure were representative and whether the quality of slides used for diagnosis was satisfactory. We selected scoring system[6] which was used to evaluate the grading and staging of liver damage, and also performed inter-observer variability analysis. The biopsy results were compared with a patient's clinical and serological data to evaluate the role of liver biopsy in the decision-making process regarding children with chronic HBV infection.

The aim of this study was to present the inter-observer variability of liver biopsy assessment performed according to the Batts and Ludwig grading/staging system[6]. The other questions raised in our project will be discussed in further publications.

MATERIALS AND METHODS

The liver biopsy slides taken from 920 children were provided by 11 centers. The number of slides per one biopsy ranged from 1 to 6 (mean 2.36). Hematoxylin-eosin staining were available for all biopsies and for most of them Azan or PAS stains were available as well.

Six independent pathologists, from academic centers, participated in the study. All of them have been collaborating with hepatology units. Before the study was started, the consensus meeting had been organized and Bats-Ludwig liver biopsy assessment scale was chosen for the study as it is widely used in Poland and has a recommendation of Polish Association of the Study of the Liver. Small sample of biopsy slides was reviewed by every pathologist at this consensus meeting and methods of biopsy assessment and results recording were discussed.

The biopsies were divided into sets, which were circulated among observers. The original identification of the biopsies was withheld and pathologists had no additional clinical data on any particular biopsy. Each observer was asked to assess representativity (size of the sample and number of portal zones) and the technical accuracy (fixation and staining) of the slides and to score grading and staging. The assessments were recorded in standard CRF and stored in a central database. For each biopsy, at least four observers' assessments were obtained.

We analyzed the score distribution for every observer and the agreement rate between different observers for the same biopsy (Table 1). Agreement of 60-66% of observers was selected as the desired minimal level of inter-observer agreement.

For each biopsy, the dispersion between maximal and minimal score for grading and staging was calculated. Satisfactory dispersion between maximal and minimal score was defined as being not greater than 1 point.

All biopsies were taken as part of a routine diagnostic procedure and the parents were asked to sign the consent for liver biopsy. This project of biopsy retrospective assessment received the approval from the Ethics Committee of Children's Health Memorial Institute in Warsaw (decision No 185/KE/2000).

RESULTS

Biopsy representativity and technical accuracy assessment

A total of 5 295 opinions on biopsy specimen representativity were available. In 83.9% of the reports, the observers stated that the specimen was representative of the tissue and could be used for grading and staging evaluation. However, the rate of representative specimens varied among different observers from 65.9% to 93.3% (Figure 1). The agreement of at least 60% of observers was reached in 97% of cases (Figure 2).

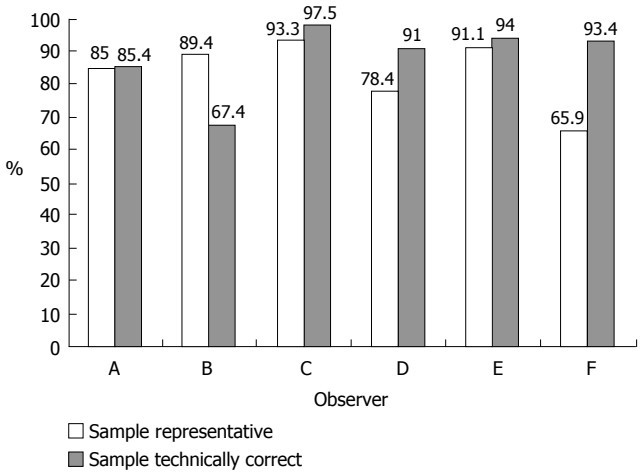

Figure 1 Observers' opinions on representativity and technical accuracy of liver biopsy specimens.

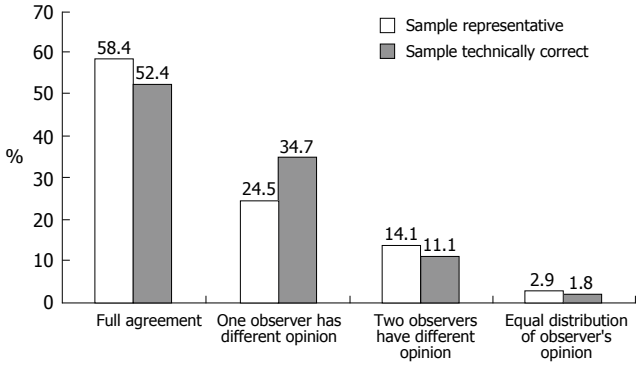

Figure 2 Inter-observer agreement in the assessment of liver biopsy specimen representativity and technical accuracy of the slides.

The opinions on technical accuracy of the biopsy slides were available in 5 296 reports and 88.1% of them showed that the slides were technically correct. The individual observers differed in their opinion on technical accuracy of the biopsy by between 67.4% and 97.5% (Figure 1). The desired level of agreement was reached in over 98% of cases (Figure 2).

Grading assessment

Four or more observers' assessments of biopsy grading were available for 844 biopsies and a total of 4 900 reports were analyzed. The frequency of different scoring varied among observers (Figure 3). The smallest differences in grading score frequencies among observers were noted for a score of 0 (0.3-17.2%, mean frequency for all observers: 8.1%) and 4 (0-7.8%, mean: 4.1%). The largest differences were noted for a score of 1 (13-50%, mean for all observers: 31%) and 3 (4-35.9%, mean: 22%).

The grading assessment agreement of more than 60% of the observers was obtained in 51.6% of cases, but full agreement of all observers reached in only 3.1% of cases. For 13.4% of biopsies, a large difference in observers' opinions was noted (Figure 4)

Different observers' grading score dispersion for the same biopsy equal or lower than 1 point was noted in 44.5% of cases and a dispersion of 2 points was noted in 47.3%. Substantial score dispersion among observers was noted in 8.2% of the cases (Figure 5).

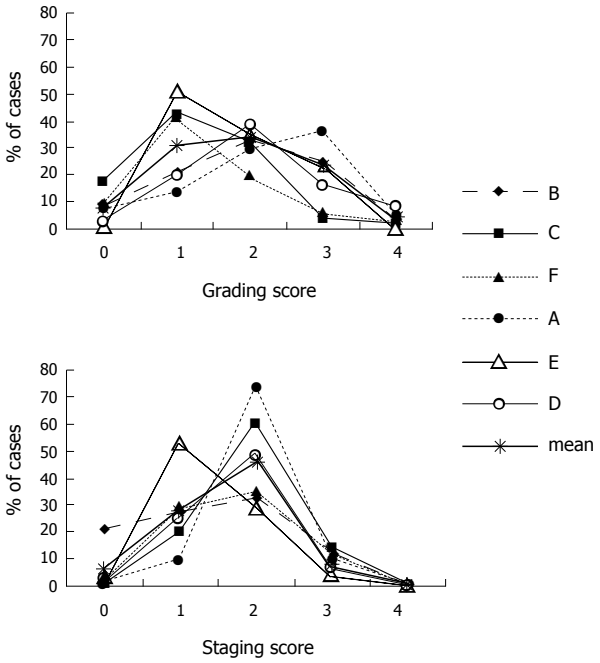

Figure 3 The frequency of different grading and staging scores for individual observer and the mean score frequency for all observers.

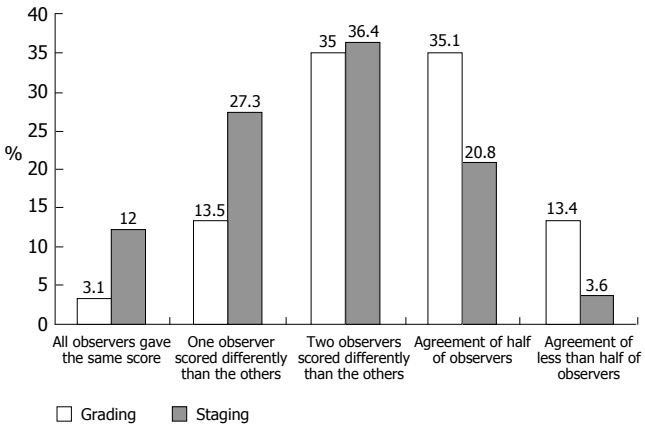

Figure 4 The agreement between different observers' grading and staging score for the same liver biopsy.

Staging assessment

Four or more observers' staging assessments were available for 843 biopsies and a total of 4 895 reports were analyzed. The frequency of different scoring varied among observers (Figure 3). The smallest differences in staging score frequency among observers were noted for a score of 4 (0-0.76%, mean frequency for all observers: 0.4%). The largest differences in score frequency among individual observers were noted for a score of 1 (9.6-52.7%, mean for all observers: 27.1%) and a score of 2 (28.4-72%, mean for all observers: 46.4%).

The staging assessment agreement of more than 60% of the observers was obtained in 75.7% of cases and

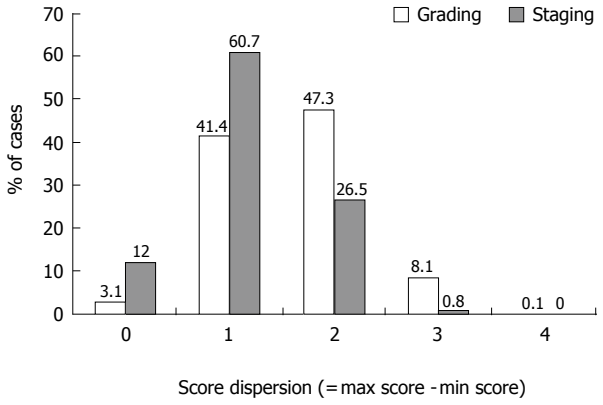

Figure 5 Grading and staging dispersion of assessments among different observers (maximal score - minimal score for the same biopsy).

full agreement of all observers reached in 12% of cases. Substantial differences in observers' opinion were noted in only 3.6% of biopsies (Figure 4).

Different observers' staging score dispersion for the same biopsy equal or lower than 1 point was noted in 72.7% of cases and dispersion of 2 points was noted in 26.5%. Substantial score dispersion among observers was noted very rarely, in only 0.8% of the cases (Figure 5).

DISCUSSION

Liver biopsy is a complex diagnostic procedure performed in many medical centers and evaluated in many laboratories. The aim of the biopsy is to obtain objective information about the condition of the liver tissue. However, there are many factors that influence the objectivity of this examination. Rousselet *et al*[8] have shown that the pathologists' experience is one of the most important factor influencing the inter-observer agreement on viral hepatitis liver biopsy assessment. The other factors that can be taken into account are: the place of liver biopsy sampling; the size of liver biopsy; fixation and staining of the liver tissue specimen; patient's age and reason for liver damage[9].

To our best of knowledge, there are no comprehensive studies addressing the inter-observer variability in liver biopsies taken from children with HBV infection. We, therefore, aimed to investigate the inter-observer error among pathologists who receive a series of biopsies taken in every day practice.

When analyzing the inter-observer variability, one must take into account the definition of agreement. Peutz *et al*[10] showed that the tolerance ±1 point markedly increases the reproducibility of staging results on the Ishak scale. The complexity of assessment scale and number of observers can also influence the variability of diagnoses. Gronback *et al*[11], in their report on a series of 46 liver biopsies analyzed independently by 5 observers, showed that the more complex the scale, the bigger the variability of results. The variability in different pathologists' assessment of the liver biopsy may lead to variability of therapeutic decisions based on liver biopsy reports. Thus, in our study, we compared the results obtained from different pathologists for the same biopsies. To provide a practical value of the results for every day life, we have resigned from sophisticated statistics. The questions that we asked were very simple but vital for the clinician who collaborates with different independent pathologists.

In our biopsy series, we demonstrated that inter-observer variability depended on the scale complexity and agreement cut-off. When the same opinion of at least 60% of observers (≥4 out of 6, or ≥3 out of 5 observers) was used as a cut-off point, the inter-observer agreement for representativity reached 97% and for technical accuracy of the biopsy reached 98%. Even with the higher cut-off, the inter-observer agreements for these two parameters were satisfactory. These positive results could be obtained in a simple scale with a two-option (yes/no) selection[12].

The Batts-Ludwig grading and staging scale is more complex. With the same cut-off as specified above (agreement of at least 60% of observers), the inter-observer agreement for grading reached in 51.6% of cases and for staging 75.7% of cases. A large number of observers was probably the reason why the agreement with the higher cut-off level was very low.

Moreover, a large number of observers was also the reason why the dispersion between the maximal and minimal score for single biopsies was high. In a four-step scale, a maximal difference of 1 point should probably be used as acceptable tolerance. This level of dispersion was achieved for grading in 44.5% of cases and for staging in 72.7%. We found a higher inter-observer agreement and a lower score dispersion for staging than for grading, showing that staging assessment is more reliable and probably more reproducible than grading assessment. This has also been confirmed by other studies[1,8].

The analysis of inter-observer grading and staging agreements and score dispersion showed that despite the same assessment scale being used by pathologists, their reports differed. This is because grading and staging assessments are essentially subjective[13] and this is not a unique situation for liver biopsy only. Similar discrepancies have been reported for other diagnostic techniques in hepatology as well[14]. However, despite the inter-observers variability, the numbers of children with extremely low and extremely high grading or staging were small. Our data showed that the vast majority of children with chronic HBV infection had mild to moderate inflammatory changes and mild to moderate fibrosis. The risk of biopsy-proven HBV-related cirrhosis in this age group is minimal. This observation confirms the clinical observations of Bortolotti *et al*[15] who could not find a progression to liver cirrhosis in children with HBV infection. On the other hand, our data indicated that the chance of completely normal liver tissue in children with chronic HBV infection is small. These observations suggest that the approach to liver biopsy should be changed. Liver biopsy should not be a mandatory examination in all children with active HBV infection, but it should be performed only in selected patients: those with persistently abnormal ALT despite HBeAg clearance, or those with initial signs of liver destruction. In everyday practice, the grading and staging scores should not replace the descriptive biopsy report, as scoring systems are not reliable and they reduce the amount of information on liver tissue. Grading and staging scores become important information in a liver biopsy

report when the series of liver biopsies for the same patient are analyzed for research or follow-up reasons, providing, however, that all the patients' biopsies are reviewed by the same pathologist on the same occasion. It seems important that both clinician and pathologist are aware of the liver biopsy limitations and they closely collaborate with each other.

In conclusion, our study shows that pathologists differ in their assessment of grading and staging of liver biopsy, but the inter-observer variability for staging is lower than that for grading. The majority of children with chronic HBV infection have mild to moderate inflammation and mild to moderate fibrosis. Thus, the value of liver biopsy as a guide for current therapeutic decision in children with chronic HBV infection is limited. The liver biopsies should rather be used as a tool for research or long-term disease dynamics assessment.

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BASIC RESEARCH

Effects of betaine on ethanol-stimulated secretion of IGF-I and IGFBP-1 in rat primary hepatocytes: Involvement of p42/44 MAPK activation

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Abstract

AIM: To evaluate the effects of betaine on the ethanol-induced secretion of IGF-I and IGFBP-1 using radioimmunoassay and Western blotting, respectively, in primary cultured rat hepatocytes.

METHODS: Hepatocytes isolated from male Sprague-Dawley rats were incubated with various concentrations of ethanol and PD98059 procedures. The hepatocytes were also treated with different doses of betaine (10^{-5}, 10^{-4}, and 10^{-3} mol/L). We measured IGF-I and IGFBP-1 using radioimmunoassay and Western blotting, respectively.

RESULTS: The ethanol-induced inhibition of IGF-I secretion was attenuated by betaine in a concentration-dependent manner in primary cultured rat hepatocytes. At 10^{-3} mol/L, betaine significantly increased IGF-I secretion but decreased IGFBP-1 secretion. In addition, p42/44 mitogen-activated protein kinase (MAPK) activity was accelerated significantly from 10 min to 5 h after treatment with 10^{-3} mol/L betaine. Furthermore, the changes in IGF-1 and IGFBP-1 secretion resulting from the increased betaine-induced p42/44 MAPK activity in primary cultured rat hepatocytes was blocked by treatment with the MAPK inhibitor PD98059. Betaine treatment blocked the ethanol-induced inhibition of IGF-I secretion and p42/44 MAPK activity, and the ethanol-induced increase in IGFBP-1 secretion.

CONCLUSION: Betaine modulates the secretion of IGF-I and IGFBP-1 via the activation of p42/44 MAPK in primary cultured rat hepatocytes. Betaine also alters the MAPK activations induced by ethanol.

Key words: Betaine; IGF-I; IGFBP-1; p42/44 MAPK; Hepatocytes; Ethanol

Lee MS, Kim MS, Park SY, Kang CW. Effects of betaine on ethanol-stimulated secretion of IGF-I and IGFBP-1 in rat primary hepatocytes: Involvement of p42/44 MAPK activation. *World J Gastroenterol* 2006; 12(11): 1718-1722

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INTRODUCTION

Betaine, an organic osmolyte, is a precursor of S-adenosylmethionine (SAMe) in the liver. It has been shown that betaine administration has the capacity to elevate hepatic levels of SAMe and to prevent ethanol-induced fatty liver[1]. Kanbak *et al*[2] reported that betaine protects the liver from ethanol-mediated hepatotoxicity in rats, and it was reported recently that it also protects the liver from ischemia/reperfusion injury, niacin toxicity, CCl_4-, hyperosmolarity-, and ethanol-induced apoptosis.

Alcohol consumption is associated with the nutritional metabolism system, which reduces hepatic glucose production and insulin secretion, and increases protein catabolism and triacylglycerol levels. The liver is predominantly responsible for ethanol metabolism and it appears to be the primary source of blood-borne insulin-like growth factor (IGF)-I. Impairment of hepatic synthesis could be responsible for at least a portion of the alcohol-induced decrease in serum levels of IGF-I. Alcohol can affect various organ conditions, thus alcohol consumption may regulate IGF, IGF binding protein (IGFBP) and the IGF-I receptor, which may be involved in growth disorders. It has been shown that IGFBPs are associated with alcohol metabolism[3,4], and we found recently that alcohol administration not only reduces the level of IGF-I, but also increases the level of IGFBP-1 in rat liver, kidney, and serum[5]. It may also contribute to the metabolic dysfunction that occurs following chronic

alcohol consumption. Acute alcohol ingestion also reduces the level of IGF-I in serum, but promptly increases serum IGFBP-1 levels in human subjects[3]. In a pilot study, we observed a reduction in the level of IGF-I in alcohol-treated primary rat hepatocytes.

Based on these previous results, we can speculate that betaine will affect IGF systems in the liver. However, the effects of betaine on the ethanol-mediated secretions of IGF-I and IGFBP-1 in primary cultured rat hepatocytes remain poorly understood. In the present study, therefore, we investigated the effects of betaine on the ethanol-mediated secretions of IGF-I and IGFBP-1 in primary rat hepatocytes.

MATERIALS AND METHODS

Materials

IGF-I antigen, and IGF-I and IGFBP-1 antibodies were purchased form Gropep (Thebarton, Australia). The mitogen-activated protein kinase (MAPK) inhibitor PD98059 was purchased from New England Biolabs (Beverly, MA, USA), and antibodies against phosphor-p42/44 MAPK, p42/44 MAPK were obtained from Cell Signaling (Beverly, MA, USA). All routine culture media were obtained from Gibco-BRL (Grand Island, NY, USA). Aqualsol, reflection X-ray film, and I^{125} (isotope) were purchased from Dupont-NEN. Immobilon-P polyvinylidene difluoride (PVDF) membranes were purchased from Millipore. Bovine serum albumin (fraction V), glycine, sodium dodecylsulfate (SDS), acrylamide, glycerol, and Tween 20 were obtained from Sigma Co. (St. Louis, MO, USA).

Isolation and culture of rat hepatocytes

Hepatocytes were isolated from male Sprague-Dawley rats weighing 200-300g by a two-step perfusion procedure with 0.5g/L collagenase that has been described previously (Seglen, 1976: Weng and Shukla, 2000). Cell viability, as assessed by exclusion of trypan blue, was 90% ± 5%. Isolated hepatocytes were plated onto collagen-coated plastic culture dishes (60 mm in diameter) at a density of 5×10^4 cells/cm^2 in William's medium E containing 100ml/L fetal bovine serum (FBS) in an incubator with an atmosphere of 50ml/L CO_2. After 3 h, the medium was replaced with FBS-free-Williams medium E and various concentrations of ethanol and PD98059 were carefully added to the cells. The dishes immediately were sealed tightly with parafilm and the cells were incubated in this way for 10-180 min at 37℃.

Cells were rinsed twice with ice-cold phosphate-buffered saline and lysis buffer was added (20 mmol/L HEPES, pH 8.8, 136 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 g/L Triton X-100, 10 mmol/L KCl, 2 mmol/L MgCl, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 1 mmol/L dithiothreitol, 1 mmol/L benzamidine, 10-B-glycerophosphate, 10 mg/L aprotinin, 10 mg/L leupeptin, and 1 mg/L pepstatin A). Cell lysates were collected and sonicated for 5 min in a VibraCell ultrasonic processor. After centrifugation of the sonicated samples at 12000 × *g* for 10 min at 4℃, the supernatant was collected and protein concentrations were estimated using a bicinchoninic acid assay kit.

IGF-I radioimmunoassay

Recombinant human IGF-I was iodinated to a specific radioactivity of 5.55 - 11.10 MBq/g with the isotope I^{125} using a modified version of the chloramin-T method (Kodak, NY, USA). The specific activity of the iodinated IGF-I was approximately 2.22 - 4.07 MBq/g protein. The iodination mixture was purified on a Sephadex G-50 column (150 cm) and pre-equilibrated with phosphate-buffered saline (0.1 mol/L, pH 7.4). Serum and tissue IGFBPs were separated using the method of Lee *et al*[6], and IGF-I immunoreactivity was determined using the method of Lee *et al*[7]. IGF-I data are expressed in terms of nanograms of pure human IGF-I per milliliter assuming equal cross-reactivity of rat and human IGF-I in the RIA. Fifty microliters of rat polyclonal IGF-I antibody diluted to 1:1,500 was added to 100 μL of each sample/standard and then incubated for 1 h at room temperature. [^{125}I]-IGF-I (20000 cpm) was then added, and the sample/standard was incubated for an additional 18 h at 4℃. Fifty microliters of horse serum (Sigma) was added to the incubated sample, which was then centrifuged at 3000*g* for 30 min. The supernatant was discarded, and the radioactivity of the precipitate containing bound [^{125}I]-IGF-I was counted in a gamma scintillation counter (Wallac, Finland). All assays were performed in duplicate. Intra- and interassay coefficients of variation for IGFs were 8% and 10%, respectively.

Western blotting

Supernatants were concentrated for 5 h using a Centricon processor (Millipore) at 4℃. Equal amounts of concentrated protein and cell lysates (20-30 μg) were separated on 100 g/L SDS-polyacrylamide gel electrophoresis (PAGE) gels. After electrophoresis, proteins were transferred to a PVDF membrane. The membrane was washed with Tris-buffered saline containing Tween 20 (TBS-T; 25 mmol/L Tris, pH 7.4, containing 137 mmol/L NaCl and 10 g/L Tween-20) and then blocked with TBS-T containing 50g/L nonfat dry milk for 2 h at room temperature. Blots were incubated with antibodies against IGFBP-1 overnight at 4℃ and then incubated with anti-rabbit horseradish peroxidase. After washing, the specific protein band was visualized using an enhanced chemiluminescence detection system (Pierce Chemical, IL, USA). For MAPK blotting, cell lysates were separated on 100 g/L SDS-PAGE and blotted with p-p42/44 MAPK antibody.

Statistical analysis

All experiments were repeated at least three times. The data obtained from this investigation were analyzed using ANOVA and Student's *t* test and are expressed as mean ± SD values.

RESULTS

Regulatory effects of betaine on ethanol-mediated IGF-I and IGFBP-1 secretion

Chronic alcohol treatment is associated with liver and kidney damage. IGF is the major growth factor that is affected by alcohol consumption. Recently, we found that

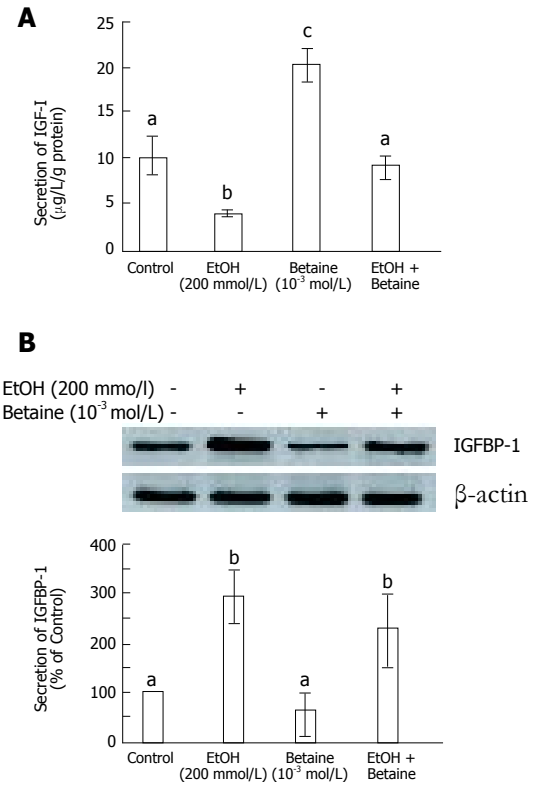

Figure 1 Betaine inhibits the ethanol-stimulated reduction in IGF-I and increases in IGFBP-1 secretion in primary rat hepatocytes (mean ± SD). **A**: IGF-I, **B**: IGFBP-1.

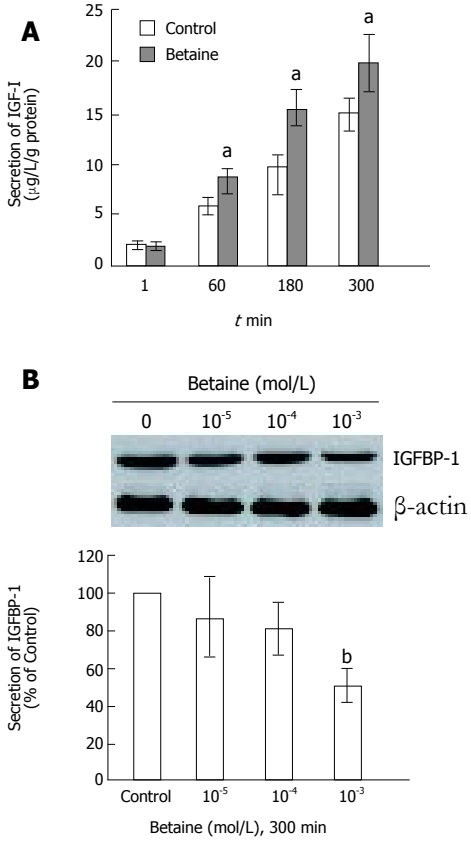

Figure 2 Betaine stimulates IGF-I secretion and decreases IGFBP-1 secretion in primary rat hepatocytes (mean ± SD). **A**: IGF-I, **B**: IGFBP-1. $^{a}P<0.05$; $^{b}P<0.01$ *vs* control.

alcohol reduces the level of IGF-I and increased that of IGFBP-1 in the serum, liver, and kidney of Sprague-Dawley rats[5]. To examine the possible effect of betaine, an osmolyte, on the regulation of alcohol-stimulated IGF-I and IGFBP-1 secretion, we measured the secretion of IGF-1 and IGFBP-I after cotreatment with betaine (for 300 min) and alcohol (for 180 min) in primary cultured rat hepatocytes. Whereas the secretion of IGF-I was significantly reduced by alcohol treatment alone, co-treatment with betaine resulted in IGF-I secretion being maintained at control levels (Figure 1A, $P<0.05$). In addition, betaine reduced alcohol-stimulated IGFBP-1 secretion (Figure 1B). Taken together, these results suggest that betaine ameliorates the alcohol-mediated secretion of IGF-I and IGFBP-1 in primary cultured rat hepatocytes.

Effects of betaine on IGF-I and IGFBP-1 secretion in primary rat hepatocytes

To evaluate the modulatory effects of betaine, we examined its effect on IGF-I and IGFBP-1 secretion in primary rat hepatocytes. Confluent cells were treated with 10^{-3} mol/L betaine for the times indicated in Figure 2. Supernatant was removed and the levels of IGF-I were measured by RIA (Figure 2A). The secretion of IGF-I increased significantly with increasing time after betaine treatment (Figure 2A, $P<0.05$ *vs* Control). To examine the effect of betaine on IGFBP-1 secretion, rat hepatocytes were treated with the concentrations of betaine indicated in Figure 2B for 300 min, and secretion of IGFBP-1 was monitored. Betaine decreased IGFBP-1 secretion in primary rat hepatocytes in a dose-dependent manner (Figure 2B). In summary, IGF-I secretion was significantly increased by betaine treatment, whereas IGFBP-1 secretion was decreased.

Involvement of MAPK in the betaine-induced stimulation of IGF-I and reduction of IGFBP-1 secretion.

To determine the molecular mechanism by which betaine stimulates IGF-I and reduces IGFBP-1 secretion in rat hepatocytes, we examined the involvement of MAPK. The effects of phosphorylation of p42/44 MAPK on the secretions of IGF-I and IGFBP-1 were monitored for 300 min after treatment with betaine alone (10^{-3} mol/L) and with betaine (10^{-3} mol/L) after pretreatment with PD98059 (10 μmol/L). Pretreatment with PD98059 not only significantly decreased betaine-stimulated IGF-I secretion (Figure 3A), but also attenuated the betaine-induced reduction in IGFBP-1 secretion (Figure 3B).

Given the effects of PD98059 on IGF-I and IGFBP-1 secretion, we performed an immunoblot analysis with antibody binding to phosphorylated MAPK 1/2 molecules. Whole lysates of cells treated with betaine were subjected to 100 g/L SDS-PAGE and blotted with phospho-p44/42 MAPK. The phosphorylation of MAPK 1/2 was increased at 60, 180, and 300 min after betaine stimulation, reaching a maximum after 300 min (Figure 4A). Betaine stimulated the phosphorylation of p42/p44 MAPK in a dose-dependent manner (Figure 4B). A significant ($P<0.05$) phosphorylation of p42/p44 MAPK was observed at a betaine concentration of 10^{-3} mol/L compared with the

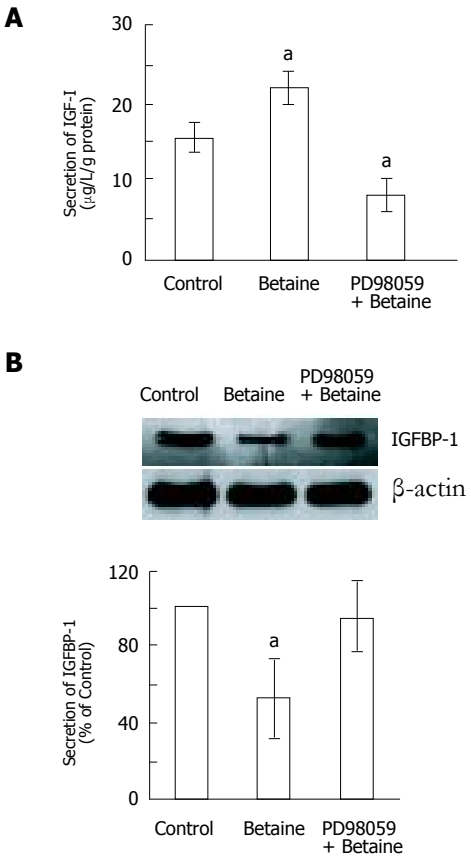

Figure 3 PD98059 attenuates the stimulation of IGF-I and attenuation of IGFBP-1 secretion effected by betaine (mean ± SD). **A**: IGF-I, **B**: IGFBP-1. $^{a}P<0.05$, *vs* control.

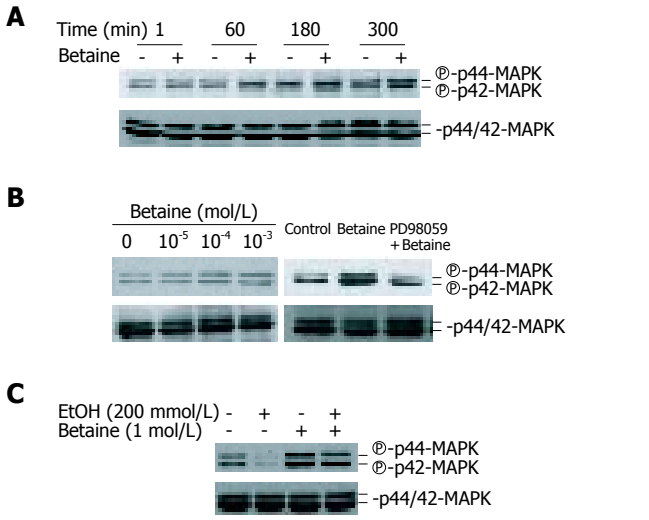

Figure 4 Betaine stimulates the phosphorylation of p42/44 MAPK in primary rat hepatocytes. **A**: Time-dependent activation of p42/44 MAPK by 10^{-3} mol/L betaine, **B**: Dose-dependent activation of p42/44 MAPK by betaine for 300 min (left panel). Treatment with betaine following pretreatment with PD98059 for 10 min (right panel), **C**: Betaine-stimulated recovery of p42/44 MAPK activation in ethanol-treated cells.

control (Figure 4B). Western blot of anti-phospho-MAPK was then reprobed with a specific antibody for MAPK 1/2. The resulting bands coincided with the immunoreactive bands of MAPK 1/2 (Figure 4B). Betaine ameliorated the activation of MAPK, although alcohol treatment inhibited the basal phosphorylation of p42/44 MAPK (Figure 4C). The effect of betaine on MAPK activation was also monitored at 300 min after treatment of the hepatocytes with betaine (10^{-3} mol/L); betaine induced the activation of p42/44 MAPK.

DISCUSSION

Alcohol abuse is associated with deleterious effects on several organs of the body, particularly the liver and brain[8]. Alcohol-induced liver damage is one of the major causes of morbidity and mortality among alcoholics, exposure to ethanol leading to both short- and long-term changes in liver function. Ethanol alters hepatic carbohydrate and lipid metabolism, as well as the synthesis of protein and DNA, which leads to liver dysfunction and cirrhosis[9]. The mechanisms and mediators underlying this liver injury, however, are not clearly understood. Kanbak *et al*[2] reported that betaine protects the liver from ethanol-mediated hepatotoxicity in rats, and it was reported recently that betaine also protects the liver from ischemia/reperfusion injury, niacin toxicity, and CCl_4-, hyperosmolarity-, and ethanol-induced apoptosis.

In the present study we found that the secretion of IGF-I, which is inhibited by ethanol, is increased by treatment with betaine, whereas that of IGFBP-1, which is increased by ethanol is reduced by betaine. It has been reported recently that lipid peroxidase activity is involved in the effects of ethanol on the secretions of IGF-I and IGFBP-1, but the underlying mechanism remains to be elucidated[10]. It has also been reported that acute ingestion of ethanol raises the plasma levels of IGFBP-1 in normal subjects[3,11]. Intake of ethanol influences the bioavailability of IGF-I in normal individuals. Barak *et al*[1] has shown that betaine, which is a naturally occurring substance, may be used as a protective agent against hepatotoxic substances such as ethanol and CCl_4. It may prevent the generation of reactive oxygen species in hepatocytes that results from treatment with ethanol, which is thought to be responsible for attenuation of IGF-I and increase in IGFBP-1 secretion.

This study also aimed to determine whether changes in IGF-I and IGFBP-1 secretion induced by betaine involve signal transduction in hepatocytes. Dose-dependent influences of betaine on the secretion of IGF-I and IGFBP-1, and on MAPK activity were observed. A significant increase in the secretion of IGF-I, a decrease in the secretion of IGFBP-1, and a significant increase in the phosphorylation of p42/p44 MAPK were observed 300 min after treatment with 200 mmol/L ethanol. The activation of p42/44 MAPK was blocked by the MAPK inhibitor PD98059 (10 μmol/L), and this greatly influenced the secretion of IGF-I and IGFBP-1.

In conclusion, the modulation of hepatocyte secretion of IGF-I and IGFBP-1 by betaine involves alteration of p42/44 MAPK activity; betaine may prevent ethanol-induced changes in IGF-I and IGFBP-1 secretion in hepatocytes.

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CLINICAL RESEARCH

Cerebral processing of auditory stimuli in patients with irritable bowel syndrome

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Abstract

AIM: To determine by brain functional magnetic resonance imaging (fMRI) whether cerebral processing of non-visceral stimuli is altered in irritable bowel syndrome (IBS) patients compared with healthy subjects. To circumvent spinal viscerosomatic convergence mechanisms, we used auditory stimulation, and to identify a possible influence of psychological factors the stimuli differed in their emotional quality.

METHODS: In 8 IBS patients and 8 controls, fMRI measurements were performed using a block design of 4 auditory stimuli of different emotional quality (pleasant sounds of chimes, unpleasant peep (2000 Hz), neutral words, and emotional words). A gradient echo T2*-weighted sequence was used for the functional scans. Statistical maps were constructed using the general linear model.

RESULTS: To emotional auditory stimuli, IBS patients relative to controls responded with stronger deactivations in a greater variety of emotional processing regions, while the response patterns, unlike in controls, did not differentiate between distressing or pleasant sounds. To neutral auditory stimuli, by contrast, only IBS patients responded with large significant activations.

CONCLUSION: Altered cerebral response patterns to auditory stimuli in emotional stimulus-processing regions suggest that altered sensory processing in IBS may not be specific for visceral sensation, but might reflect generalized changes in emotional sensitivity and affective reactivity, possibly associated with the psychological comorbidity often found in IBS patients.

Key words: Visceral hypersensitivity; Irritable bowel syndrome; Brain processing; fMRI; Auditory stimulation; Emotion

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INTRODUCTION

Visceral hypersensitivity has been shown to play a pathogenic role in the irritable bowel syndrome (IBS)[1,2]. At least in a subgroup of patients, this might be caused by altered brain processing of visceral sensation as has been suggested on the basis of brain imaging studies. Another important characteristic of IBS is the extraintestinal comorbidity. There is a high prevalence of non-gastrointestinal functional diseases, such as fibromyalgia or chronic fatigue syndrome, and also psychological disorders, such as depression and anxiety[3,4]. The frequent co-occurrence of these different disorders on the one hand, and the impact of psychological factors on all of them on the other hand[5], leads to the question whether these disorders might share a common pathogenesis such as a generalized increase in emotional and sensory sensitivity that could be involved in the alterations of sensory brain processing observed in these disorders[6-9].

Indeed, there is some evidence that altered cerebral

response patterns in IBS may not be specific for gastr-ointestinal stimuli. In patients with IBS and concomitant fibromyalgia, alterations in the central processing of painful somatic stimuli have been observed[7]. Moreover, even IBS patients without a manifest fibromyalgia may exhibit a somatic hypersensitivity with altered cerebral processing of somatic sensation[10]. These forms of somatic hypersensitivity, which are in contrast to earlier descriptions of somatic hyposensitivity states in IBS, are caused by viscerosomatic convergence mechanisms. The latter are thought to occur, because visceral and somatic sensations are both passed through the dorsal root ganglia and the dorsal columns of the spinal cord, where neural interactions have been described[11]. Therefore,any peripherally generated visceral hypersensitivity could subsequently induce a somatic hypersensitivity in the corresponding somatic dermatoma, and altered cerebral response pattern to somatic stimuli would actually reflect increased sensory input from the periphery. However, further support for cerebrally located processing alterations in IBS is provided by two studies demonstrating an increased reactivity of event-related potentials to auditory stimuli in IBS patients[12,13]. Since auditory stimuli are transmitted directly to the brain via the eighth cranial nerve, there is no peripheral connection to visceral sensory input and changes in the central reactivity could be allocated directly to the sensory processing circuits of the brain.

With this study, we aimed to test the hypothesis that IBS patients differ from healthy controls in the cerebral processing of non-visceral stimuli, detected by functional magnetic resonance imaging (fMRI). To be able to focus on the cerebral processing level, we chose auditory cues as non-visceral stimuli. The specific aims were 1) to analyze by fMRI the cerebral response patterns to non-visceral, auditory stimuli in IBS patients compared with healthy controls, and 2) to evaluate whether different brain responses are influenced by the emotional impact of the stimulus.

MATERIALS AND METHODS

Subjects

Eight right-handed patients with IBS [five females, three males; mean age, 41.3 (27-64) years; diarrhea-predominant, $n=5$, alternating stools, $n=3$] established by Rome II criteria [14] were recruited at our institution's outpatient clinic for gastrointestinal functional disorders. To exclude other causes for bowel symptoms, all patients had undergone a thorough work-up including laboratory tests, stool analysis for bacterial, fungal, or parasite infection, abdominal ultrasound, colonoscopy, lactose- and fructose hydrogen breath test. Moreover, a rectal sensitivity testing by computerized barostat was performed. The mean individual perception threshold of first sensation of the rectal barostat distension was 16.4 mmHg (SD 6.2). None of the patients had previous abdominal surgery and all patients had symptoms for more than 1 year. None of the patients used centrally acting agents to treat bowel symptoms, and peripherally acting IBS treatments were stopped 7 d prior to the study. As controls, 8 right-handed healthy volunteers [5 males, 3 females; mean age, 39.4 (24-54) years] were selected after exclusion of individuals with concomitant or previous GI-disorders by history and the IBS symptom score-questionnaire [15]. In all subjects, written informed consent as approved by the institutional ethical committee was obtained, and concomitant psychiatric disorders were excluded by using the standardized German Diagnostic Interview for Psychiatric Disorders (DIPS[16]), a structured clinical interview based on the Anxiety Disorders Interview Schedule[17].

Personality-questionnaires

For the assessment of personality characteristics, all study participants were asked to fill in the following questionnaires: The German form of the State-Trait-Anxiety-Inventory (STAI)[18] for the assessment of depression, neuroticism and complaints, the German form of the Beck's Depression Index (BDI)[19] for the assessment of depression, and the German form of the NEO-Five-Factor-Inventory (NEO-FFI)[20] for the assessment of neuroticism, openness to experience, extroversion, agreeableness, and conscientiousness.

Stimulation protocol for MRI-scanning

We used a block design of four auditory stimuli of different emotional impact: an unpleasant peep (a sound of 2000 Hz), pleasant sounds of chimes, words with emotional impact and neutral words. The part of the protocol using neutral words had served as a neutral, non-visceral control stimulus, in a previous study investigating visceral stimulation[21]. All words were chosen from a list of words that had been evaluated as neutral or emotional by a group of 20 healthy volunteers. The stimulation paradigm had been evaluated in event-related potential studies for patients with psychosomatic disorders. The stimulation phases of 48 s each were separated by resting phases and applied in the following order: peep, chimes, neutral words, chimes, emotional words, neutral words, peep.

MRI acquisition

Magnetic resonance images were collected on a 1.5 T whole body scanner (Siemens Magnetom Vision, Erlangen, Germany) with a standard head coil. A vacuum pad was used to minimize head movements. First, a T1-weighted localizer scan was recorded. Next, T2-weighted oblique scans were obtained (TR/TE 4500/128 ms, field of view 230 mm), primarily to aid Talairach transformation for data analysis. For the functional scans, an echo-planar sequence (TR/TE 4000/66 ms; flip angle 90 degrees; field of view 230 mm; matrix 128 × 128; slice thickness 6 mm, interslice gap 0.6 mm; in-plane resolution 1.8 mm × 1.8 mm) was used. One hundred and twenty images per slice were acquired. Sixteen slices adjusted at a transverse-to-coronal angle of approximately 20° covering the whole brain with the exception of the most superior frontal and superior parietal lobe, inferior temporal pole, and cerebellum (most superior z about 60 and most inferior z about -25 according to Talairach and Tournoux[22]) were obtained for all studies. Structural 3D data sets were acquired using a T1-weighted sagittal sequence with isotropic voxels (TR/TE 11.4/4.4 ms; flip angle 15 degrees; number of slices

Table 1 Personality characteristics (mean ± SD)

Personality test	Control	IBS	IBS *vs* control	Reference-population [18-20]
STAI X2	34.8 ± 6.1	41.3 ± 11.0	$P = 0.17$	35.7 ± 9.4
BDI	5.4 ± 5.3	8.7 ± 4.4	$P = 0.19$	6.5 ± 5.2
NEO: Neuroticism	1.7 ± 0.5	2.2 ± 0.6	$P = 0.11$	1.8 ± 0.7
NEO: Extroversion	2.8 ± 0.4	2.6 ± 0.3	$P = 0.24$	2.4 ± 0.6
NEO: Openness to experience	2.5 ± 0.5	2.4 ± 0.3	$P = 0.73$	2.7 ± 0.5
NEO: Agreeableness	2.4 ± 0.6	2.4 ± 0.3	$P = 0.81$	2.4 ± 0.5
NEO: Consciousness	3.0 ± 0.6	3.2 ± 0.5	$P = 0.46$	2.6 ± 0.6

STAI=State-Trait-Anxiety-Inventory, with X2=Trait-Anxiety Subtest, BDI= Beck's Depression Index, NEO= NEO-Five-Factor-Inventory

160, matrix 256 × 256, field of view 256 mm, voxel size 1 mm3).

Data analysis

For data analysis we used the Brainvoyager® software (Brain Innovation B.V, Postbus, Maastricht, Netherlands). The 2D functional data were reconstructed within the 3D structural data set. The 3D-data were then transformed into the standardized Talairach spaced brain[22]. Finally, the reconstructed data set underwent subsequent procedures of motion correction, intensity scaling and detrending.

Statistical maps were constructed using the general linear model module of the Brainvoyager software. The stimulation conditions were used as predictors, and the contrasts of each stimulation condition versus rest were analyzed. For the between group comparison, predictors were defined for each stimulus type as the interaction of the group with this stimulus type. Therefore, each predictor represented a larger signal increase of one group at the referred stimulus type.

Activated clusters were only accepted if they showed highly significant ($P < 0.001$) activation increase. In order to correct for multiple comparisons, a minimal cluster size of 6 voxels was defined.

Definition of regions of interest (RoI)

As regions of interest (RoI) for the data analysis (Table 2), we selected brain areas known to be involved in emotional processing[23]. All regions were neuroanatomically predefined by an expert neuroradiologist according to the coordinates of Talairach *et al*[22], and according to neuroanatomical visualization.

RESULTS

Subject characteristics

The personality traits were not significantly different between IBS patients and healthy controls. Results are summarized in Table 1. Descriptively, IBS patients had higher values for anxiety ($P = 0.17$), depression ($P = 0.19$) and neuroticism ($P = 0.11$) relative to healthy controls (Table 1).

Group analysis-auditory stimulation

Both groups responded to all stimuli with a significant activation ($P < 0.001$) in the auditory cortex, with a higher signal response to the word processing stimulus conditions and in the speech processing areas for the word stimulations (data not shown). With regard to the emotional processing regions we observed the following:

Response to unpleasant peep

To unpleasant peep, both groups responded with significant deactivations ($P < 0.001$) in the anterior cingulate cortex (ACC) and prefrontal cortex (PFC) with a relatively stronger ACC deactivation in controls, as indicated by the number of deactivated voxels. By contrast, only IBS patients responded with significant deactivations ($P < 0.001$) in the amygdala and the hippocampus (HC) (Table 2).

Response to pleasant sounds of chimes

To pleasant sounds of chimes, both groups responded with significant deactivations ($P < 0.001$) in the PFC and the posterior cingulated cortex (PCC) with a relatively stronger PCC deactivation in controls, as indicated by the number of deactivated voxels. Only controls responded with deactivations ($P < 0.001$) in the Insula and HC, and with significant activations ($P < 0.001$) in the ACC and a different region of the PFC. In IBS patients, on the contrary, the ACC was significantly deactivated ($P < 0.001$) (Table 2).

Response to emotional words

To emotional words, both groups responded with significant deactivations ($P < 0.001$) in the parietal sensory association cortex (PSAC), the ACC, the PFC, and the HC. However, the overall deactivation response in the ACC, PFC and HC, as indicated by number and size of deactivated clusters, was much stronger in IBS patients relative to controls (Table 2, Figure 1). By contrast, only controls responded with a significant deactivation ($P < 0.001$) in the PCC, while only in IBS patients, emotional words induced deactivations ($P < 0.001$) in the insula and moreover a significant activation ($P < 0.001$) in a different region of the PFC (Table 2).

Response to neutral words

To neutral words, both groups responded with significant activations ($P < 0.001$) in the PFC and PSAC. However, the overall activation response, as indicated by number and size of activated clusters, was much stronger in IBS patients relative to controls. Only in controls, neutral words induced a deactivation ($P < 0.001$) in the ACC. By contrast, only IBS patients responded with significant activations ($P < 0.001$) in the ACC, PCC, HC, and insula (Table 2, Figure 2).

DISCUSSION

Altered cerebral processing of visceral sensation has been proposed to play a pathogenic role in IBS. Whether these alterations are specific for visceral stimuli is currently not

Table 2 Significant activations (signal increase) and deactivations (signal decrease) induced by different acoustic stimuli

			[1]Largest cluster				
I) PEEP							
A: Control deactivation							
RoI	Side	No. of Clusters	No. of Voxels[1]	x[1]	y[1]	z[1]	% signal change*
ACC	L	1	-793	-8	37	-10	-0.53
PFC	B	7	-1506	37	35	-7	-0.52
B: IBS deactivation							
ACC	L	1	-271	-6	31	7	-0.28
PFC	B	7	-1724	23	57	8	-0.52
Amyg-dala	R	1	-452	25	1	-20	-0.33
HC	B	2	-436	33	-22	-24	-0.38
II) CHIMES							
A: Control activation							
ACC	B	1	699	-1	29	2	0.40
PFC	B	7	1957	-4	37	-6	0.39
Deactivation							
PFC	B	4	-1781	31	47	23	-0.48
PCC	L	1	-707	-5	-4	51	-0.24
Insula	R	1	-850	32	1	7	-0.25
HC	R	1	-113	26	-14	-15	-0.14
B: IBS deactivation							
ACC	B	2	-1212	2	18	44	-0.38
PCC	B	1	-159	0	-17	32	-0.36
PFC	R	3	-2095	54	13	13	-0.42
III) EMOTIONAL WORDS			[1]Largest cluster				
A: Control deactivation							
ACC	L	1	-724	-5	36	13	-0.33
PFC	B	4	-275	-4	47	-2	-0.36
PCC	L	1	-134	-1	-33	44	-0.35
PSAC	B	1	-2048	0	-62	33	-0.36
HC	R	1	-31	25	-24	-15	-0.21
B: IBS activation							
PFC	L	1	609	-46	29	-1	0.25
Deactivation							
ACC	R	1	-1207	2	40	5	-0.41
PFC	B	4	-2076	9	43	26	-0.32
PSAC	B	1	-2048	0	-46	42	-0.35
HC	B	2	-1010	24	-35	-12	-0.29
Insula	R	1	-331	34	-10	1	-0.19
IV) NEUTRAL WORDS							
A : Controls activation							
PFC	R	1	45	26	55	6	0.40
PSAC	R	1	45	26	-42	53	0.05
Deactivation							
ACC	L	1	-118	-3	31	-6	-0.44
B : IBS activation							
ACC	R	2	223	1	6	46	0.19
PFC	B	8	1408	0	46	-11	0.18
PSAC	B	4	257	26	-65	41	0.08
HC	B	2	652	27	-27	19	0.14
PCC	L	2	102	-2	-39	37	0.12

RoI= region of interest, R=right, L=left, B= bilateral, ACC= anterior cingulate cortex, PCC= Posterior Cingulate Cortex, PFC=prefrontal cortex, PSAC=parietal sensory association cortex, HC=hippocampus, x+y+z=coordinates of the Talairach space.

clear. In this study, we analyzed cerebral processing of non-visceral, auditory stimuli using fMRI. In a separate protocol, all IBS patients included in this study also underwent rectal stimulation by balloon distension adapted to the individual rectal perception thresholds, which were lower in IBS patients than in controls[21]. In IBS patients, decreased ACC and PFC activation with subliminal and supraliminal rectal stimuli and increased HC activation with supraliminal stimuli suggested disturbances of the associative and emotional processing of visceral sensation[21]. Our current findings demonstrate that IBS patients exhibit differences in the central processing of auditory stimuli of different emotional impact in brain regions involved in emotional sensory processing. Our main observations were: 1) To distressing auditory stimuli (peep and emotional words) both controls and IBS patients responded with significant deactivations in emotional processing regions demonstrating stronger responses in a greater variety of brain areas in IBS patients. 2) To pleasant auditory stimuli (chimes) IBS patients also responded with significant deactivations only, while in controls significant activations in the ACC and PFC were observed. 3) To neutral auditory stimuli (neutral words) IBS patients, unlike controls, responded with large significant activations in a variety of emotional processing regions. Taken together, IBS patients reacted more intensively with deactivations following emotional auditory stimuli, not differentiating, unlike controls, between distressing or pleasant sounds, and more intensively reacted with activations to neutral auditory stimuli.

Thus, this fMRI study indicates alterations of auditory sensory processing in IBS patients in brain regions participating in emotional stimulus processing. The cerebral processing differences involve both activation and deactivation responses. However, the specific cerebral function of deactivations observed in fMRI is still not completely understood and has been widely discussed[24]. Within the context of BOLD (Blood Oxygen Level Dependent) responses, on which the fMRI technique is based on, an activation reflects an increase in regional oxyhemoglobin that is believed to result from an increased blood flow coupled to neural activation ("neurovasular coupling"). In contrast, a "deactivation" reflects a stimulation-induced regional decrease of oxyhemoglobin of which the functional meaning is unclear. Vascular steal mechanisms or "neurovascular decoupling" resulting in reduced oxyhemoglobin have been suggested as possible cause of signal decrease[24]. In this case, the signal decrease would also reflect a neural *activation*. On the other hand, there is some evidence that the signal decrease could actually reflect a reduction of neural activity[25-27]. In fact, it has been proposed, that deactivations might contribute to cortical protection from incoming stimuli, for example in the context of chronic repetitive subconscious somatic stimuli[28] or in the context of sleep preservation[26,27]. A study demonstrating that the same auditory stimulus can induce either activation or deactivation responses of the amygdala just depending on the psychosocial background of the subjects[29],also supports a distinct neuro-functional meaning of deactivations. However, the uncertain functional meaning of deactivations to date makes the detailed interpretation of our findings difficult and we therefore restrict the following discussion to a rather descriptive evaluation of the dem-

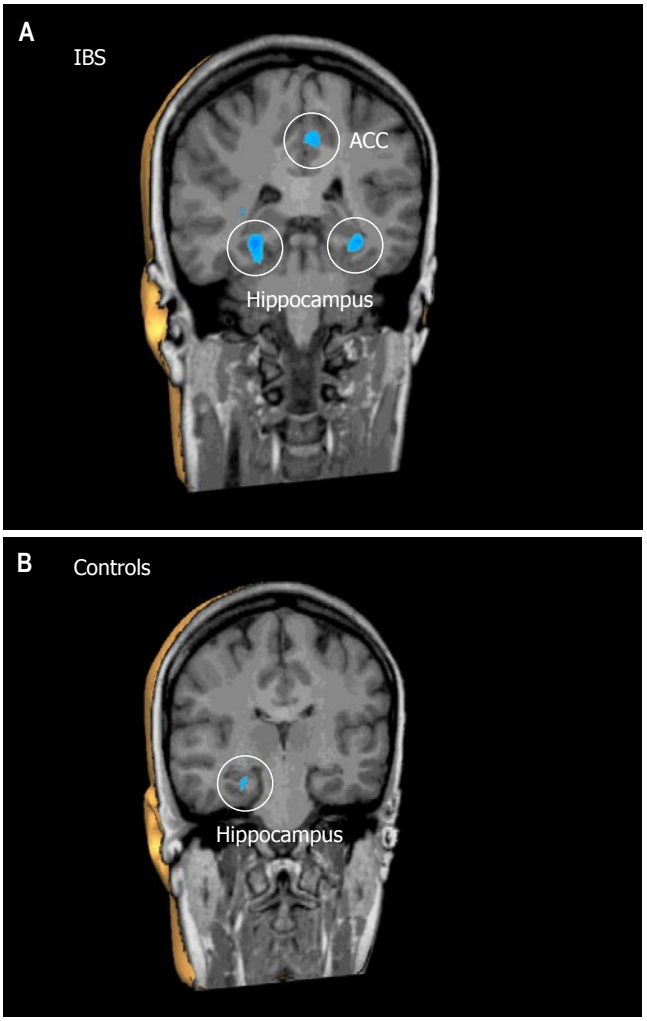

Figure 1 Auditory stimulation with emotional words induced significant deactivations of the hippocampus bilateral, and of the anterior cingulated cortex (ACC) in IBS patients, but only a very small deactivation of the right hippocampus in controls ($P < 0.001$).

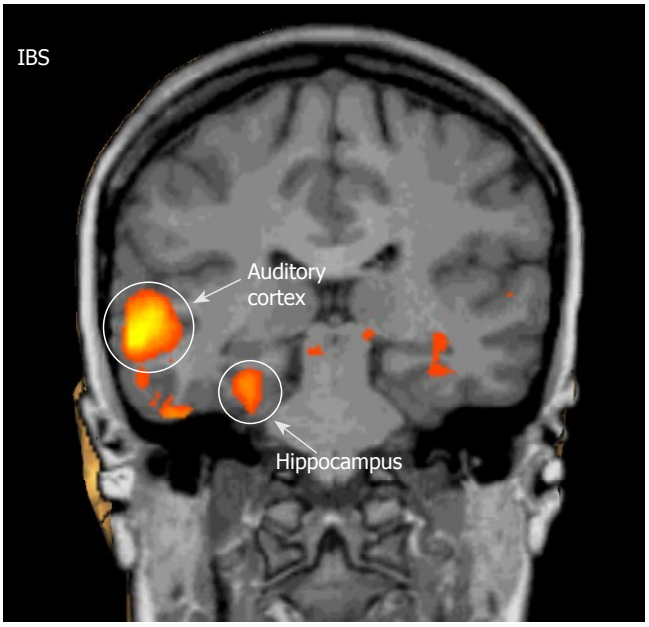

Figure 2 In IBS patients, auditory stimulation with neutral words induced significant activation of the hippocampus ($P < 0.001$).

onstrated brain processing differences.

Though both IBS patients and controls responded to distressing emotional stimuli mainly with deactivations, the deactivated regions in IBS patients were larger and involved a greater variety of emotional processing regions, among them parts of the limbic system like the amygdala or the hippocampus. In general, these observations could suggest a greater emotional reactivity to distressing stimuli in IBS patients as compared with healthy controls. This is in line with previous literature that demonstrate a central hyperreactivity of IBS patients to rectal stimuli in the presence of distressing auditory or visual stimuli[30-32].

Interestingly, IBS patients responded to pleasant sounds with similar deactivation patterns as to distressing stimuli whereas controls also reacted with activations. It is conceivable that an underlying alexithymia, a disorder characterized by the difficulty to recognize or express emotions and found to be significantly increased in IBS patients[33], could possibly accord for this finding. However, this has to remain speculative, because alexithymia was not specifically assessed in this study.

Neutral words, as expected, did hardly induce any reaction in emotional processing centers in healthy controls. By contrast, IBS patients responded with significant activations in these brain regions. These findings could indicate that IBS patients have a generalized increase in the sensitivity and reactivity of emotional processing brain regions even in response to neutral stimuli. Interestingly, the reaction to neutral stimuli is limited to activation-responses, while the emotional stimuli induced deactivation patterns in the same brain regions. This provides further evidence that activations and deactivations represent different processing functions depending on the emotional impact of the stimulus[29].

Overall, our findings indicate that IBS patients relative to controls show a greater cerebral reactivity to auditory stimuli with a larger involvement of limbic structures suggesting a higher sensitivity of emotional processing brain regions. Thereby, our results underline earlier observations by Blomhoff *et al* and Berman *et al*[12,13,34] who demonstrated a cerebral hyperreactivity to both emotional and neutral auditory stimuli in IBS patients. While altered cerebral processing in IBS has previously been demonstrated in the context of visceral stimulation[6,35,36], the current findings suggest that differences in cerebral stimulus processing in IBS might not be restricted to visceral sensation. Rather it is conceivable that differences in the emotional state might be an underlying factor. In fact, Blomhoff could demonstrate that the hyperreactivity to auditory stimuli is especially found in IBS patients with concomitant phobic anxiety disorders[34]. Accordingly, in our study, higher levels of anxiety, depression, and neuroticism in the IBS patient group (though differences were not reaching the level if significance possibly due to small sample sizes) could account for some of the observed differences in brain activation responses.

We acknowledge that this study has several limitations: The sample size of $n = 8$ for each group is rather small, and the bowel dysfunctions are heterogeneous in the IBS group. There is some evidence that diarrhea- versus

constipation-predominant IBS patients have different cerebral response patterns to rectal stimulation[37]. Whether this could also affect the cerebral processing of auditory stimuli is unknown. Furthermore, it is conceivable that the increased scores for anxiety, neuroticism and depression can alone account for the different stimulus processing of patients versus controls independent of IBS status. Future studies should include more patients, compare also to other psychologically distressed patients free of IBS, assess further psychometric variables such as alexithymia and allow for different subgroup analyses to further elucidate the nature of altered sensory processing in IBS.

Overall, the current observations of altered cerebral response patterns to neutral and emotional auditory stimuli in IBS patients indicate that altered emotional stimulus processing in IBS may not be specific for visceral sensation, but might reflect a generalized increase in emotional sensitivity and affective reactivity. This could account for the frequent association of IBS with psychological or extra-intestinal functional disorders[3].

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CLINICAL RESEARCH

Effect of IBD sera on expression of inducible and endothelial nitric oxide synthase in human umbilical vein endothelial cells

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Abstract

AIM: To study the expression of endothelial and inducible nitric oxide synthases (eNOS and iNOS) and their role in inflammatory bowel disease (IBD).

METHODS: We examined the effect of sera obtained from patients with active Crohn's disease (CD) and ulcerative colitis (UC) on the function and viability of human umbilical vein endothelial cells (HUVEC). HUVECs were cultured for 0-48 h in the presence of a medium containing pooled serum of healthy controls, or serum from patients with active CD or UC. Expression of eNOS and iNOS was visualized by immunofluorescence, and quantified by the densitometry of Western blots. Proliferation activity was assessed by computerized image analyses of Ki-67 immunoreactive cells, and also tested in the presence of the NOS inhibitor, 10^{-4} mol/L L-NAME. Apoptosis and necrosis was examined by the annexin-V-biotin method and by propidium iodide staining, respectively.

RESULTS: In HUVEC immediately after exposure to UC, serum eNOS was markedly induced, reaching a peak at 12 h. In contrast, a decrease in eNOS was observed after incubation with CD sera and the eNOS level was minimal at 20 h compared to control (18% ± 16% *vs* 23% ± 15% $P<0.01$). UC or CD serum caused a significant increase in iNOS compared to control (UC: 300% ± 21%; CD: 275% ± 27% *vs* 108% ± 14%, $P<0.01$). Apoptosis/necrosis characteristics did not differ significantly in either experiment. Increased proliferation activity was detected in the presence of CD serum or after treatment with L-NAME. Cultures showed tube-like formations after 24 h treatment with CD serum.

CONCLUSION: IBD sera evoked changes in the ratio of eNOS/iNOS, whereas did not influence the viability of HUVEC. These involved down-regulation of eNOS and up-regulation of iNOS simultaneously, leading to increased proliferation activity and possibly a reduced anti-inflammatory protection of endothelial cells.

Key Words: Crohn's disease; Human umbilical vein endothelial cells; Inflammatory bowel disease; Nitric oxide synthase; Ulcerative colitis

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INTRODUCTION

The two major forms of inflammatory bowel disease (IBD), ulcerative colitis (UC) and Crohn's disease (CD) are characterized by chronic inflammatory ulceration of the intestine. In an early[1], and in some recent studies[2, 3], the contribution of mucosal microvascular dysfunction is implicated in the development of CD. Histological analy-

Table 1 Data of patients with ulcerative colitis and Crohn's disease

Group	*n*	Age (yr)(range)	M/F	Endoscopic activity score	Clinical activity score	CRP (mg/L)	Treatment
UC	21	27 (18-33)	3/8	G_1: 4 G_2: 9 G_3: 8	4≤UC-DAI	>10	5-ASA
CD	18	31 (23-45)	13/5	8≤CDEIS≤14	150≤CDAI≤450	>10	5-ASA
Control	16	29 (25-39)	8/8				

G_{0-3}: Endoscopic grading of UC (Br Med J 1964; 1:89); CDEIS: Crohn's disease endoscopic index of severity (Gut 1989; 30:983-89); UC-DAI: Ulcerative colitis disease activity index (Br Med J 1955; 2:1041); CDAI: Crohn's disease activity index (*Gastroenterology* 1979; 77:843)

sis of IBD colonic mucosa indicated structural changes of the vascular system. In CD, arterial thickening and the appearance of an increasing number of capillaries were observed[1, 3]. Dysfunction in the vasodilatory capacity of microvessels from areas of chronic inflammatory damage was also shown in IBD[2]. Inflammatory cytokines modify and regulate the level of NO, and they are also involved in vascular endothelial response to inflammatory injury[3, 4]. Apoptosis of endothelial cells induced by proinflammatory cytokines and reactive oxygen species is counteracted by the endothelial nitric oxide (NO). The suppression of apoptosis may contribute to the anti-inflammatory and pro-angiogenic effect of endothelial NO[5].

The endothelial form of nitric oxide synthase (eNOS) plays a significant protective role against experimental colitis[6]. Decrease of eNOS immunoreactivity in the endothelium is suggested as a putative cause of arterial thickening and the appearance of an increasing number of capillaries in the mucosa of Crohn's colitis[3]. Moreover, weak eNOS immunoreactivity in the endothelium together with structural alteration of the vascular wall, both found in biopsies of Crohn's patients suggest that malfunction of local blood supply may also contribute to the development of the disease[3]. Human umbilical vein endothelial cells (HUVEC) are frequently used as a specific model for studying endothelial function. HUVECs are also sensitive to pro-inflammatory stimuli induced by interleukins. NO is produced in HUVEC constitutively by eNOS and by inducible nitric oxide synthase (iNOS) in the case of different stimuli[7, 8]. NO also contributes to the regulation of apoptosis, cell differentiation and proliferation (angiogenesis) in this cell type[9-11].

The changes in the activity of eNOS and iNOS as well as their role in the endothelium of IBD patients have not been examined in detail. In the present study, we examined the effect of sera from patients diagnosed with UC and CD on cultured HUVEC. The expression and localization of eNOS and iNOS were determined in highly confluent and in semi-confluent HUVEC cultures, and the apoptotic/necrotic characteristics and proliferation activity upon distinct treatments were also assessed.

MATERIALS AND METHODS

Collection of blood sera

Blood was sampled for examination from patients who had relapsing UC or CD. Among 21 UC patients, 16 were on their 1st relapse (mean time from diagnosis [*mtd*] was 1 yr), and 5 on 2nd relapse (*mtd* = 3 yr), whereas all the 18 CD patients were on their 1st relapse (*mtd* = 4 yr). Control sera were obtained from 16 healthy volunteers. They received no systemic or locally applied corticosteroids, and their medication was stable for the last four weeks before sampling. All the patients were on 5ASA oral therapy. Patients diagnosed as having UC had left side colitis (Table 1). Crohn's patients had colonic localization and a non-stricturating, non-penetrating form of the disease (Table 1) according to the Vienna classification (1998), and an elevated C-reactive protein level (CRP>10 mg/L), without abscess or sign of infection (all of the patients had negative abdominal ultrasound). In cases which raised the suspicion of abdominal or pelvic abscess, CT or MRI (to exclude the presence of perianal / perirectal abscesses) was performed. Five milliliters of blood were taken from patients of UC, CD and control subjects respectively, placed into collecting tubes, and centrifuged at 2000 r/min for 10 min. The sera were removed and mixed, and kept frozen at -80℃ until use.

Preparation and culturing of HUVEC

Freshly isolated umbilical cords were obtained from the Department of Obstetrics and Gynecology (University of Debrecen, Medical and Health Science Center). Umbilical cords were immersed into a sterile flask containing culture medium (components are described below) and carried to the cell culture room. Both ends of the cords were bound and the ends of the umbilical vein were cannulated. The vein vessel was washed through the cannulas by sterile PBS, followed by Hanks' Balanced Salt Solution (HBSS), and M199 media (all purchased from Sigma, Budapest, Hungary). Then, the vein vessel was filled with 1 g/L collagenase in M199, and placed in a CO_2 incubator for 12 min at 37 ℃. Endothelial cells were isolated in a sterile centrifuge tube from the vessel by washing twice with 20 mL M199 medium. Cell suspension was centrifuged at 1000 r/min for 15 min, and the supernatant was discarded and the cells were resuspended in the culture medium.

The cells (2×10^9 cells/L) were inoculated into a plastic culture dish. The culture medium contained the following components: 500 mL/L normal human serum, 20 g/L HEPES, 10 g/L L-glutamine, 10 g/L amphotericin, 10 g/L penicillin, 450 mL/L sterile filtered M199. One day after inoculation, dishes were washed with 37 ℃ HBSS, and put into the culture media again. After 4-5 d of culturing, dur-

ing which time cells grew extensively over the entire space of the dish, cells were washed with HBSS, then digested with 1 mL tripsin-EDTA (tripsin : EDTA = 1 : 25). The cells were suspended in 1 mL culture medium and collected into a sterile flask, then mixed well. The cells were inoculated into the culture dish and the cell propagation and collection procedure were repeated again as described above. Then, cells were passed onto culturing plates at about 2 $\times 10^9$ cell/L concentration. Cell monolayers that reached 20%-30% or 70%-80% confluency were used for the experiments. The cells were kept in 50% normal, 50% UC or 50% CD sera dissolved in culturing medium for 2, 4, 8, 12, 20, 24, 36, and 48 h, then processed for visualization of eNOS or iNOS.

Western blot

After removal of the culture media, cells were washed with ice-cold PBS, then harvested by a cell scraper into 50 μL RIPA lysis buffer (0.01 mol/L NaH_2PO_4, 10 g/L Nonident P-40, 10 g/L Na-deoxycholate, 1 g/L Na-dodecyl sulfate (SDS), 0.15 mol/L NaCl, 2 mmol/L EDTA), containing 100 kU/L protease inhibitor mix (Sigma-Aldrich, Budapest, Hungary). Cell lysis was accelerated by continuous shaking. Five microliters of this mixture were added to 45 μL RIPA buffer for protein measurement. All protein solutions were frozen and kept at -80 ℃ until use.

The protein concentration of the lysates was determined by the BCA method using BSA as standard in an ELISA reader at 540 nm. Data were analyzed by GraphPad Prism software using linear regression to determine the protein concentration (g/L). Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the conditions of Laemmli (1970) on 100 g/L acrylamide gels in a Bio-Rad MiniProtean apparatus[3]. Samples for immunoblot analysis were diluted in the RIPA buffer, dissolved in an equal volume of 2 ×SDS sample buffer (100 mmol/L Tris pH 6.8, 2 g/L bromophenol blue, 200 mL/L glycerol, 200 mL/L β-mercaptoethanol) and boiled for 5 min. From each solution, aliquots (30 μg protein) were applied to SDS-PAGE and electrophoresed at 120 V for 1 h. Separated proteins were blotted onto a nitrocellulose membrane (Pharmacia, Vienna, Austria) at 100 V for 90 min during the continuous mixing and cooling of the transfer buffer (192 mmol/L glycine, 25 mmol/L Tris-base, 200 mL/L methanol). Membranes were rinsed twice in 0.1 mol/L PBS (pH 7.6) containing 1 g/L Tween 20 (PBS-Tween), then blocked for 1 h with 50 mL/L non-fat dried milk in PBS-Tween. After 3 × 5 min of washing with PBS-Tween, the membranes were incubated overnight at 4 ℃ with the anti-eNOS (diluted into 1 : 5000, Cat. No.: 482726, Merck-Calbiochem, Darmstadt, Germany) or anti-iNOS (1 : 2000, Cat. No.: AB5384, Chemicon, Temecula, CA) polyclonal antibodies raised in rabbits. After washing with PBS-Tween for 3 × 5 min, membranes were incubated with horseradish peroxidase conjugate of anti-rabbit IgG (diluted into 1 : 5000 in PBS-Tween, Vector, Bulingame, CA) for 30 min. After washing the membranes subsequently with PBS-Tween for 2 ×5 min, and once with PBS for 5 min. The blots were developed with the enhanced chemiluminescent (ECL) reagent kit (Pierce, Rockford, IL) and the immunoreactions were visualized on X-ray films.

Densitometry of the blots was performed and the scans were analyzed by the Volume Analyse feature of the Molecular Analyst Software (Bio-Rad, Hercules, CA). The densities for the NOS bands were normalized in each blot to the NOS-IR band obtained from the sample of 2 h treatment with normal serum and expressed as percentage of this control. Means of the percentages obtained from three independent experiments were presented in figures as mean ± SEM. The significance of the data between groups (normal, UC, CD) was tested by analysis of variance (ANOVA). Differences were considered significant for values of $P < 0.01$.

Immunofluorescence

Cells were cultured on coverglasses coated with sterile filtered 100 g/L gelatine in PBS. Cultures showing 20%-30% confluence were used for detection of proliferation, cell distribution and apoptosis/necrosis characteristics, whereas those with 70%-80% confluency were used for visualization of NOS isoforms. After treatments, cells were fixed in an ascendant ethanol gradient (500 ml/L, 700 mL/L, 960 mL/L), then rehydrated with PBS. For specific detection of eNOS and iNOS proteins, non-specific binding sites of primary antibodies were blocked by incubation of the fixed cells for 1 h with 50 mL/L normal goat serum in PBS also containing 1 g/L bovine serum albumin (BSA), 1 g/L Triton-X 100 and 0.1 g/L Na-azide (referred to as solution-A below). NOS primary antibodies used for immunocytochemistry were the same as those used for immunoblotting. Both eNOS and iNOS antibodies were applied at 1 : 200 dilution in solution-A overnight at 4 ℃. After extensive washing with PBS (3×5 min), cells were incubated with FITC conjugated anti-rabbit IgG diluted to 1 : 40 in solution-A for 30 min at room temperature. Cells were washed for 3 ×5 min with PBS and cover glasses were mounted on microscope slides (with one drop of PBS-glycerol (1 : 1) solution on them).

For evaluation of the proliferation activity, HUVECs were incubated in a medium containing 500 mL/L normal, 500 mL/L UC or 500 mL/L CD sera, or in a medium containing 500 mL/L normal sera plus 10^{-4} mol/L nitro-L-arginine methyl-ester (L-NAME, Sigma-Aldrich), an NOS inhibitor. Cells were exposed to a monoclonal antibody (at 1 : 100 dilution) specific for the proliferation marker Ki-67 antigen (clone No. MIB-1, Dako, Denmark) for 2 h at room temperature. In this case, FITC-conjugated anti-mouse IgG (1 : 40 in solution-A, Dako) was used as a secondary antibody. For testing of the effect of normal and IBD sera on the viability of HUVEC, apoptotic/necrotic cells were assessed by the annexin-V-biotin kit (Cat. No. 1828690, Boehringer-Mannheim, Germany). Briefly, after removal of the incubation media and washing with PBS, cells were labeled with the annexin-V-biotin/propidium iodide solution (1 : 50 annexin-V-biotin stock solution diluted in a 0.01 mol/L HEPES buffer, pH 7.4, containing 0.14 mol/L NaCl, 0.005 mol/L $CaCl_2$, and 1 mg/L propidium iodide) for 15 min. After subsequent washing with HEPES, cells were fixed with methanol-ethanol (1 : 1) solution for 1 min, and labeled with 5 mg/L avidin-fluorescein for 15 min. After consecutive washing with

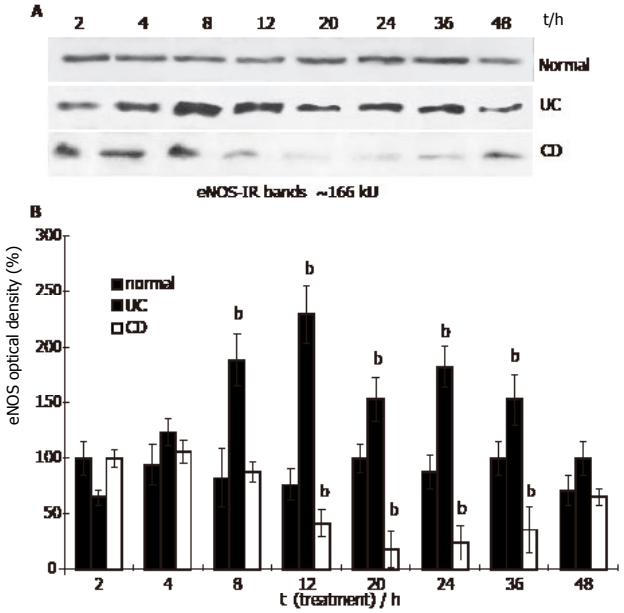

Figure 1 eNOS content in HUVEC treated with IBD sera (Western blot. Mean ± SE, $n = 3$; $^bP<0.01$ *vs* normal).

HEPES and PBS, cells were put on microscope slides as described above. Preparations used for the assay of proliferation activity and assessment of apoptosis/necrosis were stained prior to covering with 10 g/L toluidin blue in PBS. The latter labeled the cell nuclei and helped to determine total cell numbers for calculation of apoptotic/necrotic indices. The integrity of the cytoskeleton was examined by visualization of actin filaments by incubation with 0.2 mg/L phalloidin-FITC (Sigma-Aldrich) for 1 h.

Examination was carried out and digital images were captured with an Olympus AX-70 fluorescence microscope. In each case, the brightness-contrast of the images was set in the same manner by the Jasc software Paintshop Pro 7.0. Images were taken from Ki-67 immunoreactive (IR), annexin-labeled, and propidium iodide reactive cell nuclei, respectively. The same sites of the preparations were also captured in bright visual illumination to count the toluidin blue stained nuclei (total cell number). The images were quantitatively analyzed by the computer image analysis software Olympus analysis 2.11 (SiSoft), developed and validated by Olympus (Tokyo, Japan). At least 10^3 cells were counted for each data point and the ratios of proliferative, apoptotic and necrotic cells were given as the percentage of total cell numbers.

Statistical analyses

Statistical analyses were performed to compare the data obtained from the different treatments groups of IBD and normal sera using the ANOVA test. $P<0.05$ was considered as significant.

RESULTS

eNOS

Western blots from HUVEC lysates detected eNOS immunoreactive bands at approximately 160 kU, corresponding to the molecular mass of human eNOS (Figure 1A).

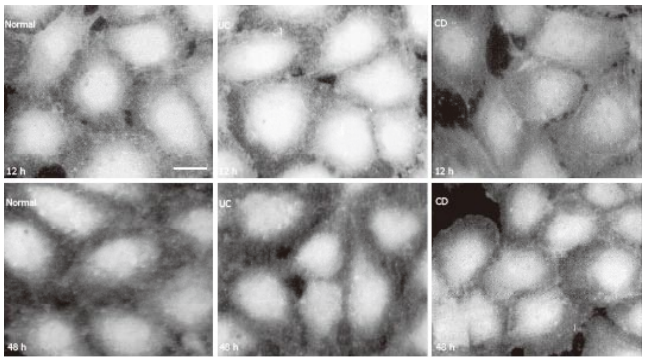

Figure 2 eNOS in HUVEC (immunoreaction was visualized by FITC. bar: 10 μm).

HUVEC was cultured in a medium containing 500 mL/L normal human serum. The amount of eNOS did not change significantly during the experimental period (0-48 h). However, in the presence of 500 mL/L UC serum, eNOS level increased after 8 h, and peaked at 12 h (relative optical density value was 188% ± 23% and 229 %± 26%, $P < 0.01$, respectively). At 12 h, eNOS level appeared to have doubled compared to the normal level at 2 h (100% ± 15%). Over the 12 h period in the presence of 500 mL/L UC serum, eNOS level began to decline (36 h, 153% ± 23%, $P < 0.01$), and reached control level at 48 h (100%± 8%). In the presence of 500 ml/L CD serum, the level of eNOS was close to normal until 8 h, and decreased after 12 h (41% ± 12%, $P < 0.01$), reaching the lowest level at 20-24 h (18% ± 16% and 23% ± 15%, $P < 0.01$, respectively), and then slowly increased rising close to normal at 48 h (65% ± 8%, Figure 1A, B).

Localization of eNOS in HUVEC by immunofluorescence revealed that the enzyme mostly accumulated in the nuclear area of the cytoplasm and was also detected at the periphery of the cells (Figure 2). Normal human serum had no effect on the distribution and intensity of eNOS immunoreactivity in HUVEC. Prolonged treatment (12 h) with UC serum extended eNOS immunoreactive area and simultaneously enhanced slightly the intensity of eNOS immunofluorescence in HUVEC. In contrast, in the presence of CD serum (after more than 4 h), eNOS immunoreactivity seemed to contract toward the centre of the cells and its immunofluorescence intensity decreased (Figure 2). At the end of the experimental period (48 h treatment), no visible difference could be detected in eNOS immunofluorescence of HUVEC in the presence of normal, UC or CD sera.

iNOS

Western blots in HUVEC lysates detected faint iNOS immunoreactive bands at around 130 kU, corresponding to the molecular mass of human iNOS (Figure 3A). HUVEC was cultured in the presence of 500 mL/L normal human serum, the relatively low level of iNOS expression did not change significantly during the 48 h incubation period. In the presence of 500 mL/L UC serum, iNOS level increased at 4 h (relative optical density value was 225% ± 20%, $P < 0.01$), peaked at 8 h (300% ± 21%, $P < 0.01$), and then declined to about normal level at 48 h (117% ± 15%). In the presence of 500 mL/L CD serum

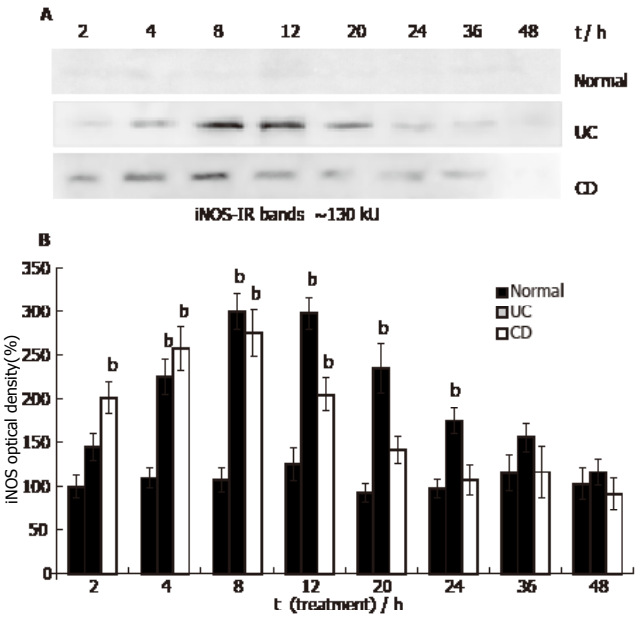

Figure 3 iNOS content in HUVEC (Western blot. Mean ± SE, n=3; ^bP<0.01 *vs* normal).

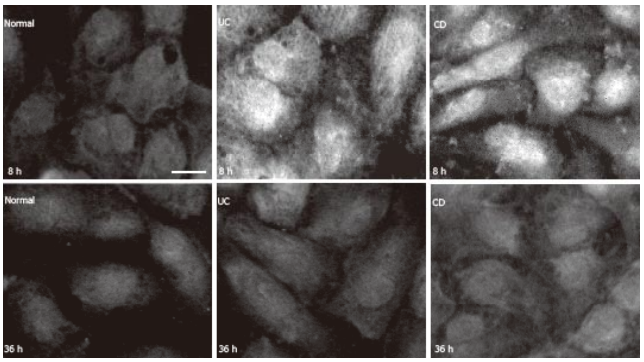

Figure 4 iNOS in HUVEC (immunoreaction was visualized by FITC. bar: 10 μm).

the iNOS level in HUVEC increased after 2 h (201% ± 19%, $P < 0.01$) and peaked at 8 h (275% ± 27%, $P < 0.01$). From 12 h iNOS level declined and was not distinguishable from the control during the 24-48 h period (at 48 h: 92% ± 18%, Figure 3A, B).

In the presence of 500 mL/L normal serum, HUVEC exhibited faint iNOS immunofluorescence (Figure 4). Expression of iNOS in HUVEC by immunofluorescence could obviously be detected in the presence of IBD sera during the 4-12 h period. Figure 4 shows that iNOS was dispersed in the cytoplasm, accumulated in certain areas showing a high intensity of fluorescent signal. Both UC and CD sera significantly increased iNOS immunofluorescence at the 4-12 h period. Before and after this time interval, iNOS could not be detected unequivocally in HUVEC by immunofluorescence (Figure 4).

HUVEC proliferation activity

Ten to fifteen percent of HUVEC was observed to be in the state of proliferation in the cultures with 20%-30% confluency at the beginning of the experiments as revealed by Ki-67 immunostaining (Figure 5). During the 2 to 48 h period no difference was detected in the ratio of Ki-67-IR/toluidin blue stained cell nuclei in cultures in

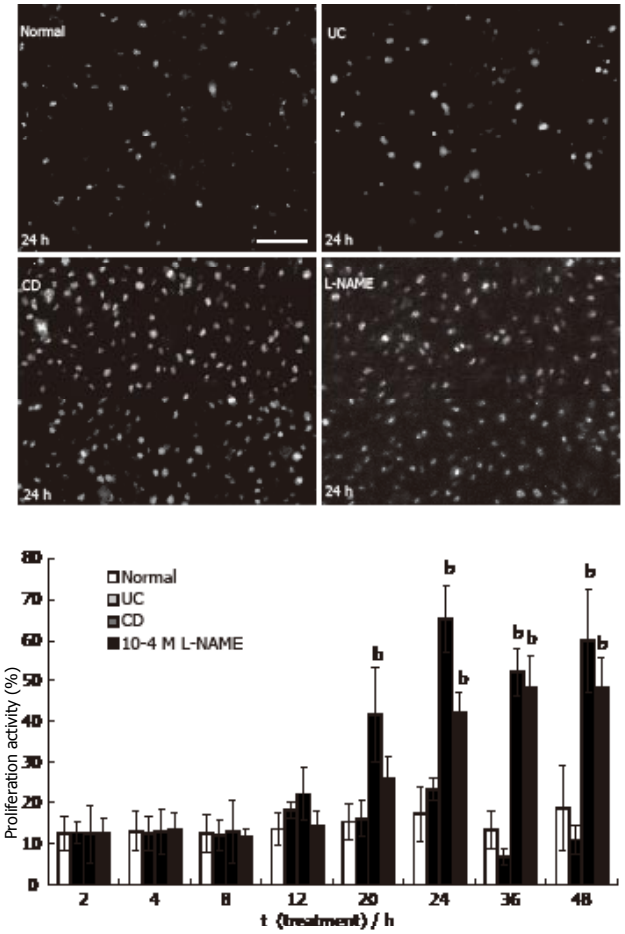

Figure 5 Proliferation activity of HUVEC (Ki-67 immunoreactivity was visualized by FITC. bar: 50 μm. mean ± SE, n = 3; ^bP<0.01 *vs* normal).

the presence of 500 mL/L normal (at 48 h: 19% ± 10%) or 500 mL/L UC sera (at 48 h: 10% ± 3%). In the presence of 500 mL/L CD serum a significant increase in the number of proliferating cells was apparent at 20 h (41% ± 12%, $P < 0.01$). About 50%-65% of HUVEC nuclei exhibited Ki-67 immunofluorescence in the presence of CD serum for 24-48 h (Figure 5). The presence of 10^{-4} mol/L L-NAME in the medium containing 500 ml/L normal serum, increased the proliferation activity significantly from 24 h (42% ± 5%, $P < 0.01$) to 48 h (48% ± 7%, $P < 0.01$).

HUVEC viability

By the annexin-V-biotin method, early apoptotic cells revealed fluorescent signals only on the cell membrane (Figure 6A), whereas necrotic cells showed propidium iodide binding to DNA (nuclear fluorescent signal, Figure 6B). Both signals could be detected in late apoptotic cells (Figure 6C). During the experimental period (2-48 h), the number of apoptotic cells was low (1%-3%) in the presence of 500 mL/L normal serum, but a continuous increase of apoptotic cell numbers was observed in the presence of 500 mL/L CD serum (2%-5%, with a maximum level at 36 h), which proved not to be significant (Figure 7A). In the presence of UC serum the apoptotic index did not differ from that of the control. Incubation of HUVEC with normal or CD sera slightly increased the number of necrotic cells during the examination period (2-48

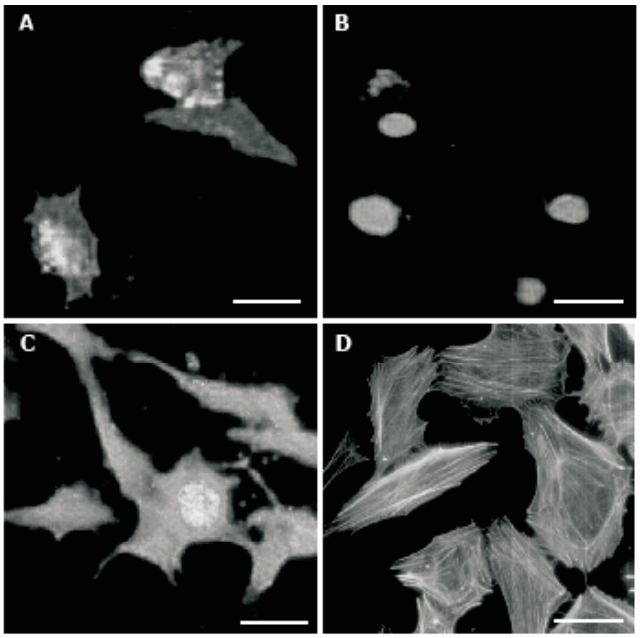

Figure 6 HUVEC viability. **A**: Early apoptotic cells; **B**: necrotic cells; **C**: Late apoptotic cell; **D**: Phalloidin conjugated with TRITC labeled actin filaments. bars: 10 μm.

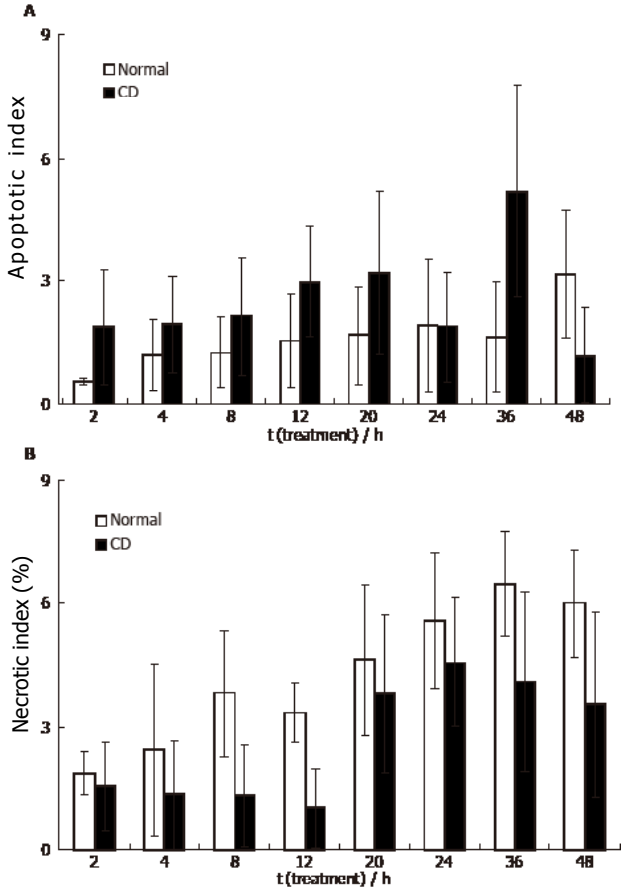

Figure 7 HUVEC 48 h incubation with CD sera (mean ± SE, *n* = 3).

h), but no significant changes were observed between the necrotic indices (Figure 7B). Actin filaments visualized by phalloidin-FITC seemed to be intact in cells independent of the media used and the time of incubation (Figure 6D).

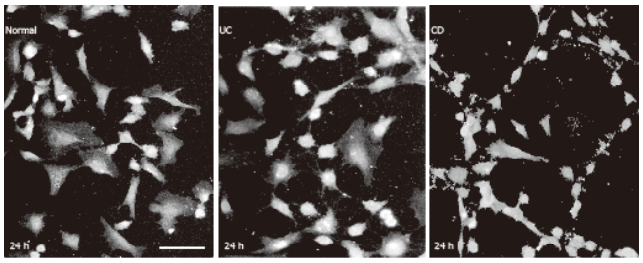

Figure 8 HUVEC visualized by eNOS immunofluorescence in culture. bar: 50 μm.

Shape and structure of HUVEC

HUVEC cultures of 20%-30% confluency were used also to investigate the effect of IBD sera on the distribution and shape of the cells. Cells visualized by eNOS immunofluorescence were partly found to be accumulated in islands exhibiting polygonal shape and attached to each other. In addition, single cells exhibiting long processes were also present. In the presence of 500 ml/L normal or 500 ml/L UC sera cells did not change in distribution and shape over the 48 h experimental period. However, in the presence of 500 mL/L CD serum, after 20 h cells were distributed in a two dimensional reticular network, which changed the shape of the culture reminiscent of the appearance of early vascular vessels in tissues (Figure 8).

DISCUSSION

In this study, we investigated the effect of sera from patients with active UC or CD on the expression and localization of eNOS and iNOS in HUVEC cultures. Induction of both iNOS and eNOS was observed in the presence of UC serum. In contrast, upon addition of CD serum to HUVEC media a transient increase in the expression of iNOS was observed in parallel with a marked decrease in the expression of eNOS. The number of apoptotic and necrotic cells was not altered significantly, and the structural integrity of the cytoskeleton (revealed by staining of the actin filaments) remained intact during the exposure of the cells to either CD or UC serum.

It has been shown previously that NO might enhance or reduce the cascade reaction of chronic inflammation in humans and animal models[12-14]. We found opposite changes in the expression of eNOS in HUVEC exposed to UC and to CD sera. The generation of eNOS is modified by elevated levels of cytokines (Th1 in CD) and the cytokine-induced expression and presentation of endothelial cell adhesion molecules[15]. UC and CD sera are known to contain a distinct cytokine and pro-inflammatory molecule profile. It was reported that TNF-α reduced eNOS protein expression in a time dependent manner in human endothelial cells[16, 17]. Moreover, TNF-α downregulated eNOS mRNA by decreasing its stability[18]. In our experiment, the low eNOS level in the presence of CD serum, could be due to TNF-α, which is a key cytokine in Th1 mediated inflammation in CD. Inflammation could result in functional and antigenic changes of the endothelium, including eNOS down-regulation and consequently increased expression of leukocyte adhesion molecules (ICAM, VCAM, E-selectin)[19-23]. At the same time, a lag in the fall of enzyme

activity was observed which may reflect a greater stability of eNOS protein compared to its mRNA, or may involve posttranslational modification(s) in the increase and stabilization of the enzyme activity[9]. Leukocyte-endothelial binding is exacerbated in an eNOS deficient state and injury during ischemia-reperfusion is more severe[24, 25].

A high amount of iNOS is generated during inflammation which can be harmful, worsening the course of disease in the animal colitis model[26]. In Crohn's colitis, human intestinal microvascular endothelial cells may lack the capacity to express iNOS, which makes them susceptible to leukocyte mediated injury[27, 28]. In UC, it has been shown that there is an elevation in iNOS level which is well correlated with disease severity[29]. Elevated iNOS activity was demonstrated in the mesenteric microvascular endothelial cells in active UC, indicating a close relationship between vascular activation and the pathogenesis of UC[30]. Stimulation of HUVEC with pro-inflammatory cytokines (Il-1β, TNF-α) or hormones (eg. relaxin, estrogen) induces the expression of iNOS and the NO produced increases the biosynthesis of prostaglandins and other mediators[31-33]. The maximal level of iNOS detected in primary cell cultures was not related to lipopolysaccharide contamination. It did not affect cell proliferation and was decreased when cells became confluent[34]. Dual modulation of NOS expression was detected upon estradiol treatment in a concentration dependent manner; estradiol in nanomolar range could induce eNOS, whereas in micromolar range it could evoke iNOS expression[35]. In previous studies, INF-γ in conjunction with TNF-α, or together with IL-1β, increased NOS activity, whereas these cytokines alone had different effects on NOS expression[9, 36, 37]. Up-regulation of iNOS by IBD sera may be an adaptive response of HUVEC to this culture environment.

The effect of UC and CD sera on eNOS expression could be of special interest and may be implicated in the distinct pathophysiological and morphological features of the two diseases. Inhibition of NO production *in vivo* results in reduced VEGF induced angiogenesis and vascular permeability[38]. Recent studies have provided evidence that basic fibroblast growth factor (bFGF), which is an important component of CD serum, induced angiogenesis by different mechanisms[39]. An impaired VEGF response was suggested in CD with down-regulation of the VEGF receptor (VEGF-Ets-1) and the angiogenic cascade supporting the vascular hypothesis of the disease[40-43]. The deficiency in eNOS expression and concomitant alteration of VEGF-induced angiogenesis and vascular permeability could be an important step in the pathomechanism of CD. However, bFGF, the most potent angiogenic molecule in CD has an NO independent activity[39, 44]. In addition, the pathomechanism of UC appears to be VEGF independent[39, 44]. The reciprocal reaction of cytokines and angiogenic molecules results in changes in the expression of eNOS and iNOS and causes an imbalance between the two isoforms which may play a role in the dysfunction of angiogenesis in IBD.

Angiogenesis is an important pathophysiological process in chronic inflammation and it includes proliferation, migration, and tube formation of endothelial cells. This process is tightly regulated by different angiogenic factors, and one of them is NO which has a pro-angiogenic effect[45, 46]. HUVEC, in the presence of CD serum, but not in the presence of UC serum, showed morphological changes reminiscent of vascular like structures, forming loop like rearrangement of the cells. It was shown recently that reactive oxygen species (ROS) have a putative pro-angiogenic activity *in vivo* possibly via iNOS up-regulation[47]. ROS also play a significant role in proliferation through regulation of eNOS activity, whereas antioxidants stimulate NO production and proliferation of HUVEC[48]. However, it was suggested also that antioxidants may enhance the activity of NOS[49, 50]. This dual effect could have a role in the pro-angiogenic and proliferation activity of HUVEC.

Recent data revealed that NO, by regulating mitochondrial respiration and cytochrome c release, triggers a defensive mechanism against cell death induced by pro-apoptotic stimuli[51-53]. In the present study, HUVEC incubated in the presence of CD serum exhibited a low expression level of eNOS, which could explain the slightly elevated apoptotic index. It also suggests a defective anti-apoptotic mechanism. The TNF-α level is known to be high also in CD serum and this could alter cell viability by inducing apoptosis in an NO independent pathway[54]. In contrast, in control and UC sera treated cells, the higher eNOS level may result in protection against apoptosis.

Our present results correlate well with our previous immunohistochemical findings through biopsy studies, and suggest the presence of a cytokine composition, which is able to evoke changes in the eNOS/iNOS ratio in endothelial cells in the blood of IBD patients. Simultaneous down-regulation of eNOS and up-regulation of iNOS, together with the increase of cell proliferation activity may reduce the anti-inflammatory capacity of endothelial cells. The latter may contribute to the initiation of pro-inflammatory cascade reactions leading to endothelial barrier dysfunction and causing structural changes in the microvessels in Crohn's mucosa.

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RAPID COMMUNICATION

Role of bile acids, prostaglandins and COX inhibitors in chronic esophagitis in a mouse model

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Abstract

AIM: To develop a new experimental model of esophagitis that serves a complementary tool to clinical investigation in an insight into the mechanism of the damage to the esophagus mucosa by aggressive factors, and role of COX inhibitors in this process.

METHODS: The study was conducted in 56 male mice. Animals were divided into seven groups: (1) perfused with HCl, (2) perfused with HCl and physiologic concentration of pepsin (HCl/P), (3) perfused with similar HCl/P solution enriched with conjugated bile acids (glycho- and tauro-sodium salts) designated esophageal infusion catheter under the general anesthesia, (4) perfused as in group 2 treated with indometacin, (5) perfused as in group 2 treated with NS-398, (6) perfused as in group 3 treated with indometacin, and (7) perfused as in group 3 treated with NS-398. The esophagus was divided into 3 parts: upper, middle and lower. The PGE2 concentration was measured in all parts of esophagus using RIA method. Esophagus of sacrificed animals was macroscopically evaluated using a low power dissecting microscope (20×). Specimens, representing the most frequently seen changes were fixed, stained with H&E and assessed microscopically using the damage score, and inflammatory score.

RESULTS: The macroscopic changes were significantly severer in HCl/P than those in HCl animals (77%) and in HCl/P/BA group (43%). In HCl/P NS-398 group we noticed significantly less changes than those in not treated group (42%) and in analogical group treated with indomethacine (45%). In HCl/P/BA INDO group we observed significantly severer changes than that in not treated group (52%). We noticed less changes in HCl/P NS-398 than that in group with indomethacine (46%). In HCl/P/BA NS-398 group we had less changes than that in indometacin group (34%). The microscopic changes observed in HCl/P/BA INDO group were severer than that in not treated group (48%). Esophagitis index in HCl group was significantly lower than in HCl/P and also HCl/P/BA group (32% and 33 %). In HCl/P/BA/INDO group the esophagitis surface was larger than that in not treated group (33%). In HCL/P group the surface of esophagus with ulceration was significantly larger (10-fold) than that in HCl/P/BA group. The PGE2 concentration was significantly higher in HCl/P group than in HCl/P/BA group. The PGE2 concentration in lower part of esophagus was also significantly higher in middle than those in HCl and HCl/P/BA groups. In upper part of esophagus the PGE2 concentration was significantly higher in HCl/P/BA group than that in group treated with indomethacine (46%). We also observed higher PGE2 concentration in middle part of esophagus in HCl/P/BA group than those in group treated with indomethacine and in group treated with indometacin and NS-398 (by 52% and 43% respectively).

CONCLUSION: Pepsin is the pivotal factor in the development of chronic esophageal injury. Bile acids diminish chronic esophageal injury induced by HCl/P, indicating its potential negative impact on pepsin proteolytic potential, pivotal for mucosal injury in low pH. The role of selective COX inhibitors is still unclear, and needs more investigations. This novel chronic experimental esophagitis is an excellent model for further study on the role of cytokines in genetically modified animals.

Key words: Bile acids; ProstaglIndins; Oesophagitis mouse model

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INTRODUCTION

Primary gastroesophageal reflux disease (GERD) is an ac-

id-related disease in majority of patients. However, there is evidence that in some patients with GERD reflux of duodenal contents into the stomach and esophagus may be involved in the disease[1, 2].

Chronic GERD may induced Barrett`s metaplasia[3]. This clinical situation has increased risk for the development of esophageal adenocarcinoma and is considerate to be a premalignant condition[4]. The complications in Barrett's esophagus was accompanied with presents of duodenal juice in gastroesophageal refluxate (GER)[3]. In the patients with esophagitis, Barrett`s esophagus strictures compared to patients with minimal injury the concentration of bile acids in refluxate was significantly higher [5]. The concentration of bile was significantly higher in patients with early adenocarcinoma arising in Barrett`s esophagus, compared to GERD patients, esophagitis group and asymptomatic volunteers[6]. Clinical trials have begun in order to assess the efficacy of selective COX-2 inhibitors to prevent the progression of Barrett's esophagus to adenocarcinoma. Bile salts and acid are likely to early induce COX-2 in this sequence, although other factors, such as proinflammatory cytokines, inducible nitric oxide synthase and growth factors such as TGF-beta, are potential COX-2 inducers in the esophagus[7]. In animal studies it has been shown that reflux of gastric contents with addition of duodenal juice into the esophagus may lead to esophageal adenocarcinoma[2]. The carcinogenetic effect of duodenal contents on gastric mucosa was clearly demonstrated[8].

Bile acid may induce mucosal injury by two mechanisms. The detergent properties of bile salts may destabilize membranes and increase permeability, disrupt cellular homeostasis and potentially result in cell death[9]. Bile acids may also create cytotoxic effect through cellular absorption of bile salts, witch is dependent upon the salt`s ionization[10].

COX-1 activity is constitutive in the rabbit esophageal mucosa, but both COX-2 and COX-1 activity are increased under the impact of acidified pepsin. Treatment with the COX-2 inhibitor DFU is associated with improvement of mucosal damage, which may have therapeutic implications[11].

PGE2 plays the important role in development of Barrett`s esophagus and adenocarcinoma of the esophagus. The concentration of PGE2 was significantlly higher in high grade displasia cells and also in adenocarcinoma cels of esophagus [12].

Our new experimental model of chronic esophagitis seems to be very useful tool to determinate the role of HCl/P/BA, major components of duodenogastroesophageal reflux, and the role of COX inhibitors on pathological changes in mucosa of the esophagus during refluxate episodes.

MATERIALS AND METHODS

The study was conducted in 56 male mice (CD1 strain from Charles River) according to study protocol approved by Animal Research Committee at KUMC. Animals were divided into seven groups: (1) Animal perfused with HCl (100 mmol/l, pH1.1), (2) Animals perfused with HCl (100 mmol/l, pH1.1) and physiologic concentration of pepsin (0.5 mg/l of HCl) (HCl/P), (3) Animals perfused with similar HCl/P solution enriched with conjugated bile acids (glycho- and tauro-sodium salts) designated esophageal infusion catheter under the general anesthesia, (4) Animals perfused as in group 2 treated with indometacin (5 mg/kg b.w. s.c.), (5) Animals perfused as in group 2 treated with NS-398 (10 mg/kg b.w. p.o), (6) Animals perfused as in group 3 treated with indometacin (5 mg/kg b.w. s.c.), and (7) Animals perfused as in group 3 treated with NS-398 (10 mg/kg b.w. p.o). The total perfusion time per day for each mouse was 1.5 h. At the end of experimental procedure the animals were sacrificed under prolonged metoxyflurane anesthesia, esophagus was removed, opened and evaluated microscopically after stained with Alcian blue (0.1%, pH 5.8), using a low power dissecting microscope (20×) with stage micrometer for measurement of the area of macroscopic damage. The macroscopic changes was evaluated based on macroscopic score: 0 - no changes; 1 - erosion - max 3, size - 3-6 mm; 2 - erosion - 6 and up, size - 6-9 mm; 3 - ulcer without perforation with small hemorrhagic areas; 4 - ulcer with perforation and large hemorrhagic areas.

Specimens, representing the most frequently seen changes were fixed, stained with hematoxylin and eosin, and assessed microscopically using the damage score [15]: 1-normal esophagus, 2-submucosal edema or separation of epithelial layer, 3-focal areas of intramural hemorrhage or partial epithelial loss, 4 - large areas of hemorrhage or complete epithelial desquamation; and inflammatory score[16]: 0 - no infiltration, 1-very mild infiltration, 2-mild infiltration, 3 - moderate infiltration, 4 - marked infiltration.

The concentration of PGE2 was measured in 1/3 upper, 1/3 middle and 1/3 lower parts of esophagus using RIA kit (*Amersham, Arlington Heights, Illinois*).

Statistical analysis was performed with S-Stat (Jandel Sci. Co).

RESULTS

The macroscopic score was significantly higher in animals perfused with HCl/P than those in groups with HCl/P/BA and with HCl (3.69 ± 0.23 *vs* 2.58 ± 0.25 and 3.69 ±0.23 *vs* 2.08 ± 0.11, $P < 0.05$). The macroscopic score was significantly lower in group HCl/P/NS-398 than that in not treated group (2.13 ± 0.21 *vs* 3.69 ± 0.23, $P < 0.05$) and also lower than that in analogical group treated with indometacin. In HCl groups, the microscopic changes were less evident in groups with HCl and HCl/P/BA than that in HCl/P (2.63 ± 0.38 *vs* 3.90 ± 0.10 and 2.64 ± 0.27 *vs* 3.90 ± 0.10, $P < 0.05$, respectively). The microscopic score was the same in HCl group and in HCl/P/BA group. The microscopic changes were significantly severer in group HCl/P/BA/INDO than that in not treated group (3.90 ± 0.10 *vs* 2.64 ± 0.27, $P < 0.05$) and also than that in analogical group treated with NS-398 (3.90 ± 0.10 *vs* 2.58 ± 0.14, $P < 0.05$). We noticed significantly higher score of microscopic changes in group HCl/P/INDO than that in group treated with NS-398 (3.92 ± 0.07 *vs* 2.12 ± 0.13, $P < 0.05$). Inflammation of esophagus in HCl group was significantly lower than that in HCL/P group (2.63 ± 0.24 *vs* 3.90 ± 0.10, $P < 0.05$). The inflammation score in HCl/P/BA group was also lower than that in HCl/P (2.23 ± 0.26 *vs* 3.90 ± 0.10, $P < 0.05$). Inflammation was less evident in HCl/P/NS-398 than those in not treated group and in group treated with indometacin (2.21 ± 0.11 *vs* 3.90 ± 0.10 and

Table 1 Macroscopic and microscopic changes in mice esophageal mucosa (mean ± SE)

Model	Grades of acroscopic changes	Grades of microscopic changes	Grades of inflammation	Surface of esophagitis (% of all esophagus)	Ulcers of esophagus (mm^2)
HCl	2.08 ± 0.11	2.63 ± 0.38	2.63 ± 0.24	15.50 ± 2.02	0 ± 0.0
HCl/P	3.69 ± 0.23[a]	3.90 ± 0.10[a]	3.90 ± 0.10[a]	23.00 ± 2.31[a]	7.09 ± 2.17[a]
HCl/P/BA	2.58 ± 0.25[c]	2.64 ± 0.27[c]	2.23 ± 0.26[c]	23.46 ± 3.85[a]	0.71 ± 0.49[ac]
HCl/P/INDO	3.90 ± 0.08	3.92 ± 0.07	3.86 ± 0.11	25.12 ± 2.14	8.11 ± 2.31
HCl/P/NS-398	2.13 ± 0.21[cg]	2.12 ± 0.13[g]	2.21 ± 0.11[cg]	19.12 ± 1.34	5.12 ± 2.13
HCl/P/BA/INDO	3.92 ± 0.06[e]	3.90 ± 0.10[e]	3.93 ± 0.03[e]	31.17 ± 2.45[e]	1.35 ± 0.11[e]
HCl/P/BA/NS-398	2.32 ± 0.11[g]	2.58 ± 0.14[g]	2.42 ± 0.13[g]	21.23 ± 1.21[g]	0.98 ± 0.27

[a]P<0.05 *vs* HCl; [e]P<0.05 *vs* HCl/P/BA; [g]P<0.05 INDO; [c]P<0.05 *vs* HCl/P.

2.21 ± 0.11 *vs* 3.86 ± 0.11 respectively, $P < 0.05$). Inflammation score was higher in HCl/P/BA/INDO than those in not treated group and in group treated with NS-398 (3.93 ± 0.03 *vs* 2.23 ± 0.26 and 3.93 ± 0.03 *vs* 2.42 ± 0.13 respectively, $P < 0.05$). Esophagitis index in HCl group was significantly lower than those in HCl/P and HCl/P/BA groups (15.50 ± 2.02 *vs* 23.00 ± 2.31 and 15.50 ± 2.02 *vs* 23.46 ± 3.85 % of all esophagus surface, $P < 0.05$). Surface area of esophagitis was significantly larger in HCl/P/BA/INDO group than that in not treated one (31.17 ± 2.45 *vs* 23.46 ± 3.85, $P < 0.05$) and also larger than that in group treated with NS-398 (31.17 ± 2.45 *vs* 21.23 ± 1.21, $P < 0.05$). In the HCl group of animals we did not observed any ulceration of the esophagus. In HCL/P group the surface of esophagus with ulceration was significantly larger than that in HCl/P/BA group (7.09 ± 2.17 *vs* 0.71 ± 0.49 mm^2, $P < 0.05$). The surface of ulceration in esophagus was significantly larger in HCl/P/BA/INDO group than that in not treated group (1.35 ± 0.11 *vs* 0.71 ± 0.49, $P < 0.05$). All data are showed in Table 1.

In the HCl group the concentration of PGE2 in middle part of esophagus was significantly higher than in lower part (1 027 ± 166 pg/mg of protein *vs* 378 ±69 pg/mg of protein, $P < 0.05$). We also observed the higher concentration of PGE2 in the middle part of esophagus than that in lower one in animals from HCL/P/BA groups (1 264 ± 134 pg/mg of protein *vs* 332 ± 59 pg/mg of protein, $P < 0.05$). In the HCL/P/BA group the concentration of PGE2 was significantly higher in the middle part of esophagus than that observed in the HCl/P (1 264 ± 134 pg/mg of protein *vs* 766 ± 95 pg/mg of protein, $P < 0.05$). The concentration of PGE2 in upper part of esophagus in HCl/P/BA/INDO group was significantly lower than that in not treated group (553 ± 50 *vs* 807 ± 111 pg/mg of protein, $P < 0.05$). We noticed lower PGE2 concentration in middle part of esophagus in HCl/P/BA treated with indometacin and NS-398 than that in not treated analogical group (614 ± 64 *vs* 1264 ± 134 and 733 ± 67 *vs* 1 264 ± 134 pg/mg of protein respectively, $P < 0.05$). All data are shown in Table 2.

Table 2 Concentration of pge2 in mouse esophagus

Model	1/3 upper part	1/3 middle part	1/3 lower part
HCl	801 ± 103	1027 ± 166[a]	378 ± 69[a]
HCl/P	674 ± 107	766 ± 95[c]	405 ± 39
HCl/P/BA	807 ± 111[e]	1264 ± 134[aceg]	332 ± 59[a]
HCl/P/INDO	500 ± 59	569 ± 60	388 ± 35
HCl/P/NS-398	576 ± 34	663 ± 59	324 ± 32
HCl/P/BA/INDO	553 ± 50	614 ± 64	324 ± 26
HCl/P/BA/NS-398	607± 50	733 ± 67	368 ± 43

Mean ± SE pg/mg of protein; [a]P<0.05 middle *vs* lower; [e] P<0.05 *vs* INDO; [g]P<0.05 *vs* NS-398; [c]P<0.05 HCl/P *vs* HCl/P/BA.

DISCUSSION

In our current study we demonstrated the significantly increase of macroscopic damage score in esophageal mucosa in animals perfused with HCl/P when compared with HCl and HCl/P/BA group. In addition in group perfused with HCl/P the microscopic changes were significantly remarkable compared to that in HCl and HCl/P/BA perfused animals. Inflammation of esophagus in HCl/P group was evidently severer than that in HCl only perfused animals. Inflammation of the esophageal mucosa in group with perfusion mimicking duodenogastroesophageal reflux was significantly higher than in HCl and HCl/P/BA perfused groups. The total surface of esophagitis in HCl/P perfused animals was significantly larger than that in HCl perfused group of animals. We observed wider surface of esophagitis in group perfused with HCl/P/BA – mimicking duodenogastroesophageal reflux, than in HCl/P perfused animals but the differences was not significant. However, the surface of esophagus with esophagitis in group mimicking the duodenogastroesophageal reflux was significantly larger than that observed in HCl perfused group. In group of mice with perfusion of esophagus with HCl we did not find any ulceration. We demonstrated in group with HCl/P significantly larger surface of esophagus with ulcer (10-fold) than in HCl/P/BA group. In our study we also found the significant decrease of macroscopic damage in esophageal mucosa in group of animals perfused with HCl/P and treated with NS-398. Macroscopic score in this group was lower than that in analogical group treated

with indomethacine. We also observed this phenomenon in microscopic and inflammatory scores. We noticed that animals perfused with HCl/P/BA and treated indomethacine had higher values of all macro- and microscopical scores than in not treated group. In addition, HCl/P/BA group of mice treated with NS-398 showed lower values of all macro-and microscopical scores than that observed in animals treated with indomethacine.

It was recently documented that in patients with reflux esophagitis the concentration of bile acids in refluxate is significantly higher than that in asymptomatic volunteers[15,16].

Chronic GERD may induced Barrett`s metaplasia[3]. This clinical situation has increased risk for the development of esophageal adenocarcinoma and is considered to be a premalignant condition[4]. The concentration of bile was significantly higher in patients with early adenocarcinoma arising in Barrett`s esophagus, compared to GERD patients, esophagitis group and asymptomatic volunteers[6].

In our previous clinical study we demonstrated that perfusion with acid, pepsin and bile acids, mimicking the duodenogastroesophageal reflux episodes increased the esophageal protective components secretion in asymptomatic volunteers, and less evidently in GERD patients.

There are some surgical experimental model of esophagitis, Barrett`s esophagus and also adenocarcinoma of esophagus[17,18]. In animal studies it has been shown that reflux of gastric contents with addition of duodenal juice into the esophagus may lead to esophageal adenocarcinoma[19]. The carcinogenetic effect of duodenal contents on gastric mucosa was clearly demonstrated [8]. The higher concentration of PGE2 in esophagus may be connected with deeper impact of bile acids on the esophagus wall, and induction of COX-2 in the esophagus muscle cells [5]. The role of COX-2 inhibitors in that phenomenon is still unclear and need more experiments.

Our new experimental model of esophagitis in mice mimicking the clinical scenario of gastroesophageal or duodenogastroesophageal reflux seems to be a useful tool to investigate some pathological problems in esophageal pathophysiology.

In conclusion, pepsin is the pivotal factor in the development of chronic esophageal injury. Bile acids diminish chronic esophageal injury induced by HCl/P, indicating its potential negative impact on pepsin proteolytic potential, pivotal for mucosal injury in low pH. The COX-2 inhibitors are much more active than not selective inhibitors in patients with esophageal mucosa injury, especially during duodenogastroesophageal reflux scenario. This novel chronic experimental esophagitis is an excellent model for further study on the role of cytokines in health and disease of the esophageal mucosa in genetically modified animals.

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RAPID COMMUNICATION

Expression of Ets-1 proto-oncoprotein in gastrointestinal stromal tumors, leiomyomas and schwannomas

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Abstract

AIM: Gastrointestinal stromal tumors (GISTs) are rare. GISTs differ from other mesenchymal tumors of the gastrointestinal tract (e.g. leiomyomas and schwannomas). The purpose of this study was to investigate the role of Ets-1 in the growth and differentiation of GISTs.

METHODS: Twenty-eight GISTs, nine leiomyomas and six schwannomas were examined by immunohistochemical staining method for Ets-1 in this study. Specimens were selected from surgical pathology archival tissues at Nagasaki University Hospital.

RESULTS: Ets-1 protein was expressed in the cytoplasm of cells in all of these tumors. Immunohistochemical staining revealed that 27 GISTs (96.4%), six leiomyomas (66.7%), and five schwannomas (83.3%) were positive for Ets-1. Ets-1 expression was statistically different between GISTs and leiomyomas ($P < 0.005$). However, there was no correlation between Ets-1 expression and clinical risk categories.

CONCLUSION: Ets-1 plays an important role in the growth and differentiation of GISTs, leiomyomas and schwannomas.

Key words: Ets-1; GISTs; Leiomyoma; Schwannoma; Immunohistochemistry; Risk category

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are rare mesenchymal tumors of the gastrointestinal tract that may occur from the oesophagus to the anus, including the omentum. These tumors have a wide clinical spectrum from benign, incidentally detected nodules to frankly malignant tumors[1]. Small GISTs are often detected incidentally during surgery for other conditions, during gastroscopy, or on routine X-ray[1,2]. GISTs may present with bleeding, perforation, pain, obstruction or a combination of these symptoms[3-6]. The mechanisms of tumorigenesis, progression and differentiation of GISTs are unknown. Traditionally, all primary mesnchymal spindle cell tumors of the gastrointestinal (GI) tract were uniformly classified as smooth muscle tumors (e.g., leiomyomas, cellular leiomyomas or leiomyosarcomas). Tumors with epithelioid cytologic features were designated leiomyoblastomas or epithelioid leiomyosarcomas[7]. Recently, Sircar *et al* postulated that GISTs origin-ate from Cajal cells in the gastrointestinal tract and differ from leiomyomas and schwannomas, which are of mesenchymal cell origin[8]. Cajal cells are thought to be gastrointestinal pacemaker cells that regulate intestinal motility[9]. GISTs are characterized by frequent expression of the bone marrow leukocytic progenitor cell antigen CD34[10] and the c-kit proto-oncogene[8,11,12]. Ets-1 was characterized originally as the v-ets retroviral gene, one of two oncogenes (v-myb and v-ets) of the avian leukemia retrovirus, E26[13]. The ets family of genes encodes transcription factors for mesodermal cell development during embryogenesis[14, 15].

Ets-1 plays a role in the regulation of physiological processes such as cell proliferation and differentiation[16]. Ets-1 also is expressed in astrocytes and vascular smooth muscle cells[17,18] but its expression has not been reported in Cajal cells. Increased Ets-1 expression was observed in several tumors in our previous studies[19-22]. We reported

Table 1 Ets-1 immunohistochemistry in intestinal stromal tumors. *n* (%)

	n	-	+	++
GISTs	28	1 (3.6)	4 (14.3)	23 (82.1)[a]
Leiomyomas	9	3 (33.3)	3 (33.3)	3 (33.3)
Schwannomas	6	1 (16.7)	1 (16.7)	4 (66.7)

[a]$P<0.005$ *vs* leiomyomas.

that Ets-1 is correlated with the progression of carcinoma cells of the stomach, pancreas, and thyroid and cells of astrocytic tumors[19-22]. These studies suggest that Ets-1 is involved in tumor growth and differentiation. However, there are no data concerning Ets-1 expression in GISTs, leiomyomas or schwannomas or the role of Ets-1 in the etiology of these tumors. The purpose of this study was to investigate the expression of Ets-1 in GISTs.

MATERIALS AND METHODS

Samples

A total of twenty-eight GISTs included 24 cases from the stomach and four from the small intestine. Nine leiomyomas included four from the oesophagus, two from the stomach and three from the large intestine, and five schwannomas included four from the stomach and one from the large intestine. Specimens were selected from surgical pathology archival tissues at Nagasaki University Hospital between 1999 and 2004. The GISTs were 0.8 - 12.0 cm in diameter, the leiomyomas were 0.1 - 4.5 cm, and the schwannomas were 0.6 - 5.0 cm. In this study, GISTs were defined as expressing both c-kit and CD34 surface antigens. GISTs were classified by risk categories, mitosis counts and tumor size[23]. The number of mitoses was determined by counting 50 high-power fields (HPF, x400) in Nikon (Tokyo, Japan) E400 microscope. Leiomyomas were defined as expressing α-smooth muscle actin (SMA) but not c-kit, CD34 and S100-protein. Schwannomas were defined as expressing S100-protein but not c-kit, CD34 and SMA. Tumor identification/classification was determined by two independent pathologists (T. Nakayama and I. Sekine).

Immunohistochemical staining

The subcellular localization of Ets-1 was determined in GISTs using a monoclonal antibody directed against the unique middle sequence of Ets-1 and this antibody was devoid of any cross-reaction with other proteins in the Ets family. Formalin-fixed and paraffin-embedded specimens were cut into 4 μm thick sections, deparaffinized and preincubated with normal bovine serum to prevent non-specific binding. The sections were incubated overnight at 4°C with the primary monoclonal antibody to human Ets-1 (1 g/L; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and then with a horseraddish peroxidase conjugated goat anti-mouse IgG antibody (0.4 g/L; Santa Cruz Biotechnology, Santa Cruz, CA). The reaction products were resolved using diaminobenzidine (DAB; DAKO Ltd., Glostrap, Denmark). Negative controls involved replacing the primary antibody with non-immunized mouse serum and human gastric cancer tissue served as the positive control[19]. Ets-1 expression was classified into three categories depending upon the percentage of cells stained and/or the intensity of staining: -, 0% to 10% tumor cells positive; +, 10% to 50% tumor cells positive; and ++, >50% tumor cells positive.

Table 2 Ets-1 immunohistochemistry and risk categories in GIST. *n* (%)

	n	-	+	++
Total	28	1 (3.6)	4 (14.3)	23 (82.1)
Risk categories				
High	4	0 (0.0)	0 (0.0)	4 (100.0)
Intermediate	5	0 (0.0)	0 (0.0)	5 (100.0)
Low	14	1 (7.1)	3 (21.4)	10 (71.4)
Very low	5	0 (0.0)	1 (20.0)	4 (80.0)
Mitosis counts (per 50 HPF)				
<2	14	1 (7.1)	3 (21.4)	10 (71.4)
2-5	6	0 (0.0)	1 (1.7)	5 (83.3)
6-10	3	0 (0.0)	0 (0.0)	3 (100.0)
10<	5	0 (0.0)	0 (0.0)	5 (100.0)
Tumour size (cm)				
<2	5	0 (0.0)	1 (20.0)	4 (80.0)
2-<5	18	1 (5.6)	3 (16.7)	14 (77.8)
5-<10	4	0 (0.0)	0 (0.0)	4 (100.0)
10<	1	0 (0.0)	0 (0.0)	1 (100.0)

Statistical analysis

The Stat View II program (Abacus Concepts, Inc., Berkeley, CA) was used for statistical analyses. Analyses comparing the degree of Ets-1 expression in GISTs, leiomyomas and schwannomas were performed using the Mann-Whitney's test.

RESULTS

The results of immunohistochemical staining for Ets-1 are summarized in the Table 1. Ets-1 expression was heterogeneous in GISTs and localized to the cytoplasm (Figure 1). Twenty-three of the GISTs (82.1%) were strongly positive, four (14.3%) were positive and one (3.6%) was negative for Ets-1. Similarly, four of the schwannomas (66.7%) were strongly positive, one (16.7%) was positive and one (16.7%) was negative. However, only three of the leiomyomas (33.3%) were strongly positive, three (33.3%) were positive and three (33.3%) were negative. There is a statistical difference in Ets-1 expression between the GISTs and the leiomyomas ($P<0.005$). Positively stained cells, i.e. those classified as ++ or +, were found in 96.4% of the GISTs, 66.7% of the leiomyomas and 83.3% of the schwannomas. Ets-1 was expressed in the cytoplasm of cells in all three tumors. However, normal stromal cells and smooth muscle cells showed faint or focal positivity of expression. GISTs were classified by risk categories, mitosis counts and tumor size in Table 2. In risk categories, all nine cases of high and intermediate groups were strongly expressed Ets-1 protein. In mitosis counts, all eight cases with over 6 mitoses per 50 HPF strongly expressed Ets-1. In tumor size, all five cases with over 5 cm strongly expressed Ets-1. However, there was no correlation between

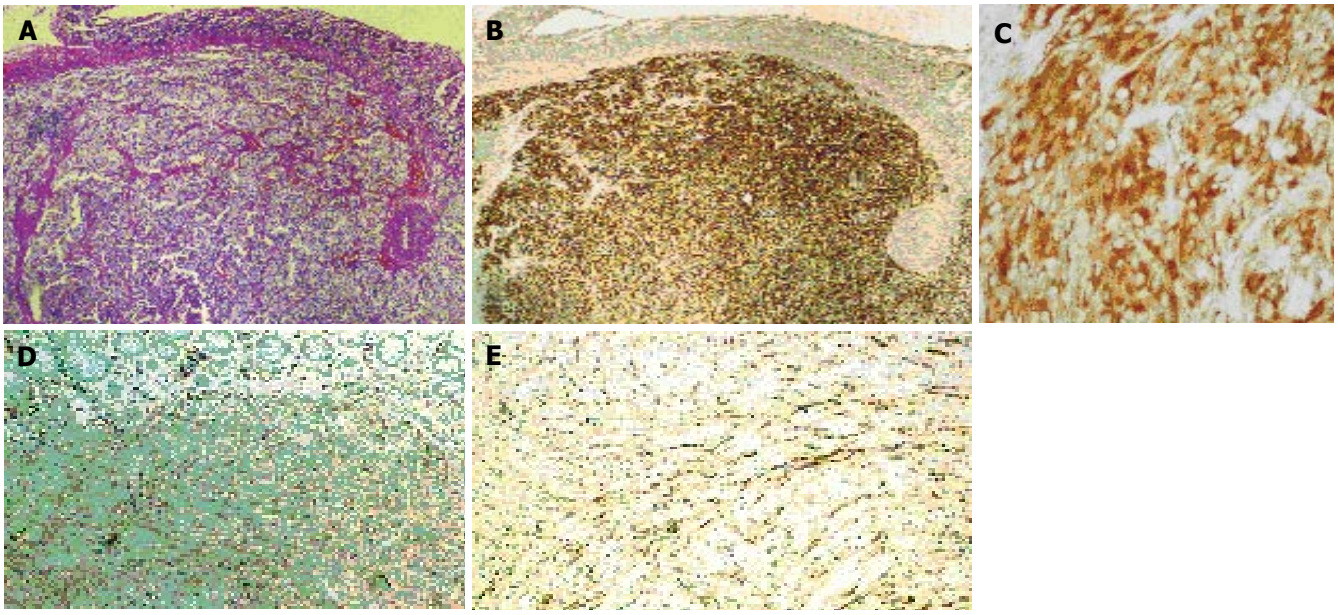

Figure 1 Ets-1 expression in GISTs (**A**-**C**), Leiomyomas (**D**) and Schwannomas (**E**). (magnification; **A**, **B**:x20, **C**: x 200, **D**, **E**: x100).

Ets-1 expression and each classification.

DISCUSSION

GISTs are known to originate from the Cajal cells of the neural crest[8] and schwannomas are thought to originate from the peripheral nerve sheath cell[24]. In this study, Ets-1 expression was higher in GISTs and schwannomas than in leiomyomas. Ets-1 expression has been reported in neural cells and astrocytes[22], but not yet in Cajal cells, cells that are all of neurons origin. Vascular smooth muscle cells also express Ets-1[18]. These findings suggest that Ets-1 may play a role in neural differentiation of intestinal stromal tumors. Previous studies have demonstrated Ets-1 expression in several tumors and normal stromal cells[19-22,25]. Furthermore, Ets-1 has been shown to play a role in the proliferation and/or differentiation of stromal cells[25]. We have shown already that Ets-1 may function as a growth factor in several tumors[19-22]. However, there have been no studies of Ets-1 expression in GISTs, leiomyomas and schwannomas, or of the potential role of Ets-1 in the growth of these tumors. Our results demonstrate substantial levels of Ets-1 expression in the cytoplasm of GIST, leiomyoma and schwannoma cells. These results suggest that Ets-1 may play a role in the growth and/or differentiation of intestinal tumors.

Ets-1 regulates the expression of many proteins, such as matrix metalloproteinases, urokinase type-plasminogen activator and parathyroid hormone-related peptide (PTHrP), which promote tumor growth and/or progression[26,27]. In our previous study, PTHrP and its receptor were found to be highly expressed in GISTs, leiomyomas and schwannomas[28]. Ets-1 may promote tumor growth and/or progression through regulating the expression of these proteins.

In recent studies, mutations affecting c-kit that cause constitutive tyrosine kinase activation have been shown to be important for the pathogenesis of GIST[29,30]. Joensuu *et al* reported a patient in whom STI-571 (imatinib, Gleevec), a tyrosine kinase inhibitor, was effective against a GIST[31]. And STI-571 has proven to be remarkably efficacious in heavily pretreated GISTs patients with advanced disease in phase II clinical trials[32]. The expression of the Ets family protein is upregulated by the activation of tyrosine kinase through the mitogen-activated protein kinase pathway[33]. Ets-1 expression may be upregulated by the c-kit/tyrosine kinase pathway.

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RAPID COMMUNICATION

Roles of adipocyte derived hormone adiponectin and resistin in insulin resistance of type 2 diabetes

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Abstract

AIM: To detect plasma levels of new adipocyte derived hormone adiponectin and resistin in type 2 diabetes patients and to explore their potential roles in insulin resistance in type 2 diabetes.

METHODS: According to the body mass index (BMI), 60 type 2 diabetes patients were divided into two groups, one group was non-obese diabetes patients with BMI < 25Kg/M^2 (30 cases) and the other group was obese diabetes patients with BMI > 25Kg/M^2 (30 cases). There were 28 healthy persons in the control group. ELISA technique was employed to determine the plasma adiponectin and resistin concentrations. The fasting blood glucose, insulin and blood lipid were detected respectively by electrocheminescence immunoassay and immunoturbidimetric assay. Insulin resistance index and insulin sensitive index were calculated by the homeostasis model assessment (HOMO).

RESULTS: The levels of plasma adiponectin were decreased significantly in diabetes group compared to that in control group (non-obese: 8.58 ± 0.86, obese: 6.22 ± 1.34 *vs* 10.53 ± 1.47 $P < 0.05$); moreover, adiponectin concentration in obese diabetes group was significantly decreased compared to that in non-obese diabetes group (6.22 ± 1.34 *vs* 8.58 ± 0.86, $P < 0.05$). The levels of plasma resistin were increased significantly in diabetes group compared to that in control group (obese: 18.64 ± 4.65, non-obese: 24.05 ± 9.07 *vs* 14.16 ± 5.25, $P < 0.05$, $P < 0.05$); furthermore, the levels of resistin in obese diabetes group were increased significantly compared to that in non-obese diabetes group ($P < 0.05$). Plasma adiponectin was correlated negatively with BMI, blood glucose, insulin resistance index and triglyceride (respectively, $r = -0.55$, $P < 0.01$; $r = -0.51$, $P < 0.05$; $r = -0.52$, $P < 0.05$; $r = -0.39$, $P < 0.05$), while it was positively correlated with insulin sensitive index (r=0.45, $P < 0.05$). Conversely, plasma resistin correlated positively with BMI, blood glucose, triglyceride and insulin resistance index (respectively, $r = 0.40$, $P < 0.05$; $r = 0.52$, $P < 0.05$; $r = 0.46$, $P < 0.01$; $r = 0.27$, $P < 0.05$), and negatively correlated with insulin sensitive index ($r = -0.32$, $P < 0.05$).

CONCLUSION: Plasma adiponectin and resistin are associated with the disorder of metabolism of glucose and lipid in diabetes. The relationship between these hormone and insulin sensitivity suggests that they may take part in the development of insulin resistance of type 2 diabetes.

Key words: Adiponectin; Resistin; Adipocyte; Type 2 diabetes

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INTRODUCTION

The prevalence of obesity is rapidly increasing with the changes of life style and structure of meals. The high prevalence of obesity has led to an increase in medical conditions that accompany obesity, especially type 2 diabetes (also known as non-insulin dependent diabetes mellitus, NIDDM), hypertension, cardiovascular disease (CVD), and certain cancers. Perhaps most importantly, obesity generally confers a significantly increased rate of mortality when compared with individuals of normal body weight[1].

Adipose tissue was once considered to be an inert storing depot for energy, in the form of triglyceride. However, in recent years, a line of evidence has

Table 1 Subject characteristics and plasma glucose, insulin, and lipid levels

Parameter	Control group	Non-obese diabetes group	Obese diabetes group
n	28	30	30
Age (yr)	47±11	48 ± 8	52 ± 9
Gender (M/F)	14/14	14/16	16/14
BMI (kg/m^2)	24.03±4.05	23.13±1.62	27.25±1.71^{ac}
FPG (mmol/L)	4.97±0.56	9.9±2.03^a	10.03±2.08^a
FINS (mU/L)	6.25±4.22	9.52±7.81	8.72±4.64
IR	0.50±0.35	0.98±0.59^a	1.22±0.53^a
ISI	-3.61±0.35	-4.09±0.59^a	-4.33±0.54^a
TCH (mmol/L)	4.79±1.07	5.79±1.42^a	5.61±1.79^a
TG (mmol/L)	1.48±1.74	2.45±1.46^a	3.05±1.92^a
NEFA (mmol/L)	0.71±0.18	0.95±0.21^a	1.13±0.24^a

BMI: body mass index; FPG: fasting plasma glucose; FINS: fasting insulin; IR: insulin resistance; ISI: insulin sensitive index; TCH: total cholesterol; TG: plasma triglyceride; NEFA: non-esterified fatty acids. $^aP<0.05$ *vs* control group; $^cP<0.05$ *vs* non-obese diabetes group.

demonstrated a much more complex function of adipose tissue, as an endocrine organ that releases hormones into the blood stream to take part in their potential implication in insulin resistance, obesity and diabetes[2]. The recent boom of interest in adipocyte derived factors has resulted in identification of a large group of adipocyte specific proteins, such as adiponectin, acylation stimulating protein, resistin, leptin, etc[3]. These adipocyte derived hormones are presently subject to intensive research concerning their involvement in the regulation of adipose tissue physiology, and in particular, their potential implication in insulin resistance, obesity and diabetes[4].

Both adiponectin and resistin are new hormones secreted exclusively from adipose tissue. They have important biological activity on glucose and lipid metabolism; moreover, they can affect insulin resistance. The present study was carried out based on the hypothesis that plasma adiponectin and resistin would play some roles in type 2 diabetes.

MATERIALS AND METHODS

Subjects and fasting blood samples

A total of 88 subjects were recruited from outpatient clinics at the Tongji Medical Centre, TongJi Hospital, Wuhan, Hubei province, China. All participants gave informed consent and the study was approved by Tongji Hospital Ethics Committee. Type 2 diabetic patients ($n=60$) were recruited at the time of their initial screening at the Endocrinology Clinic. Non-diabetic control subjects ($n=28$) were normal healthy adults recruited at their yearly checkups. Diabetes was defined in conformity with 1999 World Health Organization. According to the obesity criteria established in 2000 of Asia area, the diabetes patients were divided into two groups: (1) Non-obese diabetes group: BMI <25 ($n=30$); (2)Obese diabetes group: BMI $\geqslant 25$ ($n=30$). The type 2 diabetic participants had not taken any medication (including antidiabetic medication or herbal preparations) before we collected the blood samples. Subjects in diabetic and control groups were confirmed to have no known disease (including cardiovascular disease, thyroid disease, hypertension or any other acute and chronic disease condition) or any current infectious condition.

Table 2 Plasma adiponectin and resistin levels in diabetes patients divided by body weight

	Control group	Non-obese diabetes group	Obese diabetes group
Adiponectin (mg/L)	10.53±1.47	8.58±0.86^a	6.22±1.34^{ac}
Resistin (mg/L)	14.16±5.25	18.64±4.65^a	24.05±9.07^{ac}

$^aP<0.05$ *vs* control group; $^cP<0.05$ *vs* non-obese diabetes group.

Blood samples were drawn after an overnight fast from an antecubital vein. Fasting plasma samples were used for measurement of all parameters. The samples were centrifuged, aliquoted and immediately frozen at -80℃ for analyses of lipoproteins and proteins

Analytical procedures

Plasma samples were analyzed for concentrations of adiponectin, resistin, insulin, glucose, non-esterified fatty acids (NEFA), triglycerides (TG), total cholesterol (TCH). Plasma triglycerides (TG) were measured by GPO-PAP method and total cholesterol was measured by COD-PAP method. The plasma NEFA concentration was determined by colorimetric enzymatic assay (WAKO Chemicals, Tokyo, Japan).

Plasma glucose was determined by glucose-oxidase method (AVE-852 half-auto biochemical analyzer). Plasma insulin was measured by electrocheminescence immunoassay (Elecsys 1010, Roche Instrument Center AG).

Plasma adiponectin was measured by ELISA (B-Bridge International, Phoenix, AZ, USA) and plasma resistin also measured by ELISA (BioVendor Laboraory Medicine, USA).

Calculation

Body mass index (BMI) was calculated as weight (kg) per height (m^2). Insulin resistance index (IRI) was calculated by homeostasis model assessment, HOMA-IR as (fasting insulin IU/L) × (fasting glucose mmol/L) / 22.5 as previously reported by Matthews[5]. Insulin sensitivity index (ISI) was calculated as 1/[(fasting insulin IU/L) × (fasting glucose mmol/L)].

Statistical analysis

All results are displayed as mean ± SE (standard error of mean) unless stated otherwise. ANOVA analyses were used to compare means among the groups and correlations were calculated using Pearson correlation coefficient or multiple regression analysis. $P<0.05$ was considered statistically significant for all analyses.

RESULTS

The age and gender had no significant differences between diabetes group and control group. Subject characteristics are presented in Table 1.

The results of adiponectin and resistin are shown in

Table 3 Correlation analysis of fasting plasma adiponectin and resistin with the other parameters

	Adiponectin		Resistin	
Parameter	*r*	*P* value	*r*	*P* value
BMI	-0.55	0	0.4	0
FPG	-0.51	0	0.52	0
FINS	0.15	0.16	0.1	0.39
IR	-0.52	0	0.46	0
ISI	0.45	0	-0.32	0
TCH	-0.15	0	0.07	0.53
TG	-0.39	0.02	0.27	0.01
NEFA	-0.45	0	0.29	0

BMI: body mass index; FPG: fasting plasma glucose; FINS: fasting insulin; IR: insulin resistance; ISI: insulin sensitive index; TCH: total cholesterol; TG: plasma triglyceride; NEFA: non-esterified fatty acids. $^{a}P<0.05$ *vs* control group; $^{c}P<0.05$ *vs* non-obese diabetes group.

Table 2. There was a significant decrease in adiponectin in diabetes group as compared to that in control group ($P<0.05$). Furthermore, adiponectin in obese diabetes group was lower than that in non-obese diabetes group ($P<0.05$), while plasma resistin levels of non-obese diabetes group and obese diabetes group were apparently increased as compared to that in control group ($P<0.05$). Moreover, resistin level of obese diabetes group was also apparently higher than that of non-obese diabetes group ($P<0.05$).

There were significant differences in blood lipid parameters, including TG, TCH and NEFA, as given in Table 1. Cholesterol and triglyceride in non-obese diabetes group and obese diabetes group were apparently higher than that in control group ($P<0.05$), but there was no significant difference between non-obese diabetes group and obese diabetes group. The level of fasting insulin was not significantly different among the three groups ($P>0.05$). Fasting plasma glucose in diabetes group was apparently higher than that in control group ($P<0.05$), and there was no significant difference between non-obese and obese diabetes groups.

Pearson correlation analysis was used to identify the factors that most closely related to the hormones adiponectin and resistin, as shown in Table 3. Plasma adiponectin correlated negatively with BMI, blood glucose, insulin resistance index and triglyceride (respectively, $r=-0.55$, $P<0.05$; $r=-0.51$, $P<0.05$; $r=-0.52$, $P<0.05$; $r=-0.39$, $P<0.05$); whereas it was positively correlated with insulin sensitive index ($r=0.45$, $P<0.05$). Plasma resistin was correlated positively with BMI ($r=0.39$, $P<0.05$), fasting plasma glucose ($r=0.37$, $P<0.05$) and triglyceride ($r=0.41$, $P<0.05$); and it was correlated positively with insulin resistance index ($r=0.49$, $P<0.05$), but negatively with insulin sensitive index ($r=-0.34$, $P<0.05$). There was no significant correlation between plasma resistin and insulin and cholesterol, but there was significant correlation between plasma adiponectin and cholesterol ($r=-0.15$, $P<0.05$).

DISCUSSION

Recent clinical and experimental data have radically modified the concept of adipose tissue as one solely devoted to energy storage and release. Adipose tissue is also an important endocrine organ. It can release hormones into the blood stream in response to specific extra cellular stimuli or changes in metabolic status. The factors secreted by adipose tissue are actively involved in energy homeostasis in that they signal to the brain, the pancreatic β-cells, the liver and skeletal muscle in order to adapt to changes in energy stores by modulating feeding, insulin secretion and insulin sensitivity [6]. Secreted factors may include leptin; steroids and glucocorticoids; peptide hormone precursors, e.g. angiotensinogen; complement factors, e.g. adipsin, acylation-stimulating protein (ASP) adiponectin; pro-inflammatory cytokines, e.g. tumor necrosis factor α (TNF-α), interleukin 6 (IL-6); resistin; etc. Most of these products probably act through paracrine or autocrine mechanisms[7].

Adiponectin, also known as adipose most abundant gene transcript 1 (apM1), adipoQ and adipocyte complement related protein of 30 Kdaltons (ACRP30) was originally identified as the product of a highly induced gene after 3T3-L1 differentiation. It is a relatively abundant plasma protein (around 0.01% of total plasma proteins) that is exclusively synthesized and secreted by white adipose tissue (WAT)[8]. Adiponectin has many metabolic actions involving peripheral tissue and the regulation of energy homeostasis, particularly energy expenditure. The new adipocykin can decrease plasma glucose, increase clearance of a glucose load and ameliorate insulin resistance in mouse models with normal (C57Bl/6J), reduced (ob/ob, db/db) or absent (lipoatrophic mice) adiponectin pathways[9]. Acute administration in mice reduces elevated postprandial NEFA resulting from ingestion of a high-fat test meal or lipid intravenous injection[10]. Daily administration of adiponectin in mice on a high-fat/ high-sucrose diet induces marked and sustainable weight loss without affecting food intake[11]. Adiponectin decreases muscle and liver TG content, increases gene expression related to muscle NEFA uptake and utilization, increases muscle NEFA oxidation, and enhances hepatic insulin-mediated suppression of glucose production[12]. Adiponectin knockout mice demonstrate insulin resistance after a glucose load or after a high-fat/ high-sucrose diet (2 wk), delayed NEFA clearance and decreased expression of NEFA transporters in muscle[13]. It is apparent that adiponectin is very important for the metabolism of lipid and glucose, as well as insulin sensitivity.

Adiponectin is the only WAT derived hormone whose levels are downregulated in obesity. Human, primate and mouse models of obesity and/or insulin resistance (Type II DM and ob/ob mice) have reduced circulating adiponectin concentrations and expression in WAT[14]. In a recent study in adolescents, plasma adiponectin was shown to correlate with insulin sensitivity but was inversely proportional to plasma triglyceride and intra-myocellular lipid, suggesting its functional link to insulin resistance[15]. During weight-reduction or prolonged negative energy balance, circulating adiponectin is elevated in human[16]. We found that plasma adiponectin was decreased significantly in diabetes group, especially in obese diabetes group. Moreover, plasma adiponectin correlated negatively with insulin resistance

index, triglyceride and correlated positively with insulin sensitive index. It is well known that adiponectin improves insulin sensitivity of the whole body, so we speculated the decreased adiponectin in type 2 diabetes may be one of the reasons to cause insulin resistance. The change of plasma adiponectin was correlated with the development of insulin resistance in diabetes. It is unclear why adiponectin levels were decreased in obese diabetes with more fat tissue which can produce adiponectin. Some researchers speculated that the suppression of adiponectin production in obesity and diabetes may be mediated by an autocrine negative feedback inhibition in WAT, for instance, lower expression of adiponectin mRNA was found in cultured human visceral WAT and microarray study demonstrated that adiponectin expression is suppressed with the development of obesity and diabetes in mice[17].

Resistin is another protein identified recently as a hormone secreted by adipocytes which leads to insulin resistance *in vivo* and *in vitro* and might therefore be an important link between obesity and diabetes[18]. 3T3-L1 cells can secrete abundant resistin into the medium and its mRNA is induced (reduced?) markedly during adipocyte differentiation of 3T3-L1 cells[19]. When these adipocytes were exposed to specific resistin antibody, the insulin-stimulated glucose uptake was increased significantly. Consistent with the results, treatment of 3T3-L1 adipocytes with purified resistin reduced insulin-stimulated glucose uptake[20]. These studies suggest that resistin functions as a signal to decrease insulin-stimulated glucose uptake. Administration of anti-resistin antibody also can improve blood glucose and insulin action in mice with diet-induced obesity[21]. Resistin gene and protein expression were increased in human abdominal adipose tissue. In this study we found that plasma resistin levels of non-obese diabetes group and obese diabetes group were apparently higher than that in control group. Furthermore, resistin levels in obese diabetes group were increased as compared to non-obese diabetes group. Meantime plasma resistin was correlated positively with insulin resistance index. Al-Harithy *et al*[22] had similar result in their study on type 2 diabetes. It was well known that resistin levels were increased in differentiation of adipocytes, obesity and people on high fat diet?, and it is increased in abdominal vs. thigh adipose tissue. There is a potential link between central obesity and type 2 diabetes and/or cardiovascular disease[23]. Functionally, resistin impairs glucose tolerance, insulin action, and increases hepatic glucose production, while a neutralizing antibody normalizes glucose levels and tissue glucose uptake[24]. It suggests that increased plasma resistin may be correlated with the development of insulin resistance in type 2 diabetes.

Type 2 diabetes mellitus is characterized by target-tissue resistance to insulin. It is strongly linked to obesity as over 80% of sufferers are obese[25]. While the molecular basis for this link has remained a mystery. Insulin resistance is the core pathogenic factor for diabetes. In addition, it is also strongly associated with obesity, hypertension and cardiovascular disease, etc. The new adipocytes derived hormones adiponectin and resistin may be an important link between increased fat mass and insulin resistance and disorder of metabolism of lipid and glucose in diabetes. Adipocytes have become the center for the study of the association of insulin resistance and type 2 diabetes[26]. Studies of the roles of adiponectin and resistin will shed new light on prevention and treatment of type 2 diabetes, and open a new field for the development of new drugs to improve insulin resistance.

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RAPID COMMUNICATION

Chronic hepatitis B serum promotes apoptotic damage in human renal tubular cells

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Abstract

AIM: To investigate the effect of the serum of patients with chronic hepatitis B (CHB) on apoptosis of renal tubular epithelial cells in vitro and to study the role of hepatitis B virus (HBV) and transforming growth factor-β1 (TGF-β1) in the pathogenesis of hepatitis B virus associated glomerulonephritis (HBV-GN).

METHODS: The levels of serum TGF-β_1 were measured by specific enzyme linked immunosorbent assay (ELISA) and HBV DNA was tested by polymerase chain reaction (PCR) in 44 patients with CHB ,and 20 healthy persons as the control. The normal human kidney proximal tubular cell (HK-2) was cultured together with the sera of healthy persons, CHB patients with HBV-DNA negative(20 cases) and HBV-DNA positive (24 cases) for up to 72 h. Apoptosis and Fas expression of the HK-2 were detected by flow cytometer.

RESULTS: The apoptosis rate and Fas expression of HK-2 cells were significantly higher in HBV DNA positive serum group 19.01±5.85% and 17.58±8.35%, HBV DNA negative serum group 8.12±2.80% and 6.96 ± 2.76% than those in control group 4.25±0.65% and 2.33 ± 1.09%, respectively ($P < 0.01$). The apoptosis rate and Fas expression of HK-2 in HBV DNA positive serum group was significantly higher than those in HBV DNA negative serum ($P < 0.01$). Apoptosis rate of HK-2 cells in HBV DNA positive serum group was positively correlated with the level of HBV-DNA ($r = 0.657$). The level of serum TGF-β_1 in CHB group was 163.05 ± 91.35 μg/L, significantly higher as compared with 81.40 ± 40.75 μg/L in the control group ($P < 0.01$).

CONCLUSION: The serum of patients with chronic hepatitis B promotes apoptotic damage in human renal tubular cells by triggering a pathway of Fas up-regulation. HBV and TGF-β_1 may play important roles in the mechanism of hepatitis B virus associated glomerulonephritis.

Key Words: Renal tubular epithelial cells; HBV; TGF-β_1; Apoptosis

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INTRODUCTION

Hepatitis B (HBV) viruses are well-recognized causes for chronic hepatitis, cirrhosis, and even for hepatocellular carcinoma. Apart from liver disease, these viral infections are known to be associated with a spectrum of extrahepatic manifestations [1]. Hepatitis B is prevalent in China and hepatitis B virus associated glomerulonephritis has been one of the common renal damages secondary to HBV infection in Chinese children [2]. The cause of HBV-GN is generally believed to be immune mediated [3]. Recent studies showing expression of HBV viral antigens in kidney tissue and HBV DNA distributed generally in the nucleus and cytoplasm of epithelial cells of renal tubules suggest direct viral-induced pathological alterations and chronic immunologic injury [4,5]. Apoptosis is an active mode of cell death that promotes cell loss during both acute and chronic renal damage [6,7,8]. Here we investigated cell apoptosis in human renal tubular epithelial cells induced by sera of patients with chronic HBV infection and explored its mechanism.

MATERLALS AND METHODS

Patients and serum samples

Forty-four patients with chronic HBV infection (mild degree) came from Department of Infectious Diseases of the Affiliated Hospital of Luzhou Medical College, China

during 2003-2004. All the patients conformed to the revised standard of the Sixth National Meeting of Infectious Disease and Parasitosis in 2000. There were 38 men and 6 women. The median age was 34 years (range: 17-62 years). According to the HBV DNA levels in serum assayed by polymerase chain reaction (Positive criteria: $>1\times10^6$ copies/L), the patients were divided into HBV DNA positive patients group (24 cases) and HBV DNA negative patients group (20 cases). Twenty healthy persons whose serum markers of HBV were negative were used as the control. The blood collected from these persons was centrifuged at 4℃ and the serum samples were stored at -80 ℃ until examination. The serum samples were in the water bath at 56 ℃ for 30 min to inactivate complement. The level of HBV DNA in the serum samples was estimated by polymerase chain reaction (Shanghai Fortune Medical Science Co. Ltd, China) and TGF-β1 protein concentration was determined by specific-ELISA (Jingmei Biotech Co. Ltd, China).

Cell culture

All experiments were performed using the human kidney proximal tubular epithelial cell line (HK-2)[9] (American Type Culture Collection, USA). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, at 37 ℃ in a humidified atmosphere containing 50 mL/L CO_2. Fresh growth medium was added to cells every three to four days until confluent.

Flow cytometry analysis

HK-2 was cultured in the presence of the serum (50 μL) of healthy persons, CHB patients with HBV-DNA negative and HBV-DNA positive for up to 72 h, respectively. The survival rate and apoptotic rate of HK-2 cells were determined by Annexin V and propidium iodide (PI)(Bio Vision, USA) staining. HK-2 cells were digested by 0.25% trypsin. Cells (5×10^5) were suspended in Binding Buffer. Annexin V (5 μL) and PI (5 μL) were then added and incubated with cells at room temperature for 5 min in the dark until detected by flow cytometry. HK-2 cells (5×10^5) suspended in binding buffer were stained at room temperature with CD95-FITC (20 μL) (Bio Vision, USA) for 20 min in the dark and evaluated by flow cytometry to determine Fas expression.

Statistical analysis

The data are expressed as means ± SD. Statistical analysis of data was performed using *t* test or *F* test, with a value of $P < 0.05$ considered to represent a significant difference.

RESULTS

Detection of HBV DNA

Serum samples of forty-four patients with chronic HBV infection were assayed by polymerase chain reaction. There are 24 cases with HBV DNA positive (9.36×10^6-1.57×10^{12} copies/L) and 20 cases with HBV DNA negative.

Detection of TGF-β1

The level of serum TGF-β1 in CHB group was 163.05 ± 91.35 μg/L, significantly higher as compared with 81.40 ± 40.75 μg/L in the control group (Table 1, $P<0.01$).

Table 1 Comparison between levels of TGF-β 1 in two groups

Group	Cases	TGF-β1 (μg/L)
Control	20	81.40 ± 40.75
CHB serum	44	163.05 ± 91.35[b]

[b]$P<0.01$ *vs* control.

Table 2 Apoptosis of HK-2 in all groups

Group	Cases	Apoptosis (%)
Control	20	4.25 ± 0.65
HBV DNA negative serum	20	8.12 ± 2.80[b]
HBV DNA positive serum	24	19.01 ± 5.85[bd]

[b]$P<0.01$ *vs* control; [d]$P<0.01$ *vs* HBV DNA negative serum.

Table 3 Expression of Fas of HK-2 cells in all groups

Group	Cases	Fas (%)
Control	20	2.33 ± 1.09
HBV DNA negative serum	20	6.96 ± 2.76[b]
HBV DNA positive serum	24	17.58 ± 8.35[bd]

[b]$P<0.01$ *vs* control; [d]$P<0.01$ *vs* HBV DNA negative serum.

Detection of apoptosis by flow cytometry

The apoptosis rate of HK-2 cells was significantly higher in HBV DNA positive serum group 19.01 ± 5.85% and HBV DNA negative serum group 8.12±2.80% than that in control group 4.25 ± 0.65%, respectively ($P < 0.01$) (Table 2, Figure 1). The apoptosis rate of HK-2 in HBV DNA positive serum group was significantly higher than that in HBV DNA negative serum group($P < 0.01$) (Table 2). Apoptosis rate of HK-2 cells in HBV DNA positive serum group was positively correlated with the level of HBV-DNA ($r = 0.657$) (Figure 2).

Detection of expression of Fas by flow cytometry

The expression of Fas of HK-2 cells was significantly higher in HBV DNA positive serum group 17.58 ± 8.35% and HBV DNA negative serum group 6.96 ± 2.76% than that in control group 2.33 ± 1.09%, respectively ($P<0.01$) (Table 3, Figure 3). The expression of Fas of HK-2 cells in HBV DNA positive serum group was significantly higher than that in HBV DNA negative serum ($P < 0.01$) (Table 3).

DISCUSSION

Apoptosis plays an essential role in the regulation of renal cell number in both healthy and diseased kidneys [10]. Efficient deletion by apoptosis of excessively damaged, or nonfunctioning renal cells and infiltrating inflammatory cells may be beneficial. However, the loss of resident renal cells by uncontrolled apoptosis is detrimental as it may in-

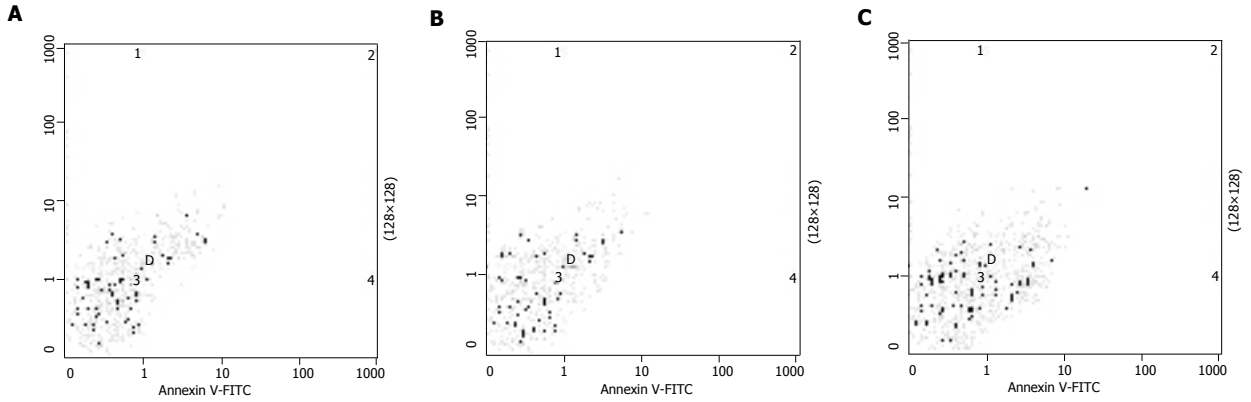

Figure 1 Flow cytometric analysis of HK-2 cells stained with Annexin V and PI. **A**: Treated with serum of healthy persons. **B**: Treated with HBV DNA negative serum. **C**: Treated with HBV DNA positive serum.

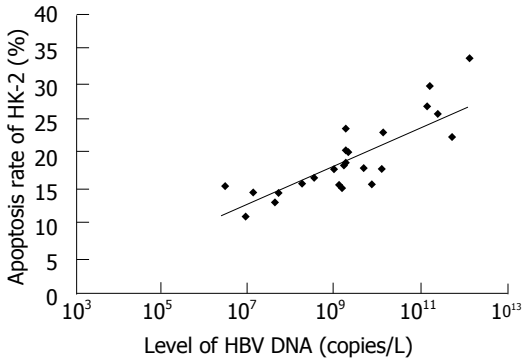

Figure 2 Relationship between apoptosis of HK-2 and levels of HBV DNA in HBV DNA positive serum.

duce a reduction of functional renal mass and lead to renal insufficiency [11-13]. Several external and internal signals can induce apoptosis, which is then effected via a number of pathways. These pathways are either the Fas/FasL pathway and downstream MAPK (mitogen-activated protein kinases) and JNK (c-Jun N-terminal kinase) signal transduction, or the RANK/RANK-L (receptor activator of NFkB) pathway via activation of the caspase cascade [14,15]. Finally, irreversible branch of these pathways is the release of cytochrome c from the mitochondria, leading to nuclear fragmentation [16,17]. Previous studies indicated that pathological factors had a toxic effect on HK-2 cells by triggering an apoptotic pathway involving caspase activation, Fas up-regulation, Fas ligand (FasL) expression, the increase of Bax, the decrease of Bcl-2 protein expression and JNK signaling transduction[18,19], thus potentially interacting with mechanisms of cell loss which have been already shown to be activated in chronic renal diseases. In this study, we showed that CHB serum induced a significant increase in apoptosis in HK-2 cells detected by flow cytometry. It suggested that CHB serum might play a crucial role in the apoptosis of HK-2 cells. Excessive apoptosis of renal proximal tubular cells might be associated with renal injury in patients with chronic HBV infection.

Hepatitis B virus associated glomerulonephritis is occurring at a high prevalence in most Asian endemic areas [20]. The detailed mechanism of renal tubular epithelial cells (RTC) injury in HBV-GN remained unclear. The cause of HBV-GN is generally believed to be immune mediated [3]. Recent studies suggest hepatitis B viral direct infection in RTC may be correlated with renal pathological alterations. HBV DNA and HBV antigen were distributed generally in epithelial cells of renal tubules and the positive results from HBV DNA in situ hybridization correlated well with HBV antigen assays[4,5,20]. The analyses implied that the more extensive the existence of HBV DNA in the nephron unit, the more severe the clinical manifestation. The duration of proteinuria in cases with HBV DNA in renal tubules was much longer than in those with no HBV DNA in renal tubules [5]. Our previous study has observed that the rate of early impairment of renal cells in HBV DNA positive patients with HBV chronic infection was higher than that in HBV DNA negative patients with HBV chronic infection[21]. In this study, we demonstrated that apoptosis of HK-2 cells stimulated by HBV DNA positive serum was significantly higher than that of HBV DNA negative serum, and the apoptosis rate of HK-2 cells was correlated with serum level of HBV-DNA positively. These data suggested that the level of HBV DNA in CHB serum may have a significant effect on the apoptosis of HK-2 cells, and supported the viewpoint that hepatitis B viral direct infection in RTC could lead to cellular apoptosis and might cause renal pathological alterations.

Epithelial cell loss characterized as tubular atrophy is considered to be a hallmark in the development of chronic renal interstitial fibrosis[22]. Large amounts of research demonstrated that the proximal tubular cell may contribute to the pathogenesis of renal interstitial fibrosis[23,24]. Although the mechanism of epithelial cell loss remains uncertain, it is conceivable to assume that tubular atrophy primarily results from apoptotic cell death under pathologic conditions[25,26]. Previous studies demonstrated that TGF-β1 markedly potentiated cell apoptosis initiated by accelerating the activation of pro-caspase-9 and -3[27]. We observed that the level of TGF-β1 significantly increased in CHB serum compared with healthy person serum. Apoptosis of HK-2 cells stimulated by HBV DNA negative serum was notablely higher than that of healthy person serum. We speculate that TGF-β1 may contribute to progressive renal dysfunction after severe tubular injury.

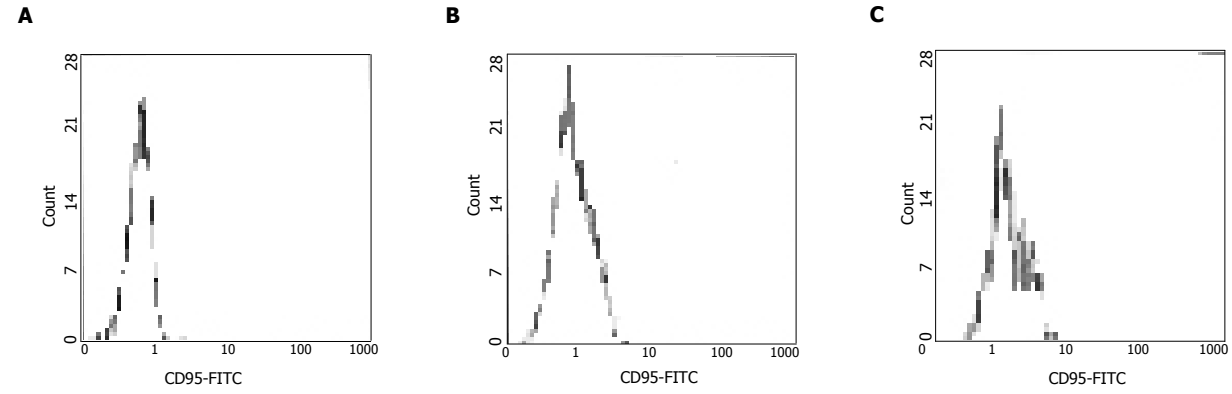

Figure 3 Flow cytometric analysis of HK-2 cells stained with CD95-FITC. **A**: Treated with serum of healthy person. **B**: Treated with HBV DNA negative serum. **C**: Treated with HBV DNA positive serum.

Fas (CD95, APO-1) is a ubiquitously expressed member of the tumor necrosis factor receptor superfamily, which mediates diverse cellular responses, including proliferation, inflammation, angiogenesis, and apoptosis[28]. Fas and FasL are exclusively expressed in RTC undergoing apoptosis. Fas and FasL expression were related to the apoptosis of RTC[29,30]. The Fas-mediated cell death of a tubular cell may be true cell suicide or fratricide in which the FasL is derived from the same or neighboring tubular cell [31]. Binding of FasL to Fas leads to receptor oligomerization and recruitment of the cytoplasmic adapter protein FADD (i.e., Fas-associated death domain protein)[8]. FADD binds caspase 8, which subsequently becomes autoactivated. The catalytically active caspase 8 then initiates the caspase cascade, which leads to the rapid demise of the cell [32]. Our study showed that Fas was clearly up-regulated in HK-2 cells stimulated by CHB serum. These results indicate that apoptosis of proximal tubule kidney cells effects by triggering a pathway of Fas up-regulation. The extracellular microenvironment of injured kidneys may sensitize parenchymal renal cells to Fas induced death, leading to cell loss in the course of acute or chronic renal damage.

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RAPID COMMUNICATION

Morphological observation of tumor infiltrating immunocytes in human rectal cancer

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Abstract

AIM: To investigate the morphological characterization of tumor infiltrating dendritic cells (TIDCs) and tumor infiltrating lymphocytes (TILs) in human rectal cancer.

METHODS: Light and electron microscopy as well as immunohistochemistry were used to observe the distributive and morphological changes of TIDCs and TILs.

RESULTS: TIDCs were mainly located in tumor-surrounding tissue. The number of TIDCs in the earlier stage was higher than that in the later stage ($P < 0.01$). TILs were mainly seen in adjacent tissue of cancers and tumor-surrounding tissue. There were more TILs in the earlier stage than that in the later stage ($P < 0.01$). Under electron microscope, TIDCs were irregular in shape and exhibited many dendritic protrusions. It isn't obvious that cancer cells perforated the basement membrane and TILs were arranged along the basement membrane in the earlier stage. In the later stage, it is explicit that cancer cells perforated the basement membrane and surrounded by TILs. There were contacts among TIDCs, TILs and tumor cell. One TIDCs contacted one or several TILs which clustered around TIDCs. Glycogen granules were seen between TIDCs and TILs.

CONCLUSION: The number of TIDCs and TILs is related with tumor progression There exist close relationships among TIDCs, TILs and tumor cell.

Key words: Rectal cancer; Tumor infiltrating dendritic cells; Tumor infiltrating lymphocytes

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INTRODUCTION

Antitumor effects of human body are mainly achieved by tumor infiltrating immunocytes, including TIDCs, TILs, natural killer(NK) cells and lymphokine-activated killer(LAK) cells. Because of their prominent role in antitumor effects, TIDCs and TILs have drawn more and more attention. DCs are the most powerful professional antigen-presenting cells (APCs) so far. Immunophenotype and function of DCs in biological therapy of tumor are the focus of immunological study [1,2]. Although studies on tumor immunotherapy involving injection of DCs cultured *in vitro* are reported [3-5], infiltrating DCs in tumor microenvironment have not been fully investigated and studies usually concentrate on the correlation between infiltrating DCs and tumor progression[6,7]. Morphological studies on the relation among TIDCs, TILs and tumor cells, are few. TILs are heterogenous lymphocytes, most of them are T lymphocytes. TILs are a new kind of antitumor effectors after LAK cells. In this study, the morphology of TIDCs and TILs in earlier and later stage of human rectal cancer was investigated, which may provide morphological evidence for antitumor effect of tumor infiltrating immunocytes.

MATERIALS AND METHODS

Patients

Eighteen rectal cancer patients surgically treated in earlier or later stage in the Second and Third Hospitals of Harbin Medical University were studied. According to their clinical manifestations and pathological features, the patients were divided into earlier stage group ($n = 8$) and later stage group ($n = 10$).

Methods

Fresh rectal cancer samples were obtained during surgery. Immediately after surgical resection, tissues were rinsed in redistilled water and then in 0.2 mol/L phosphate-buffered saline (PBS). Tumor-surrounding tissues were obtained and fixed in 3% glutaraldehyde for transmission electron microscopy or in 10% neutrally buffered formalin solution. The cut into slices, embedded in paraffin and sectioned at 4 μm for HE and immunohistochemical staining. The

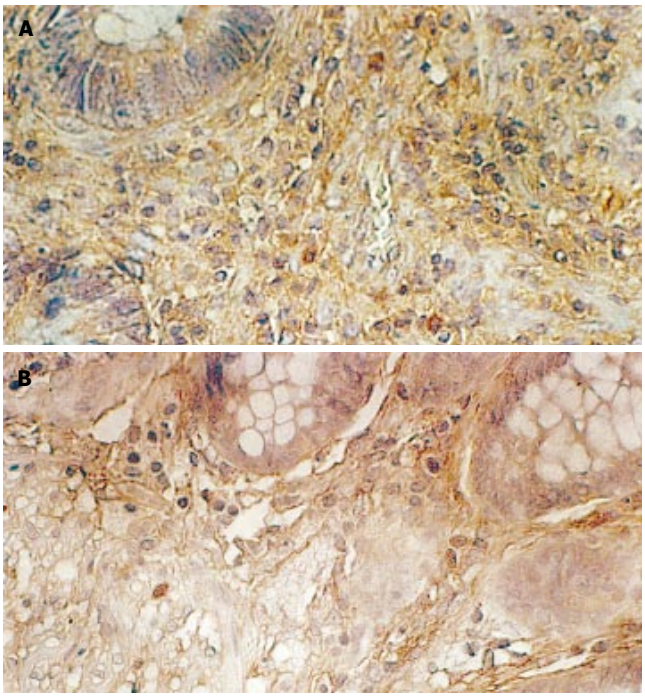

Figure 1 TIDCs in earlier (**A**) and later (**B**) stages of rectal cancer. (magnification: A, B x 400)

Table 1 Number of immunocytes between earlier and later stages (mean±SD)

	Earlier stage	Later stage	*P*-value
Number of cases	8	10	
Number of TIDCs	49.32 ± 5.48	17.02 ± 3.14	<0.01
Number of TILs	107.38 ± 13.61	48.21 ± 5.13	<0.01

deparaffinized sections were washed with PBS. Primary antibodies used were rabbit-anti human S-100 protein and CD3 (Boster Biological Technology Ltd). Secondary antibody used was mouse-anti rabbit IgG. The procedure was performed with SABC kit following the manufacturer's instructions.

Statistical analysis

Student *t* test was used for statistical analysis. $P < 0.05$ was considered statistically significant.

Cell counting

Under the light microscope, the number of infiltrating S-100 (+) and CD3 (+) cells in each section was calculated under 5 high power fields (×400) and statistically analyzed.

RESULTS

Observation of HE and immunohistochemical staining under light microscope

TIDCs were mainly seen in tumor-surrounding area, only a small number of TIDCs were located in cancer tissue scat-

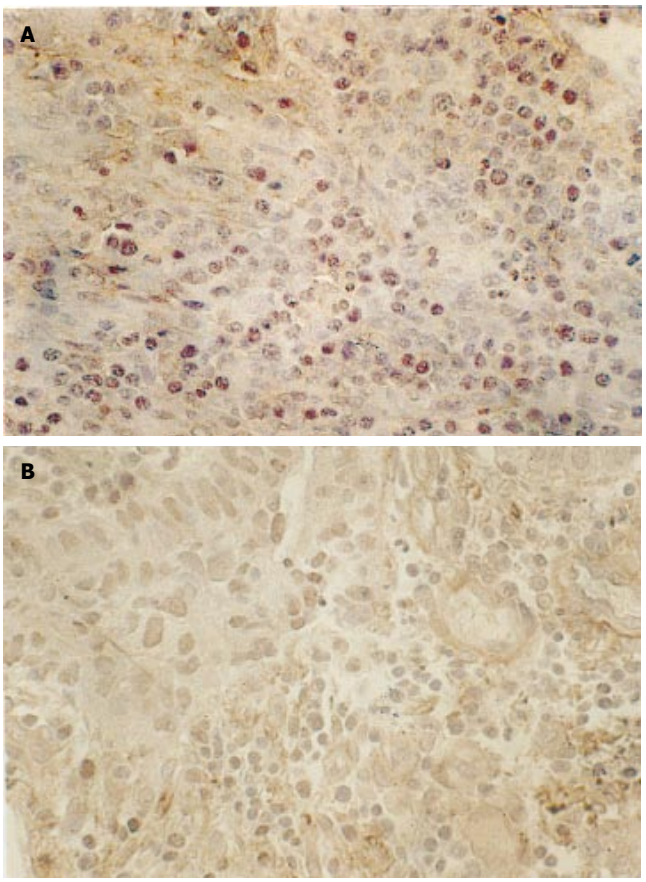

Figure 2 TILs in earlier (**A**) and later (**B**) stages of rectal cancer. (magnification: A, B x 400)

teredly by HE staining. Using anti-S-100 antibody with the SABC method, Brown S-100 (+) DCs could be observed in each section. Comparatively large DCs exhibited many dendritic protrusions, mainly located in tumor-surrounding tissue. The number of TIDCs was large in the earlier stage (Figure 1A) and comparatively small in the later stage (Figure 1B). The number of TIDCs in earlier stage was significantly higher than that in later stage and the difference was statistically significant ($P < 0.01$, Table 1).

Brown CD3 (+) TILs could be seen in each section. Circular CD3 (+) lymphocytes, little cytoplasm and large nuclei were distributed uniformly and densely. In the earlier stage, the number of TILs was large and TILs were primarily located centrally (Figure 2A). In the later stage, the number of TILs was small and TILs were scattered, only a small number of TILs were distributed centrally (Figure 2B). The number of TILs in earlier stage was significantly higher than that in later stage and the difference was statistically significant ($P < 0.01$, Table 1).

Transmission electron microscopy

Under electron microscope, TIDCs were seen in tumor-surrounding tissue. TIDCs were irregular in shape and displayed many dendritic cytoplasmic protrusions with varying width. The nuclei were irregular in shape and the nucleoli were small. Heterochromatin was highly marginated along the nuclear membrane. Mitochondria and endoplasmic reticulum were prominent in these cells. A fraction

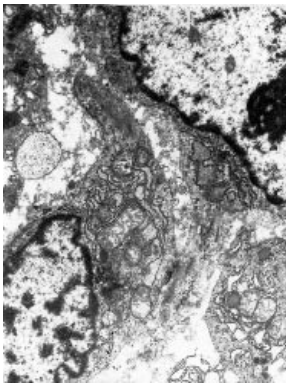

Figure 3 Morphology of TIDCs . D represents TIDCs, T represents tumor cells. (magnification x 10000)

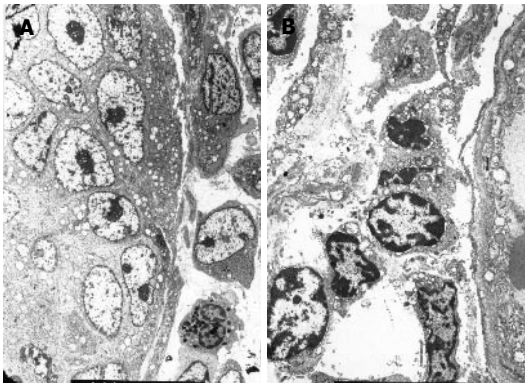

Figure 4 Lymphocytes in earlier (**A**) and later (**B**) stages of rectal cancer. (magnification: A x4000, B x2000)

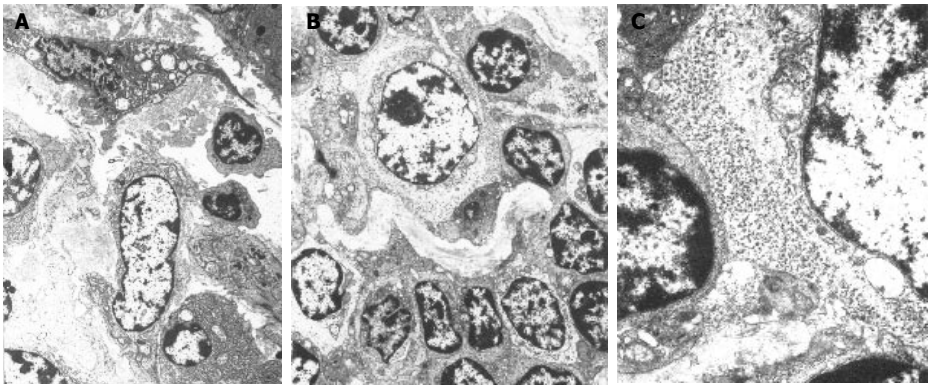

Figure 5 Relations between TILs and tumors cells (**A**), TIDCs and TILs (B) and glycogen granules (**C**). (magnification: A, x 4000, B x3500, C x15000)

of endoplasmic reticulum and some mitochondria were expanded (Figure 3).

It isn't obvious that cancer cells perforated the basement membrane. Lymphocytes were arranged serially along the basement membrane in earlier stage (Figure 4A). However, in the later stage, it is explicit that cancer cells perforated the basement membrane and were surrounded by TILs (Figure 4B). Mitochondria in cancer cells perforating the basement membrane degenerated and the cancer cells were surrounded by DCs, lymphocytes and plasma cells (Figure 5).

TIDCs were contacted with TILs in tumor-surrounding tissue, forming DC-lymphocyte clusters (Figure 5A). Clusters of glycogen granules were seen between TIDCs and TILs (Figure 5B). In addition to contacting TILs, TIDCs also contacted cancer cells at the same time. TILs could be seen surrounding the cancer nests, basement membrane of cancer was fragmentary, and TIDCs contacted cancer cells directly (Figure 5C).

DISCUSSION

DCs are the most powerful professional antigen-presenting cells (APCs)[1]. Studies on immunophenotype and function of DCs are now available[2-5]. Morphological studies on DCs and TIDCs are few. In this study, the morphology of TIDCs and correlation between TIDCs, TILs and cancer cells in human rectal cancer were investigated.

Antitumor immune responses of human body are mainly centralized in local cell-mediated immunity. The infiltrating degree of lymphocytes surrounding tumor tissue reflects the status of antitumor immune responses of human body. Activation of T lymphocytes needs the assistance of APCs. DCs are the most important APCs in human body and are closely related with the function of T lymphocytes. TILs are heterogenous lymphocytes, most of which are T lymphocytes. TILs play an important role in immunity and anti-tumor effects[8,9]. In our study, TILs were closely related with tumor immunity and mainly distributed in adjacent tissue of cancer, suggesting that localization of TILs in tumor-surrounding tissue limits the growth of cancer and confines cancer to a local site, which is the first barrier of anti-tumor effects of human body and contributes to immune response and prevents tumor spreading, which is the second barrier of anti-tumor effects of human body. Infiltration of TILs represents anti-tumor immune responses of human body. The infiltrating degree of TILs plays a decisive role in invasion and metastasis of tumor as well as prognosis of patients. In our study, the number of TILs in earlier stage of rectal cancer was significantly higher than that in later stage ($P < 0.01$), demonstrating that the number of TILs is related with the progression of rectal cancer.

It was reported that metastasis and prognosis of tumor are related with the infiltrating degree of DCs in tumor tissue [6]. Wright-Browne *et al* [7] showed that the number of TIDCs in human gastric cancer, colon cancer and carcinoma of esophagus is negatively correlated with lymph node metastasis, size of tumor and survival time, that is, the more the number of TIDCs, the better the patient's prognosis. In this study, the number of TIDCs in earlier stage was significantly higher than that in later stage ($P < 0.01$), suggesting that the number of TIDC is related with the progression of rectal cancer.

DCs play an important role in tumor immunity. The powerful antigen-presenting function of DCs can induce

efficient and specific anti-tumor immunity activated CTLs can kill tumor cells[10-12]. In this study, TIDCs were closely contacted with TILs. TILs were clustered around TIDCs and cluster of DCs-lymphocytes cluster was formed. Glycogen granules could be seen between TIDCs and TILs. All these suggest that there exists some relation between TIDCs and TILs. It has been reported that DCs mainly present antigen to lymphocytes, stimulate naïve T lymphocyte proliferation and activation to kill tumor cells [13]. The contact of TIDCs and TILs observed in our study may provide morphological evidence and suggests that TIDC can present antigen to TILs and activate TILs. Moreover, fraction of endoplasmic reticulum and some mitochondria in DCs were expanded. These data suggest that these active cells can capture and identify antigens by their cytoplasm protrusions, and subsequently transmit antigens to lymphocytes and prime anti-tumor immunity to kill tumor cells through cytotoxicity.

DCs participate in local anti-tumor immune response and have corresponding changes during oncogenesis and tumor progression, suggesting that DCs are closely related with anti-tumor immunity. DCs are not only professional APCs, but also immunological adjuvant. DCs play an important role in immunoregulation. It has been shown that antigen-sensitized DCs can induce specific CTL *in vivo* and directly inhibit or kill tumor cells [13-16]. But whether DCs can directly inhibit the growth of tumor *in vivo* or directly kill tumor cells remains unclear and should be further studied.

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RAPID COMMUNICATION

Effect of electro-acupuncture at Foot-Yangming Meridian on somatostatin and expression of somatostatin receptor genes in rabbits with gastric ulcer

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Abstract

AIM: To discuss the protective effect of electroacupuncture at the Foot-Yangming Meridian on gastric mucosal lesion, somatostatin (SS) and the expression of SS receptor genes (SSR$_1$mRNA) in rabbits with gastric ulcer and to further explore the relative specificity of meridians and viscera at gene expression level.

METHODS: Forty rabbits were randomly divided into control group (A), gastric ulcer model group (B), Foot-Yangming Meridian group (C), Foot-Shaoyang Meridian group (D) and Foot-Taiyang Meridian group (E). The gastric ulcer model was prepared by infusing alcohol into stomach. Groups C－E were treated with electro-acupuncture at points along the above meridians using meridian stimulating instruments for 7 days respectively. By the end of treatment, the index of gastric ulcer was determined, the amount of epidermal growth factor(EGF) and somatostatin was measured by radioimmunoassay (RIA). SS-R$_1$mRNA expression in gastric mucosa was determined by RT-PCR.

RESULTS: The value of EGF in model group was obviously lower(73.6 ± 14.8 *vs* 91.3 ± 14.9 pg/mL, $P < 0.01$) than that in control group**.** The index of gastric ulcer, content of SS and expression of SSR$_1$mRNA in gastric mucosa were significantly higher than those in control group (24.88 ± 6.29 *vs* 8.50 ± 2.98 scores, $P < 0.01$; 2978.6 ± 587.6 *vs* 1852.4 ± 361.7 mIU/mL, $P < 0.01$; 2.56 ± 0.25 *vs* 1.04 ± 0.36, $P < 0.01$) . The value of EGF in Foot-Yangming Meridian group was higher than that in model group(92.2 ± 6.7 *vs* 73.6 ± 14.8 pg/mL, $P < 0.01$). The index of gastric ulcer, content of SS and expression of SS-R$_1$mRNA in gastric mucosa were significantly lower than those in control group(10.88 ± 3.23 *vs* 24.88 ± 6.29 scores, $P < 0.01$; 1800.2 ± 488 *vs* 2978.6 ± 587.6 mIU/mL, $P < 0.01$; 1.07 ± 0.08 *vs* 2.56 ± 0.25mIU/mL, $P < 0.01$). Compared to the model group, the content of SS and expression of SSR$_1$mRNA in gastric mucosa in Foot-Shaoyang Meridian group decreased (2441.0 ± 488. *vs* 2978.6 ± 587.6 mIU/mL, $P < 0.05$;1.73 ± 0.16 *vs* 2.56 ± 0.25 mIU/mL, $P < 0.01$). But the above parameters in Foot-Taiyang Meridian group did not improve and were significantly different from those in Foot-Yangming Meridian group ($P < 0.05$).

CONCLUSION: Electro-acupuncture at Foot-Yangming Meridian can protect gastric mocusa against injury. The mechanism may be releted to the regulation of brain-gut peptides and the expression of SSR$_1$mRNA.

Key words: Foot-Yangming Meridian; Electro-acupuncture; Somatostatin; SSR$_1$mRNA

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INTRODUCTION

Studies have shown that needling the Foot-Yangming (stomach) Meridian can protect gastric mucosa against injury in rabbits[1-3]. This study was to further explore its mechanism and specificity in the experimental gastric ulcer model of rabbits and to compare its effect on the index of gastric mucosal lesion, brain-gut peptides and expression of SSR$_1$mRNA by investigating the correlation between Foot-Yangming Meridian and stomach.

MATERIALS AND METHODS

Main reagents and instruments

TRIZOL reagent and RT reagent kit as well as TagDNA polymerase were obtained from GZBOD BRL and Pro-

gmego Company respectively. RIA kits of EGF and SS were provided by Beijing SINO-UK Institute of Biological Technology. Hypothermia Eppedorflow-temperature centrifuge, Strategene Eagle Eye II figure recognition analytical system(made in USA), Xuanshou Meridian Unblocking Instrument (China Peace Economic Technic Counseling Corporation) were used in the study.

Preparation of experimental gastric ulcer

Gastric ulcer model was induced by ethanol[4]. In brief, the animals were perfused with 100% ethanol (2.35 mL/kg) into the stomach after fasted for 48 h. Twenty-four hours after the perfusion, the animals were allowed to have normal foods. The model group received normal saline (2.35 mL/kg) through an esophageal cannula.

Grouping and disposing

Forty New Zealand white rabbits of either sex, weighing 1 500-2 500 g were supplied by the Medical Animal Center of Hunan College of Traditional Chinese Medicine. The animals were randomly divided into 5 groups: control group (A), gastric ulcer model group (B), Foot-Yangming (stomach) Meridian group (C), Foot-Shaoyang (gallbladder) Meridian group (D) and Foot-Taiyang (bladder) Meridian group (E). NS was perfused into stomach of the control group. Dehydrated alcohol was administered intragastrically (i.g.) using a metal tube in B-E groups. C-E groups were treated with electro-acupuncture at points along the meridians of stomach, gallbladder and bladder for 7 d respectively. All the animals were killed at the end of the treatment. Gastric mucous membrane was removed for the detection of the indexes.

Location of acupoints and method of electro-acupuncture

Acupoints were selected on the basis of *Experiment Acupuncture Science*[5] and the method was chosen according to the human body[6]. For Foot Yangming (stomach) Meridian, the acupoints of "Neiting", "Jiexi", "Zusanli", "Liangqiu", "Tianshu" and "Liangmen" were chosen as stimulating points. According to the above stimulating points, acupoints in other meridians were selected at the same level of Foot Yangming Meridian in corresponding regions. For example , the acupoints of "Jiaxi","qiuxu", "Yanglingquan","Qiyangguan","Daimo","Jingmen" were selected for Shaoyang Meridian while the acupoints of "Zutonggu","Shenmo","Chengjin","Fuxi","Beishangdian","Beixiadian" were selected for Taiyang Meridian.

The method of stepwise stimulation along the meridians was used as previously described[7]. Then the instrument entered the state of making program and the single way of running was turned at the running speed of 0.5 second. The stimulation parameters included two-direction narrow pulse and continuous wave with a frequency of 50Hz and a width wave of 0.5ms. The output claps of stimulating instrument were clapped with 6 inserted needles at acupoints. Stimulating excitation was turned from low limber to body. The output intensity of instrument was controlled between "2-3" sections. Acupuncture was performed for 30 min a day for 7 d.

Index of gastric ulcer measurement

The abdomen was opened with the stomach removed 7 d after acupuncture. The stomach was then cut from pylorus to cardia. The index of gastric ulcer was calculated as previously described[8].

Determination of EGF and SS

Samples of gastric mucosa were homogenized and disposed with the test kit according to the manufacturer's instructions. EGF and SS were determined by radioimmunoassay.

Determination of SS-R$_1$mRNA

The expression of SS-R$_1$mRNA was analyzed RT-PCR. A small piece of gastric mucosa tissue was cut with a sterilized operating knife and mounted on a mortar stuffed with liquid nitrogen. After the tissue was ground into powders, the total RNA of was extracted by one step method with Trizol reagent kit. The rRNA bands of 28S, 18S and 5S were observed by electrophoresis. The proportion of A260/A280 of the total RNA in all samples was between 1.7 and 1.95. mRNA from 2 μg total RNA in samples was reversed to cDNA at 65℃ using oligo (dT)$_{18}$ as primer for 5 min. Then, 20 μL RT reaction system comprised of 20 uRNA enzyme inhibitor (offered by Promega corporation), 0.5 mmol/L dNTP, 10u AMV RT enzyme as well as 5xRT buffer. PCR was performed with the follow primers. The sense primer of SS-R$_1$ was 5' -CAAGACGACGCCACCGTGAGCCA-3', antisense primer was 5' -GGGGTTGGCACAGCTGTTG-3'. The sense primer of cyclophilin(cyc) was 5'-CCATCGTGTCATCATCAAGGACTTCAT-3' and antisense primer was 5'-TTGCCATCCAGCCAGGAGGTCT-3'. The 50 μL PCR reaction system comprised of 5 μL 10×PCR reaction buffer, 1.5 mmol/L $MgCL_2$, 200 umol/L dNTP, 5 μL cDNA template, 0.1 mmol/L specific primers, and 3u Taq DNA polymerase covered by paraffin oil. The PCR conditions for SS-R$_1$ and cyc cDNA were as follows: predenaturation at 94℃ for 2 min; denaturation at 94℃ for 30s, annealing at 60℃ for 30s, and an extention at 72℃ for 30s for 26 cycles; then a final extention at 72℃ for 5 min. Ten μL product of PCR was put in 1.5% sepharose to go on electrophoresis in ranks in different groups. The products of SS-R$_1$ and cyc were 66 bp and 216 bp, respectively. The products of electrophoresis were stained with ethidium bromide and photographed under ultraviolet lamp. The electrophoresis band was scanned by figure recognition analytical system. The proportion of SS-R$_1$/cyc was calculated as the relative expression level of SS-R$_1$mRNA.

Statistical analysis

All data were presented as mean ± SD and analyzed by the software of SPSS10.0. Statistically significant differences were calculated by analysis of variance (ANOVA). If the mean square deviation was regular, it was analyzed by LSD test. Otherwise it was analyzed by Dunnett T3 test.

RESULTS

Effect of electro-acupuncture on index of gastric ulcer

The highest gastric ulcer index was observed in the model group (24.88 ± 6.29, $P < 0.01$). After electro-acupuncture, the gastric ulcer index in stomach Meridian group was significantly lower than that in the other groups ($P < 0.01$),

Table 1 Index of gastric ulcer in different groups (mean ± SD)

Group	*n*	Index of gastric ulcer
Group A	8	8.50±2.98[b]
Group B	8	24.88±6.29[d]
Group C	8	10.88±3.23[b]
Group D	8	19.38±3.66[d]
Group E	8	24.13±1.64[d]

[b]$P<0.01$ *vs* group B; [d]$P<0.01$ *vs* group C.

Table 2 EGF and SS in gastric mucosa in different groups (mean ± SD)

Group	*n*	EGF(pg/mL)	SS(mIU/mL)
Group A	8	91.3±14.9[b]	1852.4±361.7[b]
Group B1	8	73.6±14.8[d]	2978.6±587.6[d]
Group C	8	92.2±6.7[b]	1800.2±488.1[b]
Group D	8	74.9±9.0[d]	2441.0±488.1[a c]
Group E	8	65.4±12.8[d]	2592.7±426.8[d]

[a]$P<0.05$, [b]$P<0.01$ *vs* group B; [c]$P<0.05$, [d]$P<0.01$ *vs* group C.

Table 3 SS-R1mRNA expression in different groups of rabbits with gastric ulcer (mean ± SD)

Group	*n*	SSR1/cyc
Group A	8	1.04±0.36[b]
Group B	8	2.56±0.25[d]
Group C	8	1.07±0.08[b]
Group D	8	1.73±0.16[b c]
Group E	8	2.39±0.39[d]

[a]$P<0.05$, [b]$P<0.01$ *vs* group B; [c]$P<0.05$, [d]$P<0.01$ *vs* group C.

indicating that electro-acupuncture at Stomach Meridian could significantly decrease the gastric ulcer index in rabbits with experimental gastric ulcer (Table 1).

Effect of electro-acupuncture on content of EGF and SS

The content of EGF in the control group was significantly higher than that in the other groups ($P<0.01$) except for the control group. However, the content of SS in the Stomach Meridian group was significantly lower than that in the other groups except for the control group ($P<0.05$), indicating that electro-acupuncture at Foot-Yangming Meridian could increase the content of EGF in ulcer rabbits and decrease the content of SS (Table 2).

Effect of electro-acupuncture on SS-R$_1$mRNA expression in gastric mucosa

The expression of SS-R$_1$mRNA in model group was obviously lower than that in control group and there was a significant difference ($P<0.01$). The expression of SS-R1mRNA in gastric mucosa after acupuncture treatment was Foot-Yangming Meridian group> Foot-Shaoyang Meridian group> Foot-Taiyang Meridian group. There was a significance difference ($P<0.05$), suggesting that acupuncture at Stomach Meridian had a distinct effect on inhibiting expression of SS-R1mRNA of experimental gastric ulcer (Table 3).

DISCUSSION

Cytoprotection is referred to the fact that some substances can protect epithelial cells of the digestive tract against injury. Studies showed that acupuncture confers quite good protective effect on gastric mucosal lesion[1,2]. The protective mechanism may be as follows. Acupuncture can increase the level of PGE_2 in gastric mucusa[3], inhibit release of GAS, influence metabolism of oxygen free radicles[9], increase gastric mucosal NO and NOS[10], regulate expression of Bcl-2 and Fas and depress gastric mucosal epithelial cell apoptosis[11]. Acupuncture at Stomach Meridian can decrease gastric mucosal ulcer index, thus protecting gastric mucosa against injury.

Epidermal growth factor is secreted by salivary gland, duodenum Brunner gland and pancreas[12], which can resist the destructive effect of gastric and pancreatic protease and chymotrypsin, inhibit secretion of gastric acid and gastric protease, promote proliferation of gastric epithelial cells and synthesis of DNA in gastric mucosa, prevent formation of ulcer[13-15]. Studies showed that epidermal growth factor can accelerate healing process[16-18]. Zhao *et al*[19] showed that electro-acupuncture at "Zusanli" can increase EGF in upper alimentary cannel and depress gastric acid secretion. Jin *et al*[20] reported that EGF remains normal in stress rats and electro-acupuncture can relieve the gastric ulcer index, suggesting that EGF plays an important role in protecting gastric mucusa from injury during acupuncture. In our study, acupuncture at Stomach Meridian could increase EGF in gastric mucosa of experimental gastric ulcer rabbits, suggesting that acupuncture at Stomach Meridian can promote the healing of gastric mucosal lesion by regulating EGF.

SS is the main inhibitory hormone in digestive tract and can inhibit release and activity of the gastrointestinal hormones as well as gastric acid secretion and participates in protecting gastric mocusa[21-23]. Somatostatin receptors exist in gastrointestinal tract and central nervous system. Among the 5 subtypes of SS-R, SS-R_1 is highly expressed in gastric mucosa and can inhibit secretion of gastrin, histamine and gastric acid and plays an important role in adjusting gastric acid secrection. SS can also inhibit upper gastrointestinal hemorrhage and epithelial hyperplasia[24-26]. There are reports on the change of SS in digestive ulcer[27-29]. Lin *et al*[30] found that stomach perfusion with alcohol can result in gastric mucosal lesion and increase SS, but after electro-acupuncture at Foot-Yangming Meridian, SS in gastric antrum and medulla is decreased. Wang *et al*[31] showed that under psychological stress, gastric mocusal lesion of rats is associated with level of SS, but after electro-acupuncture at Zusanli, the degree of gastric mucosal lesion is reduced and SS in blood plasma tends to decrease. Chen *et al*[32] showed that Codonapsis pilosula, one of the Chinese herbs, can increase SS in stomach and duodenum mucosa, thus promoting healing of alimentary tract ulcer, suggesting that the level of SS and expression

of SS-R_1mRNA are increased in rabbits with experimental gastric ulcer due to the process of compensation feedback.

Konturek *et al*[33] found that SS may play a role in inhibiting epithelial proliferation and mocusal healing in stomach. However, Pfeiffer *et al*[34] found that epithelial growth factor receptors and SS receptors can increase the healing process of experimental gastric ulcer. Our study showed that the level of SS and expression of SS-R_1mRNA in Foot-Yangming Meridian group were significantly lower than those in model group, suggesting that the effect of SS on cell proliferation is weakened during the process of ulcer healing[35].

The protecting effect of acupunture at Foot-Sanyang Meridian on gastric mocusal lesion indicates that the action of acupuncture at Foot-Yangming Meridian is the strongest, suggesting that Foot-Yangming Meridian is closely related with stomach. This result accords with traditional meridian theory and clinical practice of acupuncture and has confirmed once again the relative specificity between Foot -Yangming Meridian and stomach.

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RAPID COMMUNICATION

Cyclin D1 antisense oligodexoyneucleotides inhibits growth and enhances chemosensitivity in gastric carcinoma cells

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Abstract

AIM: To examine the effects of cyclin D1 antisense oligodexoyneucleotides (ASODN) on growth and chemosensitivity of gastric carcinoma cell lines SGC7901 and its mechanism.

METHODS: Phosphorothioate modified cyclin D1 ASODN was encapsulated by LipofectAMINE2000 (LF2000) and transfected into cells, the dose-effect curves and growth curves were observed. 5-FU, MTX, CDDP of different concentrations were given after transfecting cells with cyclin D1 ASODN for 24 h，the dose-effect responses were observed and IC50s were calculated. The mRNA expression of cyclin D1, thymidylate synthase (TS), thymidine phosphorylase (TP) and dihydrofolate reductase (DHFR) was detected by reverse transcription-PCR (RT-PCR) at 24 h and 48 h after transfection.

RESULTS: Dose-dependent inhibitory effect was caused by cyclin D1 ASODN in SGC7901 cells. Transfecting gastric carcinoma cells with 0.2 μmol/L cyclin D1 ASODN for 24 h could inhibit growth significantly and reduce expression of cyclin D1 mRNA. Cyclin D1 ASODN could increase the chemosensitivity to 5-FU, MTX, CDDP in cells, The IC50s of different chemotherapeutic agents in ASODN plus chemotherapy groups were significantly lower than those in controls. Transfection with cyclin D1 ASODN leaded to an increase in TS and DHFR mRNA and a decrease in TP mRNA as determined by RT-PCR at 24 h, the alterations were more significant at 48 h.

CONCLUSIONS: Cyclin D1 ASODN can decrease mRNA expression of cyclin D1，inhibit growth and enhance the chemosensitivity by changing the expression of enzymes related to metabolism of chemotherapeutic agents in SGC7901 gastric carcinoma cells.

INTRODUCTION

Cyclin D1 involves in the regulation of G1 phrase in cell cycle, with three subtypes (D1, D2, D3), cyclin D1 located in 11q13, cDNA is 4.3 Kb, is identified as the proto-oncogene and overexpressed in breast, esophageal, and hepatic carcinoma. Cyclin D1 is an important cell cycle regulatory protein that is expressed in the early G1 phase. This protein, in association with the cyclin-dependent kinases CDK4 and CDK6, mediates the phosphorylation of pRb [1]. In this study, we transfected gastric carcinoma cells with cyclin D1 antisense oliogodeoxyneucletides，to observe the effects of it on growth and chemosensitivity to 5-fluorouracil (5-FU)，methotrexate (MTX) and cisplatin (CDDP), furthermore, we examined the expression of enzymes related to metabolism of chemotherapeutic agents, including TS, TP and DHFR, to find out the underlying mechanism.

MATERIALS AND METHODS

Cell culture and reagents

Human moderately differentiated gastric adenocarcinoma cell line SGC7901 was kindly gifted by the Prof. Daiming Fan (the Fourth Military Medical University, Xi'an, China). Cells were cultured in DMEM medium (GIBCO) with 10% newborn calf serum (GIBCO) at 37 ℃ in 50mL/L CO_2. Cyclin D1 antisense oliogodeoxyneucletide 5'-GGA GCT GGT GTT CCA TGG-3' (ASODN) was complementary to the translation start site of the cyclin D1 cDNA and sense oligomers 5'-CCA TGG AAC ACC AGC TCC-3' (SODN) was used as control[2], the two sequences were synthesized and phosphorothioate modified by Shanghai Shengong Biotechnology Corp. The lyophilized ODNs was diluted by sterile water and filtered, storaged in -20 ℃. Lipofectamine2000 (LF2000, Invitrogen)-ODN complexes were prepared as described in the instruction. 5-FU, MTX and CDDP were purchased from SIGMA.

Dose-effect curves

Cells were plated in 96-well plates, SODN and ASODN were mixed with LF2000 respectively, diluted to a increasing concentration (from 0.05 to 0.5 μmol/L) and added to plates, after transfection for 24 h, the media were replaced with DMEM contain serum and cells were continue cultured for 72 h. The viability was determined by MTT assay and survival rates were calculated. The optimal concentration, able to inhibit cell growth of at least 50% *vs* control, was selected for further experiments.

Growth Curves

Cells were plated in 12-well plates, treated with LF2000-SODN or ASODN complexes at concentration of 0.2 μmol/L, after transfection for 24 h, replaced with media with serum and cultured for 6 d, cell numbers were counted everyday and the growth curves were drawn.

Chemotherapy-induced cytotoxicity

Cells were plated in 96-well plates and divided into three groups: (1) chemotherapy group, (2) SODN + chemotherapy group, (3) ASODN + chemotherapy group. After transfection with 0.2 μmol/L LF2000-ASODN or LF2000-SODN complexes for 24 h, cells were incubated with medium contain chemotherapeutic agents (5-FU, MTX or CDDP) for 72 h. Cell variability was examined by MTT assay, and dose-effect curves were drawn. The IC50s were calculated by software Prism3 (Graphpad, USA).

Reverse transcriptase polymerrase chain reaction (RT-PCR) analysis

Cells were plated in 6-well plates, transfected and cultured as described, the cells were harvested at 24h and 48h after transfection. Total RNA was isolated by TRIZOL Reagent (GIBCO). Reverse transcription was carried out on 1 μg of total RNA, 20 μL reaction system contained oligo(dT) 0.1μg, 2 mmol/L dNTPs 4 μL, Rnasin 0.1μg, MMLV 200U, incubated at 42 ℃ for 1h and enzymes were inactivated at 95 ℃ for 5min. 2.5μL RT products were used for PCR reaction, the dNTPs, sense and antisense primers (100 pmol), Taq DNA polymerase were added into reaction system of 40 μL. G3PDH was used as the control, primers and PCR condition as follow(Table1)

PCR conditions After denaturation for 4 min at 94 ℃, thirty-five cycles were accomplished as follow: Cyclin D1, 94 ℃, 60 s → 65 ℃, 1.5 min→ 72 ℃, 1.5 min; TS, 94 ℃, 30 s → 55 ℃, 30 s → 72 ℃, 30 s; TP, 94 ℃, 3 0s→ 60 ℃, 30 s → 72 ℃, 30 s; DHFR, 94 ℃, 30 s → 60 ℃, 30 s → 72 ℃, 30 s; G3PDH, 94 ℃, 30 s → 54 ℃, 30 s → 72 ℃, 30 s.

RT-PCR products (cyclin D1:514 bp; TS：208 bp; TP:353 bp; DHFR:231 bp; GAPDH: 309 bp) were analyzed by 2% agarose gel electrophroesis and visualized by EB staining, densitometric scanning of the bands was carried on and relative amount of each gene mRNA expression was estimated by normalized to the G3PDH mRNA detected in the same sample.

Statistical analysis

Results were expressed as mean±SD and the Student's *t* test was used for statistical analysis (two sides). $P < 0.05$ was taken as level of significance.

Table 1 Sequences of primers (bp)

	Primers	Length of amplification
Cyclin D1	5′-GGA TGC TGG AGG TCT GCG AGG AAC-3′	514
	5′-GAG AGG AAG CGT GTG AGG CGG TAG-3′	(332--845)
TS	5′-CAC ACT TTG GGA GAT GCA CAT ATT T -3′	208
	5′-CTT TGA AAG CAC CCT AAA CAG CCA T-3′	(853--1060)
TP	5′-ACA AGG TCA GCC TGG TCC TC-3′	353
	5′-TCC GAA CTT AAC GTC CAC CAC -3′	(491--834)
DHFR	5′-TCC ATT CCT GAG AAG AAT CGA CCT T-3′	231
	5′-CAC AAA TAG TTT AAG ATG GCC TGG G-3′	(657--887)
G3PDH	5′-TCC CTC AAG ATT GTC AGC AA-3′	309
	5′-AGA TCC ACA ACG GAT ACA TT-3′	

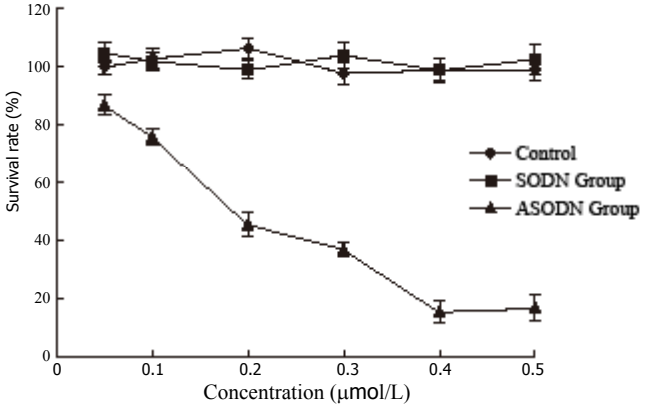

Figure 1 Dose-response curves of SGC7901 cell lines.

RESULTS

Cyclin D1 ASODN inhibits the growth of SGC7901 cells

Dose-dependent inhibitory effects were caused by cyclin D1 ASODN in SGC7901 cells， as shown in Figure 1. At concentration of 0.2 μmol/L, the inhibitory rate was 54.7% and 0.2 μmol/L was taken as optimal concentration for further experiments. Transfection with 0.2 μmol/L cyclin D1 ASODN significantly inhibited the growth of cells. IC50 in ASODN group was lower than that in the control from the third day as shown by growth curves, and there was no difference between SODN group and the control (Figure 2).

Cyclin D1 ASODN enhances the chemsensitivity of SGC7901 cells

After transfecting with cyclin D1 SODN or ASODN, cells were treated with 5-FU, MTX or CDDP in different concentrations. We found that transfection with cyclin D1 ASODN enhances the cytotoxicity of 5-FU, MTX and CDDP significantly, the IC50s in ASODN + chemotherapy groups to different chemotherapeutic agents were significantly lower than the those in chemotherapy group and SODN+ chemotherapy group. The dose-effect curves and IC50s to different treatment are shown in Figure 3 and Table 2.

Table 2 IC50 of *5-FU*, *MTX* and *CDDP* in each group ($\times 10^6$ mol/L)

	5-FU	MTX	CDDP
Chemotherapy Group	3.95 ± 0.92	1.27 ± 0.32	2.12 ± 0.22
SODN+Chemotherapy Group	4.43 ± 0.41	1.25 ± 0.10	1.88 ± 0.07
ASODN+Chemotherapy Group	0.98 ± 0.15 [ac]	0.38 ± 0.04 [ac]	0.52 ± 0.07 [ac]

[a]P<0.05 *vs* Chemotherapy Group; [c]P<0.05 *vs* SODN+ Chemotherapy Group.

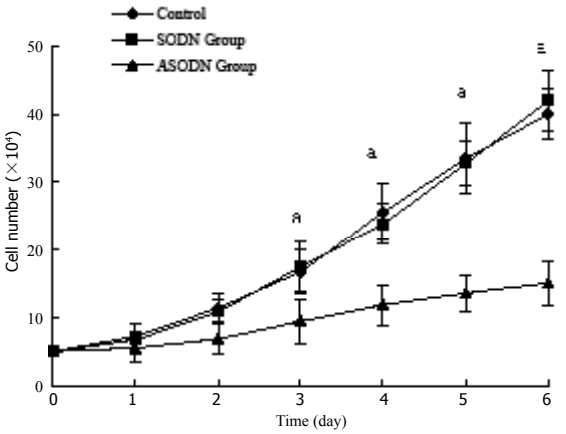

Figure 2 Growth curves of SGC7901 cell lines. [a]$P<0.05$ *vs* control.

Effect of cyclin D1 ASODN on mRNA expression of cyclin D1 and enzymes related to chemotherapy

Relative cyclin D1, TS, TP and DHFR mRNA expression is quantified by densitometric analysis, and illustrated in Figure 4. Comparing with the control, the cyclin D1 mRNA expression reduced to 26.3% and 50.0% at 24 h and 48 h, respectively. In comparison with the control, TS mRNA levels were reduced to 83.5% and 48.4% at 24 h and 48 h, respectively. TP mRNA levels were significantly increased to 124.6% and 148.3% at 24 h and 48 h, respectively. DHFR mRNA levels were reduced to 52.3% and 37.5% at 24 h and 48 h, respectively.

DISCUSSION

The use of antisense oligonucleotides as selective inhibitor of gene expression has become an important tool in current laboratory research and clinical trails. In our study ASODN were phosphorothioate modified and encapsulated by LipofectAMINE2000, to enhance the efficiency of transfection and prolong the time of action. We found dose-dependent inhibitory effects were caused by cyclin D1 ASODN in SGC7901 cells, the percentage of inhibition were 54.7% in cells treated with 0.2 μmol/L cyclin D1 ASODN for 24 h, and the growth of cells in ASODN group were also inhibited as shown by growth curves. Furthermore, cyclin D1 ASODN markedly reduced mRNA expression of cyclin D1. It suggested that cyclin D1 ASODN could inhibit the growth of gastric carcinoma cells through decreasing the cyclin D1 expression. The expression of cyclin D1 mRNA at 48h elevated a little, the lost of inhibitory effect of ASODN may be due to digestion by nucleases existed in cells.

5-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP), metabolic product of 5-FU, forms a tight-binding complex with TS and thereby blocks DNA synthesis process. Inhibition of TS by FdUMP is one of main mechanism underlying 5-FU action, the degree of inhibition of TS and the persistence of inhibition are essential factors for maximal in vivo growth inhibition by 5-FU[3]. TP is an enzyme that involved in pyrimidine nucleoside metabolism, and able to catalyze the conversion of 5-FU to 5-fluoro-2'-deoxyuridine (5-FdUR), which is the first step in one pathway for the metabolic activation of 5-FU. The 5-FdUR can

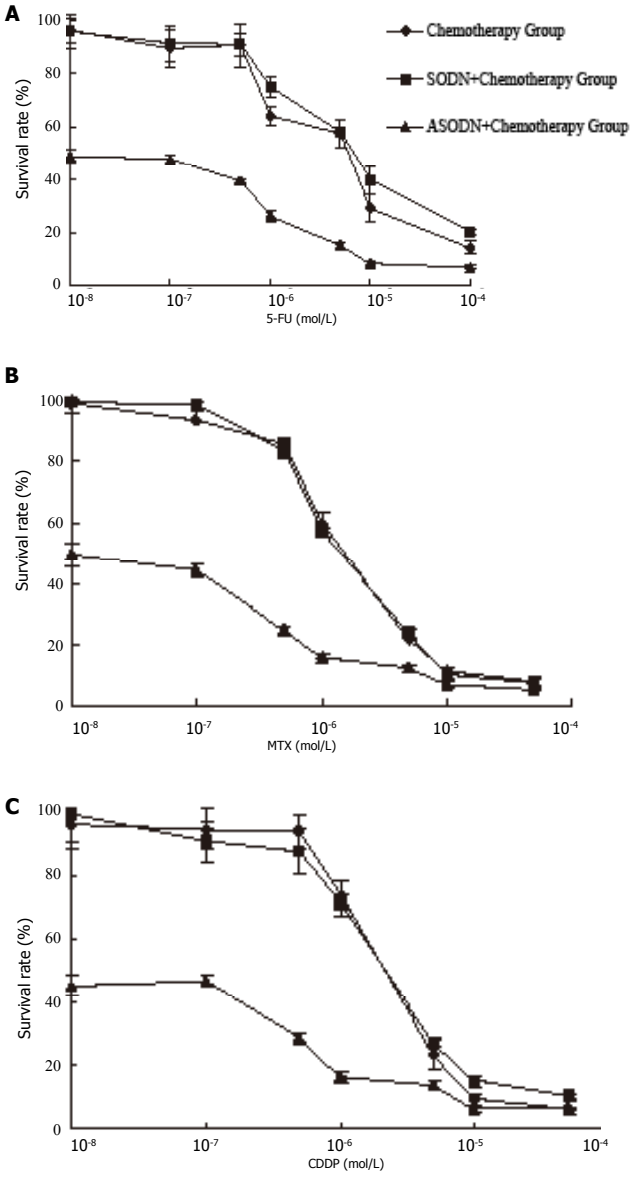

Figure 3 Effect of Cyclin D1 ASODN on chemosensitivity in SGC7901 cells. **A**: 5-FU; **B**: MTX; **C**: CDDP.

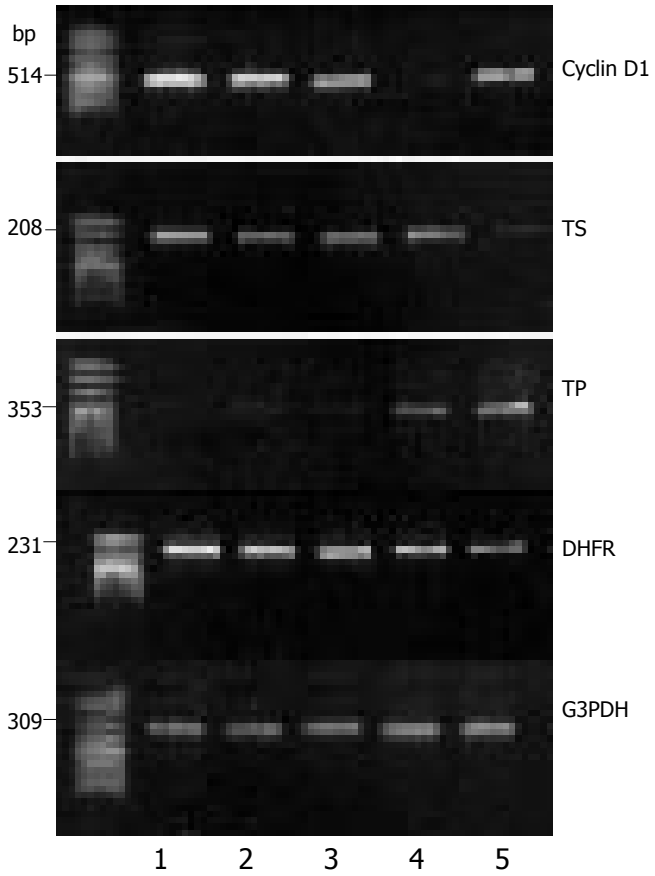

Figure 4 Effect of Cyclin D1 ASODN on mRNA expression of Cyclin D1, TS, TP and DHFR. 1, the Control; 2, SODN group at 24 h; 3, SODN group at 48 h; 4, ASODN group at 24 h; 5, ASODN group at 48 h.

be activated to 5-FdUMP, which blocks TS activity, and exerts antiproliferative effect.[4] The major target for MTX is the enzyme DHFR, which is important in biosynthesis of RNA and DNA. MTX has a chemical structure similar to folic acid, and can competitively inhibit DHFR activity. At 48 h after cyclin D1 ASODN transfection, the mRNA expression of TS, TP, DHFR in SGC7901 cells changed significantly in accompany with enhancement of chemosensitivity as shown in our study.

E2F may play an important role in this alteration. E2F is a cellular transcription factor, which can induce a number of genes important in the passage of cell cycle through the G1/S phase transition as well as in the initiation of DNA synthesis. When E2F binds the promoter and pRb simultaneously, the pRb-E2F complex acts as a transcriptional suppressor and completely silences the transcription of target genes. Interaction with E2F is the means through which pRb exerts its antiproliferative effect[5]. It would be expected that decreased expression of cyclin D1 by ASODN might affect pRb phosphorylation and might lead to change of level of E2F protein as a consequence of alteration in their interactions with pRb, thus change the expression of TS, TP and DHFR and enhance the chemosensitivity to 5-FU and MTX.

Warenius *et al* [6] firstly found that high cyclin D1 expression is related to CDDP resistance in the 16 human cell lines. Furthermore, CDDP resulted in significantly higher rates of cell killing in the antisense cyclin D1 transfected laryngeal squamous cell carcinoma cell lines CCL23 than parental cells, and ID50 decreased, which suggest the decreased expression of cyclin D1 may enhance the DNA-damaging effects of CDDP [7]. It is possible that the blockade of autocrine mitogenic signals by inhibiting cyclin D1 expression might also be a mechanism for increase in CDDP chemosensitivity[8]. As shown in our study, cyclin D1 ASODN can increase the cheosensitivity to CDDP in SGC7901 cells, but the exact mechanism needs further research.

Cyclin D1 ASOND could inhibit the growth of gastric carcinoma cells and change the expression of enzymes related to metabolism of chemotherapeutic agents through the influence on expression of cell cycle regulators, and enhance the chemosensitivity to 5-FU, MTX and CDDP, indicating that ASODN technology is a feasible way to enhance the efficiency of chemotherapeutic agents and provide a new strategy for combination therapy of gastric cancer.

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RAPID COMMUNICATION

Effect of drug treatment on hyperplastic gastric polyps infected with *Helicobacter pylori*: A randomized, controlled trial

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Abstract

AIM: To study the effects of drug treatment on hyperplastic gastric polyps infected with *Helicobacter pylori* (*H pylori*).

METHODS: Forty-eight patients with hyperplastic gastric polyps (3-10 mm in diameter) infected with *H pylori* were randomly assigned to a treatment group (n = 24) which received proton-pump inhibitor (omeprazole or lansoprazole), clarithromycin, bismuth citrate and tinidazole, and a control group (n = 24) which received protective agent of gastric mucosa（tepretone）. Patients underwent endoscopy and *H pylori* examination regularly before enrollment and 1-12 mo after treatment.

RESULTS: Twenty-two patients in the treatment group and 21 in the control group completed the entire test protocol. In the treatment group, polyps disappeared 1-12 mo (average, 6.5 ± 1.1 mo) after the treatment in 15 of 22 patients (68.2%) and *H pylori* infection was eradicated in 19 of the 22 patients (86.4%). However, 12 months after the study, no change in polyps or *H pylori* status was seen in any controls ($^bP < 0.01$).

CONCLUSION: Most hyperplastic gastric polyps disappear after eradication of *H pylori*.

Key words: *Helicobacter pylori*; Hyperplastic gastric polyps; Therapy

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INTRODUCTION

The risk of conversion of hyperplastic gastric polyps is very low, only accounting for 1.5%-3% [1]. Patients with gastric polyps may present with bleeding of the upper gastrointestinal tract, abdominal pain, or gastric outlet obstruction. Large gastric polyps or polyps associated with complications can be removed endoscopically or surgically. *Helicobacter pylori* (*H pylori*) infection is closely associated with hyperplastic gastric polyps and *H pylori* is present in 100% of hyperplastic gastric polyps[2-4]. Hyperplastic gastric polyps may disappear in 40-71% patients after eradication of *H pylori*[5, 6]. Since the pathogenicity of *H pylori* is different in various regions, we conducted this randomized, controlled trial to see whether hyperplastic gastric polyps disappear after eradication of *H pylori*.

MATERIALS AND METHODS

Selecting and grouping of patients

Forty-eight patients were from the Gastroenterology Department, First Affiliated Hospital of Zhejiang University and Red Cross Hospital of Hangzhou (26 men, 22 women, age range from 21 to 73 years, average 47 years). All patients did not receive antibiotics, bismuth, steroid and non-steroid drugs before their enrollment in the study. Hyperplastic gastric polyps (3-10 mm in diameter) were diagnosed on the basis of the examination results at least three histological samples. Patients were considered to have multiple gastric polyps (at least 5) when they were easily cut. Our criteria for hyperplastic gastric polyps included hyperplasia of the foveolar epithelium on histologic examination and infiltration of inflammatory cells into the stroma in biopsy specimens [7, 8]. Hyperplastic gastric polyps were diagnosed by two blinded pathologists. Patients with adenomatous gastric polyps, Peutz-Jegher syndrome and juvenile polyps were excluded. The diagnosis of *H pylori* infection was based the positive results of staining with Giemsa and ^{14}C-urea breath test. The patients were randomly assigned to two groups and sequentially numbered. In the treatment group (n = 24), patients received proton-pump inhibitor (omeprazole 20mg/d or lansoprazole 30 mg/d), clarithromycin (1g/d), bismuth citrate (440 mg/d) and tinidazole (1g/d), 2 weeks a course. In the control group (n = 24), patients had endoscopic examination and received protective agent of gastric mucosa (tepretone 150 mg/d). Two patients of the treatment group did not take the drugs for one course. In the control group, 2 patients lost follow-up and 1 patient took both proton-pump in-

hibitor and amoxicillin. These 5 patients exited from our study. If polyps progressed and were accompanied with malignant transformation, the study was stopped and the polyps were removed endoscopically. After completion of our study, endoscopic removal of polyps or eradication of *H pylori* was proposed for those who failed in *H pylori* eradication.

Endoscopy, histologic examination and assessment of H pylori eradication

Patients in the treatment group underwent endoscopy every 3 months after the treatment. On each occasion, biopsy specimens were taken from the same areas (three from the antrum and three from the body) for histologic examination. If patients without eradication of *H pylori* were not adapted to endoscopic examination very well, controls underwent endoscopy every 3 months after enrollment. Biopsy specimens for histologic examination were stained with Giemsa and evaluated for the presence of *H pylori*. Histologic diagnosis of the biopsied mucosa of the antrum and body was made by two blinded pathologists. The severity of activity, inflammation, atrophy, and metaplasia was graded on a scale from 1 to 4 and expressed as the histologic index according to the updated Sydney System: 1: normal, 2: mild, 3: moderate, and 4: marked [9]. Eradication of *H pylori* was confirmed by the negative results of these two tests 1-3 months after the treatment and endoscopic examination. The size and number of polyps were measured at each endoscopic examination using biopsy forceps (GIF XQ240 or GIF140, Olympus) placed near the polyp (open size: 6 mm in diameter; closed size: 2 mm in diameter). The endoscopic data on the disappearance and regression of polyps were reviewed independently by two blinded endoscopists.

Statistical analysis

All data were analyzed by unpaired *t* test (for age), Wilcoxon rank-sum test and Fisher's exact test. $P < 0.05$ was considered statistically significant. All data were coordinated by SPSS RDS and statistical analyses were done by SPSS software.

RESULTS

Comparison of baseline clinico-pathological characteristics between treatment and control groups

Twenty-two patients in the treatment group and 21 patients in the control group completed the entire study. The two groups were similar with respect to the number, age, sex, coexisting disease, as well as the number, size and distribution of polyps, histologic findings (Table 1). The two groups were comparable.

Analysis of curative effects between treatment and control groups

In the treatment group, *H pylori* was eradicated without serious side effects in 19 of 22 patients (86.3% [95% CI, 63%-99%]), and polyps disappeared in 15 of 22 patients (68.2% [95% CI, 54%-91%]) 1-12 months after treatment (Figure 1). Hyperplastic gastric polyps in the other 4 patients with successful *H pylori* eradication regressed to a certain extent, decreasing in size or number. However, in the remaining 3 patients without *H pylori* eradication, no polyps showed regression and no diminution of inflammation in the gastric mucosa 12 months after the treatment. In the control group, all patients showed no change of *H pylori* infection (0% [95% CI, 0%-21%]), no hyperplastic gastric polyp regression or disappearance (0% [95% CI, 0%-21%]) and no significant diminution of inflammation (Table 2). Polyps were enlarged or increased in number in 5 of the 21 patients. The rates of eradication of *H pylori* and disappearance of polyps in the treatment group were significantly higher than those in the control group ($P < 0.01$).

Table 1 Clinico-pathological characteristics of treatment and control groups

Characteristics	Treatment groups (n = 22)	Control groups (n = 21)
Mean age(mean±SD，yr)	49 ± 9	47 ± 8
Men, n(%)	13(59.1)	11(52.4)
Coexisting disease, n(%)		
Chronic atrophic gastritis	10(45.4)	12(57.1)
Duodenal ulcer	2(9.1)	2(9.5)
Gastric ulcer	0(0)	1(4.8)
Mean number of polyps	5.6	4.2
Mean size of polyps(mm)	6.6	7.8
Distribution of polyps, n (%)		
Body	79(64.2)	61(69.3)
Antrum	19(15.4)	15(17.0)
Angle	8(6.5)	5(5.7)
Fundus	9(7.3)	4(4.5)
Cardia	8(6.5)	3(3.4)
Histologic findings		
Inflammation	2.7, 2.4	2.6, 2.4
Activity	2.5, 2.5	2.5, 2.3
Atrophy	2.4, 2.6	2.5, 2.4
Metaplasia	0.9, 0.6	1.0, 0.6

DISCUSSION

In our study, *H pylori* was successfully eradicated in 19 of the 22 patients. The regression or disappearance of hyperplastic gastric polyps was seen in the 19 patients. The polyps disappeared in 15 of the 19 patients 1-12 months (average, 6.5 ± 1.1 mo) after the treatment. However, none of the polyps in any of the controls or in patients without *H pylori* eradication showed regression. These results strongly suggest that eradication of *H pylori* leads to regression and disappearance of hyperplastic gastric polyps. *H pylori* is the main cause of chronic active gastritis and can produce multi-virulence agents, damage the gastric mucosa, stimulate gastric body to release inflammation medium, activate various cytokines and promote inflammation reaction. *H pylori* infection damages the gastric mucosa and glandular cells, stimulates crypt epithelia and muscularis mucosa hyperplasia with eminent mucus. If the damage factors continue their existence, the pathological changes can progress to intestinal metaplasia or atypical hyperplasia and even

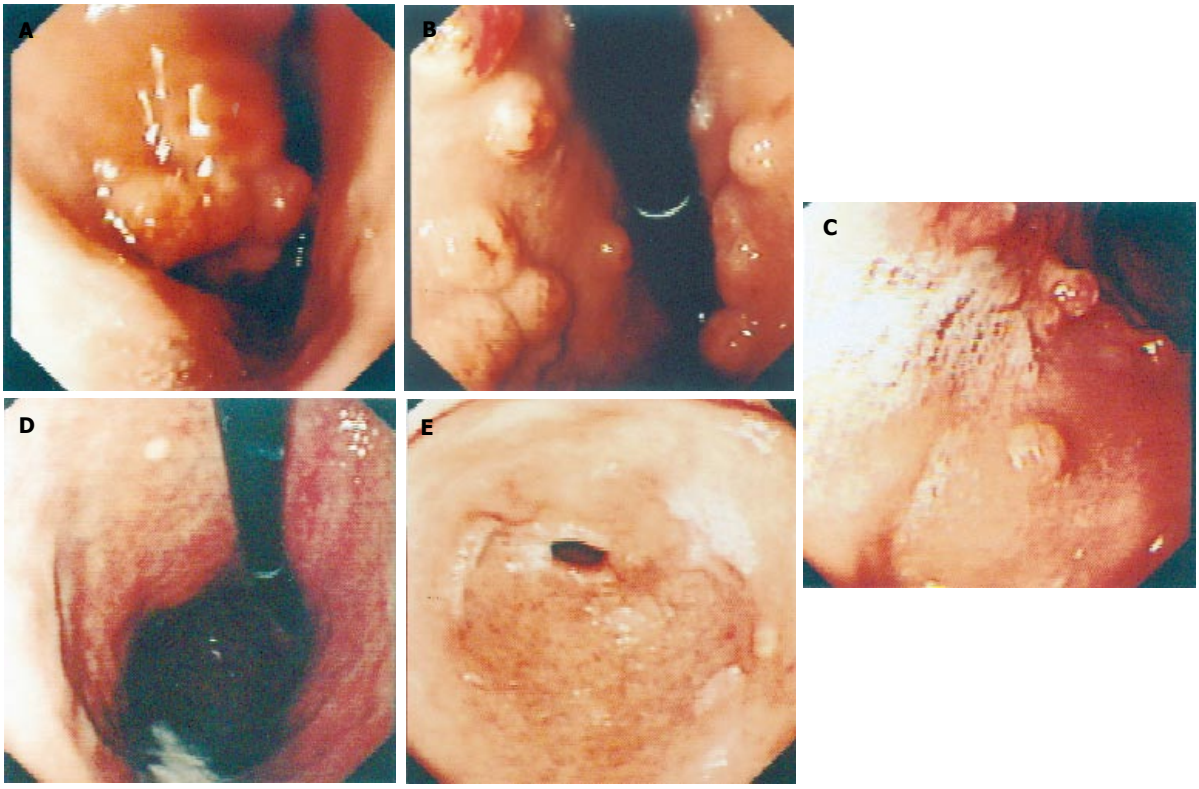

Figure 1 Effect of drug treatment on hyperplastic gastric polyps infected with *H pyori*. **A** and **B**: Multiple gastric polyps of the antrum and body; **C**: Decreased size of gastric polyps 7 mo after treatment; **D** and **E**: Disappearance of gastric polyps 11 mo after treatment.

Table 2 Inflammation status in treatment and control groups

Variable	Treatment groups (n = 22)	Control groups (n = 21)	P
Histologic findings			
Inflammation	1.6, 1.5	2.4, 2.3	<0.01
Activity	1.1, 1.3	2.4, 2.5	<0.01
Atrophy	2.2, 2.1	2.4, 2.4	>0.05
Metaplasia	0.8, 0.6	0.9, 0.7	>0.05

carcinoma. Yasunaga *et al* [10] reported that increased production of interleukin-1 beta and hepatocyte growth factor due to *H pylori* infection may contribute to thickening of the stomach by stimulating epithelial cell proliferation and foveolar hyperplasia in patients with enlarged fold gastritis, leading to formation of hyperplastic polyps.

Although hyperplastic gastric polyps did not accompany malignant transformation during the 9-12 month follow-up period in our study, *H pylori* infection is closely associated with gastric carcinoma. We therefore recommend that when hyperplastic gastric polyps are detected during endoscopy, serologic and pathologic tests should be done to detect and eradicatw *H pylori*.

Though the risk of hyperplastic gastric polyps conversing into cancer is very low, large hyperplastic gastric polyps should be snared and removed completely. Because of the malignant potential, all gastric polyps (0.5 cm or larger in diameter) should be removed. In addition, about 1 year after eradication of *H pylori* (4 patients in this study), any remaining hyperplastic polyps should be removed endoscopically because of the potential for development of cancer.

In conclusion, *H pylori* infection is related with hyperplastic gastric polyps and inflammatory cell infiltration, eradication of *H pylori* can prevent formation of hyperplastic gastric polyps.

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RAPID COMMUNICATION

Melanoma differentiation-associated gene-7, MDA-7/IL-24, selectively induces growth suppression, apoptosis in human hepatocellular carcinoma cell line HepG2 by replication-incompetent adenovirus vector

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Abstract

AIM: To investigate the effect of replication-incompetent adenovirus vector expressing MDA-7/IL-24 on tumor growth and apoptosis in human hepatocellular carcinoma (HCC) cell line HepG2 and normal liver cell line L02.

METHODS: We constructed the recombinant replication-incompetent Ad.mda-7 virus vector and infected it into the human HCC cell line HepG2 and normal liver cell line L02. RT-PCR was performed to detect the mRNA expressing in cells. by ELISA was used to detect MDA-7/IL-24 protein expression in the culture supernatant. The effect of apoptosis induced by Ad.mda-7 was confirmed by Hoechst staining and flow cytometry assay with Annexin-V and PI staining. MTT assay was used to determine growth inhibition of HepG2 cells, and cell-cycle and hypodiploidy analyses were performed by flow cytometry.

RESULTS: Recombinant replication-defective virus expressing MDA-7/IL-24 was constructed successfully. RT-PCR showed that the Ad.mda-7 could mediate the expression of the exogenous gene MDA-7/IL-24 into HepG2 and L02. The concentration of MDA-7/IL-24 protein in supernatant was 130 pg/mL and 110 pg/mL in Ad.mda-7-infected L02 and HepG2 cells, respectively. Ad.mda-7 infection obviously induced apoptosis (from 2.60±0.72% to 33.6±13.2%, $P = 0.00012$) and growth suppression in HepG2 (inhibition ratio IR = 68%) and an increase in the percentage of specific cancer cell types at the G2/M phase of the cell cycle (from 6.44% to 32.29%, $P < 0.01$), but not in L02 cells.

CONCLUSION: These results confirm selectively induction of apoptosis and growth suppression by the mda-7/IL-24 gene with replication-incompetent adenovirus vector in human hepatocellular carcinoma cell line HepG2.

Key words: Cancer gene therapy; Hepatocellular carcinoma (HCC); Apoptosis; Growth suppression; MDA-7/IL-24

Wang CJ, Xue XB, Yi JL, Chen K, Zheng JW, Wang J, Zeng JP, Xu RH. Melanoma differentiation-associated gene-7, MDA-7/IL-24, selectively induces growth suppression, apoptosis in human hepatocellular carcinoma cell line HepG2 by replication-incompetent adenovirus vector. *World J Gastroenterol* 2006; 12(11): 1774-1779

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INTRODUCTION

Primary hepatocellular carcinoma (HCC) is one of the most common lethal malignant tumors in the world[1], causing an estimated 1 250 000 deaths every year worldwide. Unfortunately, about 50% new cases are from China. The clinical therapies for HCC include surgical resection and liver transplantation, but only few HCC patients can receive these treatments. Moreover, the recurrent rate is very high even the patients received surgical treatments. Besides clinical therapies, the gene therapies for HCC are running, including transgenic therapy by antioncogenes such as p53 and Rb, anti-sense nucleotide technique, drug gene therapy such as suicide gene-like HSV-TK, tumor vaccine, and so on. However, the clinical effect of gene therapies was limited because these genes were not specific to tumor cells, which means they kill the normal cells and the tumor cells simultaneously. The research on the treatment protocol selectively killing tumor cells but not influencing normal cells has become a hot topic of research on tumor treatment[2]. Melanoma differentiation-associated gene-7 (MDA-7)/IL-24 was identified by a combination of recombinant fibroblast interferon (IFN-b) and the protein kinase C activator mezerein (MEZ) subtraction hybridization by Fisher in 1995[3], according

to the chromosomal location on 1q32, the presence of a secretory signal, its association with specific cells of the immune system, and the ability of MDA-7 protein to act as an immune modulator. Now mda-7 has been renamed IL-24[4-7].

Some studies indicated that over-expression of mda-7/IL-24 by a replication-defective adenovirus vector results in growth suppression and apoptosis in a broad range of different carcinoma cell lines, including mesotheliomas[8], osteosarcoma[8], melanoma[2,3,9,10], and carcinomas of the lung[11,12], breast[13], pancreas[14], glioblastoma[15,16] and prostate[17]. The anti-tumor effects were independent of the genomic status of p53, RB, p16[2]. Although MDA-7/IL-24 has the properties potentiality kill many different types of cancer cells, it has not any harmful effects in many kinds of normal cells. These unique potentiality of mda-7/IL-24 suggested that this gene could prove beneficial for cancer gene therapy[2]. In the present study, we investigated the impact of Ad.mda-7 on growth, cell cycle and survival of human HCC cell line HepG2 and normal liver cell line L02, resulting in selectively induction of apoptosis and growth suppression by the mda-7/IL-24 gene with replication-incompetent adenovirus vector. In these contexts, the study provides important support to the use of Ad.mda-7 for selective cancer gene therapy for HCC.

MATERIALS AND METHODS

Cell lines, transfection and culture conditions

Human HCC cell line HepG2 and normal human liver cells line L02 (gift from Dr Guanjian) were cultured in high glucose DMEM supplemented with 100 mL/L fetal bovine serum (FBS) at 37℃ in a humidified incubator containing 50 mL/L CO_2 95% in air.

Virus construction, identification and purification

The recombinant replication-defective Ad.mda-7 virus was created in our laboratory. Briefly, human MDA-7/IL-24 cDNA was directionally cloned into pSGCV to produce pSGCMV-MDA7. By using plasmid transfection method, the pSGCMV-MDA7 and adenovirus skeletal plasmid were co-transfected to HEK293 cells to construct the recombinant adenovirus vector Ad.mda-7, carrying MDA-7/IL-24 gene, by intracellular homologous recombinant. The genomes were analyzed to confirm the recombinant structure and then the virus was plaque purified and amplificated in 293 cells.

RNA isolation and RT-PCR

After cells infected with 1 000 VP/cell (virus particle/cell) of Ad.vec and Ad.mda-7, respectively, were harvested at 48 h, total RNA was extracted from cells using the Qiagen RNeasy mini kit (USA) according to the manufacturer's protocol. Primers used in PCR were designed according to the reported IL-24 cDNA sequence. The primer sequences were 5'-GGGCTGTGAAAGACACTAT-3' (forward) and 5'-GCATCCAGGTCAGAAGAA-3' (reverse). The primer sequences of β-actin were 5'-CCTTCCTGGGCAATGGAGTCCT-3' (forward) and 5'-GGAACAATGATCTTGATCTT-3' (reverse). The reaction mixture with corresponding primers was amplified through 30 cycles, each cycle consisting of denaturation at 94℃ for 30 s, primer annealing at 55℃ for 30 s, and extension at 72℃ for 30 s. Cycles were preceded by incubation at 95℃ for 3 min to ensure the full denaturation of the target gene and also an extra incubation at 72℃ for 5 min to ensure full extension of the product. The products of PCR were analyzed on 10 g/L agarose gel electrophoresis.

ELISA assay

The cell culture supernatant was collected and stored at -20℃ until use. A total of 100 μL of the supernatant was added to Microplate wells, and 200 μL of anti IL-24 (Biotin USA) was added 10 s later. After incubation at 37℃ for 30 min, the sample was washed 5 times with wash buffer, followed by addition of 200 μL of HRP to each well following an incubation at 37℃ for 30 min. Then the wells were washed 5 times, followed by addition of 100 μL of TMB and incubation in room temperature with protection from light. Twenty minutes later, 100 μL of stop solution was added, and the absorbance was read on a microplate reader at 450 nm. All experiments were performed in duplicate. Finally, we calculated the value of results with standard curve.

MTT assay to determine cell growth

Cells were seeded in 96-well tissue culture plates ($1x10^3$ cells/well) and treated with PBS, 1 000 VP/cell of Ad.mda-7 and Ad.vec (1 000 VP/cell), respectively, at the next day. At the indicated time points, the medium was removed, and fresh medium containing 0.5 mg/mL MTT (Roche Diagnostics GmbH Co., Germany) was added to each well. The cells were incubated at 37℃ for 4 h, followed by addition of about 150 μL of solubilization solution (0.01 mol/L HCl in 100 g/L SDS) to each well, and incubation of cells for a further 10 min at 37℃ with gentle shaking. The optical density of the plates was read on a microplate reader at 540 nm.

Hoechst staining test (fluorescent microscopy evaluation of cell apoptosis)

After 48 h of infection, cells were washed once with PBS and fixed in 40 g/L paraformaldehyde for 30 min at room temperature. After two washes with PBS, cells were stained for 30 min in the dark at room temperature with 0.05 mg/mL Hoechst 33258 (Sigma USA) in PBS. Nuclear fragmentation was visualized using a fluorescence microscope equipped with a UV-2A filter and Olympus BX60 photographic camera. Apoptotic cells were identified by condensation of nuclear chromatin and its fragmentation.

Apoptosis and necrosis assay (Annexin-V-PI assay)

Cells were trypsinized and washed once with complete media. Aliquots of cells (5×10^5) were resuspended in complete media (0.5 mL) and stained with FITC-labeled Annexin-V (Jinmei Co., China) according to the manufacturer's instructions. Propidium iodide (PI) was added to the samples after staining with Annexin-V

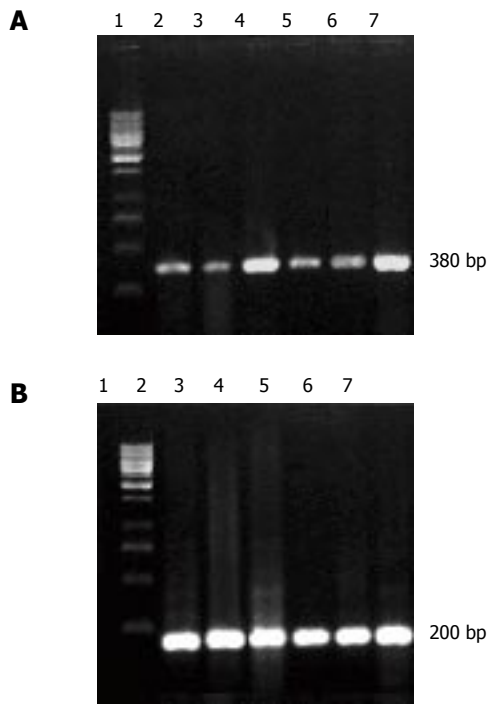

Figure 1 Expression of mda-7/IL-24 mRNA. Infection of normal human liver cells and HCC with Ad.mda-7 resulted in an expression of mda-7/IL-24 mRNA. Cells infected with 1 000 VP/cell of Ad.vec or Ad.mda-7 were harvested at 48 h, treated as described in "Materials and Methods". The total RNA was extracted and the RT-PCR was performed. (**A**) Lane 1: marker; lane 2: control L02 cells; lane 3: Ad.vec-infected L02 cells; lane 4: Ad.mda-7-infected L02 cells; lane 5: control HepG2 cells; lane 6: Ad.vec-infected HepG2 cells; lane 7: Ad.mda-7-infected HepG2 cells. (**B**) Expression of β-actin.

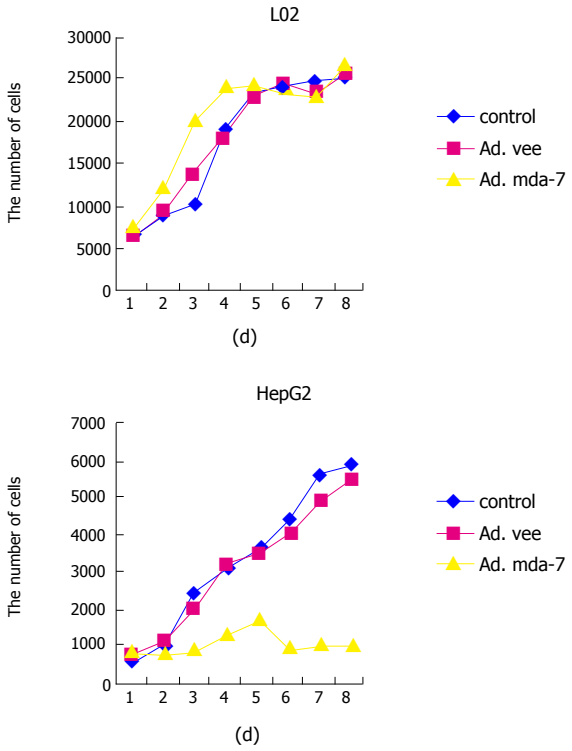

Figure 2 Ad.mda-7 expression causing in vitro inhibition of growth of HCC cells, but not normal liver cells. The various cell types were uninfected (control) or infected with 1 000 VP/cell of Ad.vec or Ad.mda-7. Cell numbers were determined over an 8-d period. Cells were seeded in 96-well plates and treated the next day as described in 'Materials and Methods'. After 24 h, the medium was removed, and cells were stained with MTT. Experiments were repeated three times in quadruplicates, and the results were presented as mean±SE.

to distinguish late apoptotic and necrotic cells. Flow cytometry (Becton Dickinson, San Jose, CA, USA) was performed immediately after staining.

Cell-cycle and hypodiploidy analyses

Cells were cultured as aforementioned. After reaching 30% confluence, the cells were treated with DMEM without FCS for 24 h for synchronization. At the next day, cells were treated with PBS, Ad.vec and Ad.mda-7, respectively. Forty-eight hours later, cells were trypsinized, washed with PBS and fixed in 700 mL/L ethanol overnight at -20℃. Cells were then washed with PBS, and aliquots of $1x10^6$ cells were resuspended in 1 mL of PBS containing 1 mg/mL of RNase A and 0.5 mg/mL of PI. After 30 min of incubation, cells were analyzed by flow cytometry using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA).

Statistical analysis

All the experiments were performed at least three times. The results were expressed as mean ± SE. Statistical comparisons were made using an unpaired two-tailed Student's t test. A $P<0.05$ was considered statistically significant.

RESULTS

mRNA expression of MDA-7/IL-24 gene

To determine the efficiency of transgenic expression, HepG2 and L02 cells were infected with Ad.vec and Ad.mda-7, and mRNA expression was detected using RT-PCR (Figure 1). The results suggested that the expression of MDA-7/IL-24 mRNA could be detected in both HepG2 and L02 cell lines infected by Ad.mda-7 but not by Ad.vec or control team. MDA-7/IL-24 gene could be expressed in cells with infected with Ad.mda-7.

Protein expression of MDA-7/IL-24

Secreting MDA-7/IL-24 protein was confirmed by ELISA assay after Ad.mda-7 infection. After 48 h of L02 and HepG2 cells infection with Ad.mda-7, the concentration of MDA-7/IL-24 protein in the supernatant was detected 130 pg/mL and 110 pg/mL, respectively, whereas no MDA-7/IL-24 protein expression was detected in L02 and HepG2 cells treated with PBS and Ad.vec.

Growth suppression and viability assays

HCC cell line HepG2 and normal liver cell line L02 were infected with Ad.mda-7 and proliferation and cell viability were determined by MTT. As shown in Figure 2, no proliferation arrest effect was observed on normal liver cell line L02 of Ad.vec, Ad.mda-7 or control groups. However, the anti-proliferative activity of Ad.mda-7 was readily apparent in HCC cell line HepG2, and the inhibition ratio was 68%.

Apoptotic effect determined by Hoechst staining

We observed that Ad.mda-7 infection induced apoptosis of human HCC cell line HepG2. As shown in Figure 3, a

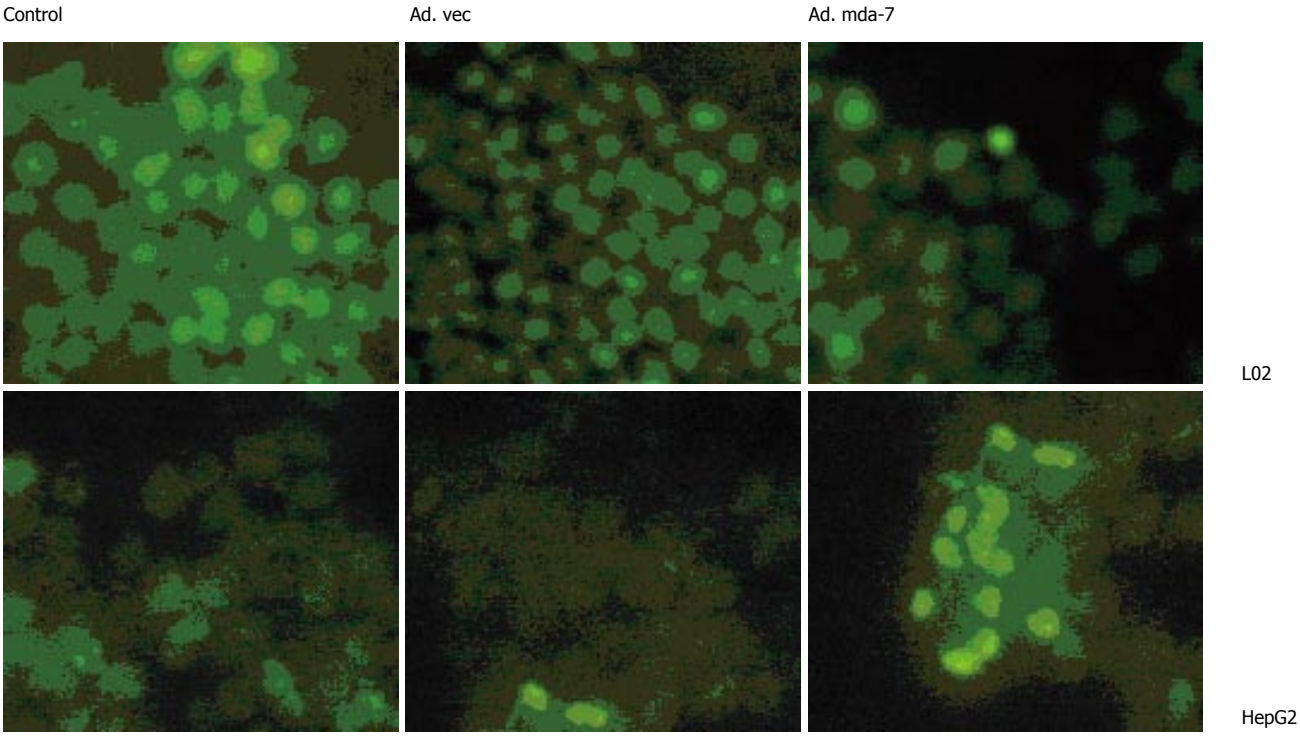

Figure 3 Over-expression of mda-7 causes apoptosis selectively in HCC cells (400×). Cells were infected with 1 000 VP/cell of Ad.mda-7 and all assays were performed 48 h after infection as described in 'Materials and Methods'. To evaluate characteristic apoptotic morphology with fluorescence microscopy, the cells were seeded on glass slides and fixed with 40 g/L paraformaldehyde in PBS for 1 h at room temperature. After washing twice with PBS, cells were stained for 30 min in the dark at room temperature with 0.05 mg/mL Hoechst 33258 in PBS. The nuclear fragmentations were visualized by using a fluorescence microscope equipped with a UV-2A filter and Olympus BX60 photographic camera. Apoptotic cells were recognized by condensation of nuclear chromatin and its fragmentation.

Table 1 Over-expression of mda-7 induces apoptosis selectively in HCC cells

	L02			HepG2		
	Control	Ad.vec	Ad.mda-7	Control	Ad.vec	Ad.mda-7
Early apoptotic cells	110±23	120±36	100±11	50±37[b]	300±85[d]	1 990±430
Late apoptotic cells	60±14	100±21	130±15	10±40[b]	200±63[d]	1 370±902
Total cells	170±28	220±45	230±16	60±72[b]	500±150[d]	3 360±132

The table shows the apoptotic cells in 10 000 cells. The percentage of early apoptotic cells (stained with Annexin-V only) and late apoptotic and necrotic cells (stained with PI) was calculated using the CellQuest software (Becton Dickinson, San Jose, CA, USA). [b]$P<0.01$ *vs* Ad.mda-7 group; [d]$P<0.01$ *vs* Ad.mda-7 group.

significantly higher apoptotic rate was observed in HepG2 cells infected with Ad.mda-7 (48%) compared to those infected with Ad.vec (1.6%) and the control group (1.1%) (counted 1 000 cells consecutively) ($P<0.005$), whereas no apparent changes were observed in normal liver cell L02, the rate of apoptosis being 1.7%, 1.9% and 2.1%, respectively ($P>0.05$). These data indicated that MDA-7/IL-24 could induce apoptosis in HepG2.

Evaluation of apoptosis effect by flow cytometry

Annexin-V and PI staining assays with flow cytometry quantified the effect of Ad.mda-7 on apoptosis induction in human HCC cell HepG2 and normal liver cell L02 (Table 1). We observed a significantly increased percentage of apoptotic HCC cells HepG2 infected with Ad.mda-7 as compared to HepG2 infected with Ad.vec and the control cells. In contrast, L02 cells did not show significantly increased apoptotic rate after being infected with Ad.mda-7. Thus, these results suggested that Ad.mda-7 infection could kill HCC cells but not normal liver cells.

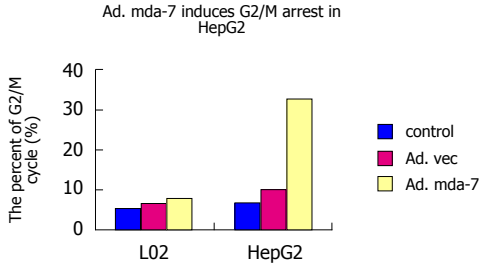

Figure 4 Induction of G2/M arrest in HCC cells HepG2, but not in normal liver cells L02 by Ad.mda-7 infection.

Cell cycle analysis by flow cytometry

Cell cycle phase assayed by flow cytometry after the fixed cells were stained with PI. As shown in Figure 4, Ad.mda-7 markedly induced a G_2/M accumulation in HepG2 cell lines, showing that the rates of G_2/M phase cells were 32.29% in Ad.mda-7 group, 10.30% in Ad.vec and 6.44% in control groups ($P<0.01$). However, the

rates of G_2/M phase cells were 7.95%, 6.65% and 5.54% in L02 cells of Ad.mda-7, Ad.vec and control groups, respectively ($P > 0.05$), demonstrating only minimal G_2/M accumulation in normal liver cells L02. Thus, these results suggested that Ad.mda-7 infection could significantly induce an increase in the percentage of HCC cell line HepG2 in the G2/M phase of the cell cycle, but not in normal liver cell line L02.

DISCUSSION

A study by Jiang *et al*[3] demonstrated that melanoma differentiation-associated gene-7 (MDA-7/IL-24) was highly expressed in melanocytes, but low expressed in melanoma cells, and because of the inducing capacity of growth arrest and differentiation in human melanoma cell line, named MDA-7. After the MDA-7/IL-24 was transfected into malignant melanoma cells, it could obviously block the growth, and promote apoptosis and differentiation, even reverse the malignancy of the melanoma[3]. Now it is clear that the MDA-7/IL-24 gene has been localized to chromosome 1q32 and its exon/intron structure has been established. Mda-7 is composed of seven exons with the first exon being noncoding. The chromosomal assignment and gene structure have been independently confirmed[4-6]. The region includes IL-10, IL-19, IL-20 and mda-7[4]. Moreover, based on structure of protein and the receptor, the gene has been reclassified and grouped into a newly recognized family of IL-10-related interleukins, IL-24[7]. Several independent studies have demonstrated that over-expression of the MDA-7/IL-24 gene, using vectors either plasmid or a replication-defective adenovirus, resulted in growth arrest and induction of apoptosis in a broad range of cancer cells, including lung cancer[11,12], breast cancer[13], pancreatic cancer[14], glioma[15,16], and prostate cancer[17]. Some signal transduction pathway and molecules have been reported as being regulated during mda-7-induced tumor suppression, including activation of the caspase cascade, PKR, p38, STAT3, PI3K, GSK-3, ILK-1, BAX, BAK, Fas, DR4, TRAIL, inducible nitric oxide synthase (iNOS), IRF-1, IRF-2 and p53[8]. Treatment of tumor cells with Ad-mda7 resulted in an increase in cells in the G2/M cell cycle phase[2]. In comparison to the antioncogenes, such as p53, the inhibitory effect of MDA-7/IL-24 on the growth of cancer cells was not related with the state of antioncogenes in these cancer cells (p53, Rb, or p16ink4)[2,11,14], so it could be used in cancer treatment more effectively without the influence in the expression of these anticancer genes in various kinds of cell lines. Surprisingly, the previous studies revealed that MDA-7/IL-24 gene had no any toxic and side effects on the normal cells, such as epidermal cells, lung fibroblasts, breast cells, prostate and lung epithelia, astrocytes, endothelia, melanocytes and so on[2,3], suggesting its selective property to malignant tumors. Therefore, MDA-7/IL-24 is considered a unique cytokine –tumor suppressor in the IL-10 family and there is no any toxic effect in normal cells, suggesting it as a perfect gene for use as a human cancer gene therapy.

In this study, the replication-incompetent adenovirus vector carrying MDA-7/IL-24 was successfully constructed and transfected into human normal liver cell line L02 and HCC cell line HepG2. Its effects on the two kinds of cells were observed, which provided the theoretical foundation for its application in the gene therapy for HCC in clinical practice. RT-PCR indicated MDA-7/IL-24 was successfully transfected into L02 and HepG2 cells with Ad.mda-7, but the control and Ad.vec groups could not show the mRNA expression. The protein expression was confirmed by ELISA assay and the effect was very different, although protein expression was seen both in L02 and HepG2. MTT assay revealed the capability of Ad.mda-7 in tumor growth arrest of HCC cell line HepG2, but not of normal liver cell line L02, indicating that MDA-7/IL-24 induces growth arrest only in HCC cells. Like the pervious studies, Ad.mda-7 induced a G_2/M accumulation in HCC cell line HepG2, but not in normal liver cell line L02. Moreover, infection with Ad.mda-7 could increase the percentage of apoptotic cells apparently in HCC cells. On contrary, no increased percentage of apoptotic cells appeared in L02 cells.

In conclusion, Ad.mda-7 can induce the gene MDA-7/IL-24 expression in normal liver cells and hepatocellular carcinoma cells. Over-expression of MDA-7/IL-24 obviously induces the apoptosis and growth suppression in hepatocellular carcinoma cell line HepG2, without any toxic effect on normal liver cell line L02. These findings provide support for future clinical applications of MDA-7/IL-24 in the gene therapy of hepatocellular carcinoma.

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CASE REPORT

Tubulovillous adenoma of anal canal: A case report

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Abstract

Tumors arising from the anal canal are usually of epithelial origin and are mostly squamous cell carcinoma or basal cell carcinoma. We present a case of benign anal adenomas arising from the anus, an extremely rare diagnosis. A 78-year-old white man presented with rectal bleeding of several months duration. Examination revealed a 4 cm friable mass attached to the anus by a stalk. At surgery, the mass was grasped with a Babcock forceps and was resected using electrocautery. Microscopic examination revealed a tubulovillus adenoma with no areas of high grade dysplasia or malignant transformation. The squamocolumnar junction was visible at the edges of the lesion confirming the anal origin of the tumor. We believe the tubulovillus adenoma arose from either an anal gland or its duct that opens into the anus. Although seen rarely, it is important to recognize and treat these tumors at an early stage because of their potential to transform into adenocarcinoma.

Key words: Tubulovillous adenoma; Anal canal

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INTRODUCTION

The anal canal is lined by transitional mucosa in its proximal one-half and by stratified squamous epithelium in the distal portion. Anal glands and ducts arise from this area and are lined by stratified columnar epithelium. The anal glands have secretary functions and help lubricate the anal canal[1]. Tumors of the anal canal are uncommon and are classified according to their tissue of origin. These consist of tumors of epithelial origin (squamous cell carcinoma, basal cell carcinoma, adenosquamous carcinoma), lymphoid tissue (lymphoid polyp, malignant lymphoma), mesenchymal tissue (fibroma, fibrosarcoma, leiomyoma, leiomyosarcoma), neural tissues (neurofibroma, ganglioneuroma), and vascular tissue (hemangioma, angiosarcoma)[2].

The most common tumor of the anal canal is squamous cell carcinoma, which arises from the squamous epithelium of the anal canal. Rarely, adenocarcinoma of the anus has been recognized and is believed to arise from the anal glands or ducts. There is a single report of multiple adenomatous polyps arising in the transitional zone of the anus in a patient with familial adenomatous polyposis (FAP), seven years after colon resection and ileo-anal anastomosis[3].

We describe a patient with a tubulovillus adenoma arising from the anal canal. These tumors are rarely encountered in patients without predisposing risk factors, such as FAP, ulcerative colitis or Crohn's disease.

CASE REPORT

The patient was a 78 years old white man who presented with history of rectal bleeding for several months. Bleeding occurred with almost every bowel movement and consisted of passage of fresh blood. The patient was otherwise healthy with no recent change in bowel habit, weight loss or reduced appetite. The patient was referred to the gastrointestinal service for work-up of rectal bleeding. His past history was positive only for hypertension. General physical examination was unremarkable. At the time of colonoscopy, rectal examination revealed a 4 cm friable mass attached to the anus by a definite stalk. The mass was located entirely outside the anal canal, with no extension into the rectum. Colonoscopy showed polyps in transverse (tubular adenoma), descending (tubulovillus adenoma with high grade dysplasia) and sigmoid colon (villous adenoma), which were removed endoscopically by the snare technique. At surgery, the anal polyp was found to be attached to the anus by a stalk. The mass was grasped with a Babcock forceps and was resected using electrocautery.

Gross examination of the specimen showed a tan-red, soft tissue mass, measuring 4.0 cm x 1.4 cm x 1.2 cm (Figure 1A). Microscopic examination revealed a tubulovillus adenoma (TVA), with nearly 30% of the surface of the polyp showing villous architecture (Figure 1B). There were no areas of high grade dysplasia or malignant transformation. The squamocolumnar junction was visible

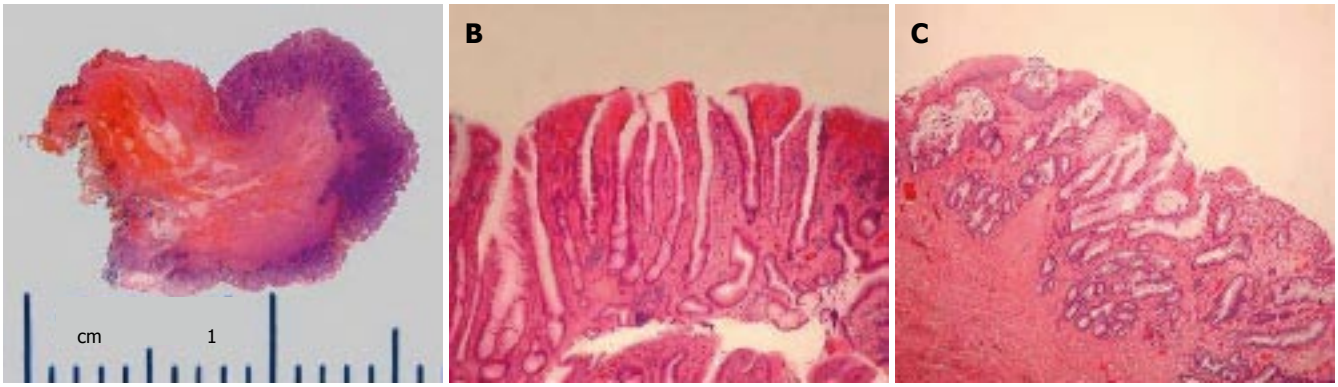

Figure 1 A: Gross appearance of the tubulovillus adenoma mounted on a glass slide. **B:** Areas with villous architecture were apparent (Hematoxylin and Eosin, original magnification x 400). **C:** The squamocolumnar junction is seen at the upper left (arrow). No atypical features were noted in the squamous component. (Hematoxylin and Eosin, original magnification x 400).

at the edges of the lesion and did not show any atypical changes (Figure 1C).

DISCUSSION

The anal canal is lined by stratified squamous epithelium and the most common tumor arising from this site is squamous cell carcinoma. Rarely, adenocarcinomas have been reported and these are believed to arise form the anal glands or their ducts which open into the anus. The current concept of the etiology of colorectal adenocarcinoma is based on the multistep genetic sequence of events which lead to the transformation of normal mucosa to adenoma and finally carcinoma[4]. That a similar sequence may occur in the development of adenocarcinoma of the anus was shown in a recent study which described the development of an invasive apocrine adenocarcinoma arising from a benign adenoma in the perianal region of a 45 year old women[5]. In our patient, the polyp was attached to the anus by a narrow stalk. Moreover, at histopathology, squamocolumnar junction was visible at the edges of the histological specimen confirming that the polyp arose from the anus and not from the rectum. We believe the site of origin of the tubulovillus adenoma in our patient was from one of the anal glands which are the only adenomatous elements in the anus. It is unclear why such tumors are not diagnosed more frequently. It is possible that because of the submucosal location of the anal glands, such adenomas are not easily visible and only become apparent when malignant transformation takes place. Clinically, it is important to recognize and treat these tumors at an early stage because of their potential to transform into adenocarcinoma.

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CASE REPORT

Acute biliary pancreatitis and cholecystolithiasis in a child: One time treatment with laparoendoscopic "Rendez-vous" procedure

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Abstract

Acute biliary pancreatitis (ABP) is rare in childhood and endoscopic sphincterotomy should be avoided in the child due to the risk of both early and late complications but, when necessary, the optimal timing between endoscopic procedure and cholecystectomy is still uncertain. A nine years old child with acute biliary pancreatitis underwent successfull laparo-endoscopic "Rendez-Vous" procedure in which endoscopic drainage of the common bile duct and laparoscopic cholecystectomy were performed simultaneously. This is the first case reported of laparo-endoscopic Rendez-Vous in a child. The excellent outcome of this patient and the review of the literature concerning other available options for the treatment of such cases suggest that this procedure offers great advantages, especially in children, of reducing the required number of treatments, the risk of ineffectiveness, the number of anaesthesia, the length of hospital stay and the risk of iatrogenic morbidity.

Key words: Acute biliary pancreatitis; Gallstones; ERCP; Laparoscopic cholecystectomy; Endoscopic sphyncterotomy

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INTRODUCTION

Acute biliary pancreatitis (ABP) is rare in childhood and endoscopic sphincterotomy should be avoided in the child due to the risk of both early and late complications but, when necessary, the optimal timing between endoscopic procedure and cholecystectomy is still uncertain. In the child the literature concerning therapeutic ERCP with ES for ABP is scarce, and ERCP, combined with ES or not, carries also the well known risk of iatrogenic acute pancreatitis. The optimal diagnostic and therapeutic approach and the timing of the procedures in children with cholecystolithiasis will often depend on the level of expertise of both the biliary endoscopist and laparoscopist. A nine years old child with acute biliary pancreatitis underwent the first successfull laparo-endoscopic "Rendez-Vous" procedure in which endoscopic drainage of the common bile duct and laparoscopic cholecystectomy were performed simultaneously. The favourable outcome of the reported case led us to deepen this issue.

CASE REPORT

A nine-year-old girl, weighing 52 kilograms, BMI of 27, was urgently admitted to our department because of pain in the right upper abdomen associated with vomiting. The only abnormal laboratory tests were the serum ALT 163 u/L (normal value 10-55), amylase 131 U/L (normal value < 110), lipase 482 U/L (normal value < 300), and ALP 168 U/L (normal value 38-126). Abdominal ultrasonography revealed multiple gallstones (3-5 millimeter) and sludge in the gallbladder and a normal common bile duct (CBD). The patient was kept NPO with intravenous fluids and analgesics and was discharged from the hospital after being asymptomatic for 24 h against medical advice, because her parents refused laparoscopic cholecystectomy (LC). The patient was followed in the paediatric outpatient clinic where metabolic or haematologic causes of cholecystolithiasis were excluded.

Three weeks later the patient was again urgently admitted because of a recurrence of abdominal pain, vomiting and a fever (38.6℃). Ultrasonography showed gallstones, a dilated CBD (8 mm) and pancreatic edema. Laboratory test showed a nine fold increased serum amylase of 998 U/L and of serum lipase 2430 U/L. Total and direct bilirubin were both increased to 2.81 mg/dl and 1.58 (normal

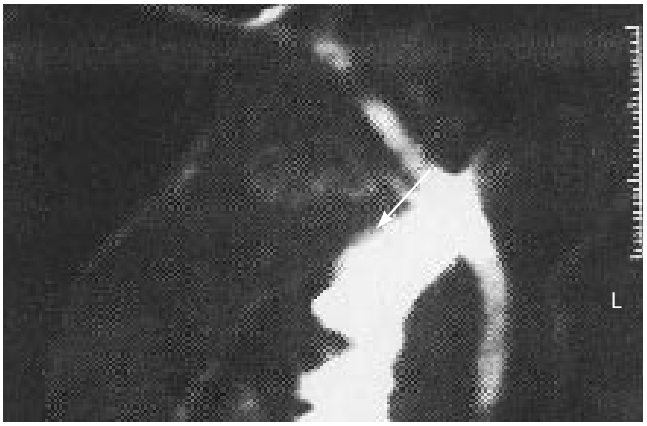

Figure 1 Cholangio-MRI showing a persistent filling defect (arrow) in the extrahepatic bile duct suspected to be biliary sludge or a stone.

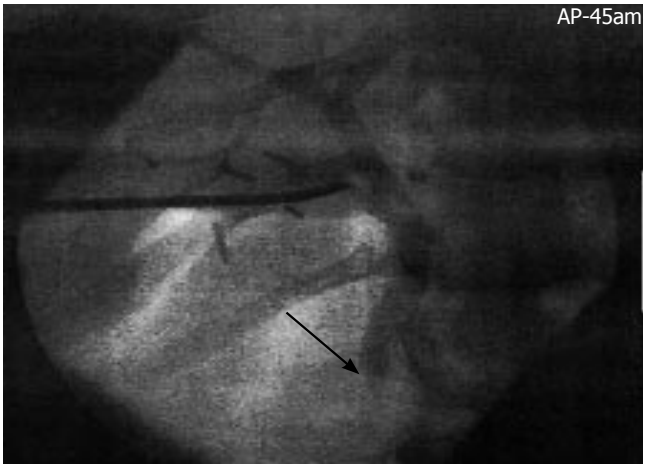

Figure 2 Intra-operative transcystic cholangiography 30 min after contrast medium injection showing the stop of contrast medium passage into the duodenum and a vanished image of the papilla of Vater (arrow) due to occluding biliary sludge.

value 1.3 and 0.5) respectively and other pathologic parameters were: gamma GT 479 U/L (normal value 78), ALT 1032 u/L, AST 584 U/L (normal value 10-45), LDH 1855 U/L (normal value 313-618), ALP 283 U/L and C reactive protein (CRP) 6.8 mg/d (normal value <1). White blood cell count (WBC) was 13 200 (normal value 6000-9000) with 91% polymorphonucleates, and blood glucose was 132 mg/dL. According to the Atlanta classification and Ranson's criteria[1] the diagnosis of mild acute biliary pancreatitis was given and a conservative treatment was started with fluids, analgesics and imipenem. The main symptoms disappeared after 12 h so that the conservative treatment was continued. After 48 h a CT scan showed a pancreatic and peripancreatic oedema that was classified as Balthasar stage B[2]. The bilirubin remained slightly abnormal (1.62 total and 0.74 direct) but serum amylase and lipase were increased to 1300 and 30560 U/L respectively, whereas CRP reduced to 4.4 mg/dL. A cholangio-MRI was performed (Figure 1) showing a filling defect in the upper portion of the CBD that was highly suspicious for a gallstone, sludge or mucinous aggregate.

Based on increased experience in adults with cholecystocholedocolithiasis, we decided to perform a laparoendoscopic "Rendez-vous" for both the endoscopic drainage of the CBD and treatment of gallstones despite the fact that there was no evidence in the literature of this procedure being performed on a child that was considered suitable, without contraindication and useful. Laparo-endoscopic "Rendez-Vous" procedure was as follows: The whole laparo-endoscopic procedure was carried out to maintain the patient in the same supine "french" position used for the laparoscopic cholecystectomy. Three trocars (10-10-5 mm) were introduced with open technique. The first manoeuvre was the preparation, encircling and clipping of the cystic duct to avoid migration of stones in the CBD. An intra-operative cholangiography was performed to introduce a Pedinelli catheter (8 fr) in the cystic duct. This showed a dilated CBD and a delayed passage of the contrast medium in the duodenum (Figure 2), but could not confirm the filling defect. The X-ray picture of the whole biliary tree remained unmodified without any flow of the contrast medium in the duodenum, up to 30 minutes after the injection and despite intravenous administration of a dose of antispastic drug (cymetropio bromide). This led us to suspect an underlying sphincter of Oddi dysfunction but a manometry was considered unnecessary because the indication for retrograde exploration of the papilla was given. During the time waiting to control the passage of the contrast medium in the duodenum, the cholecystectomy was carried out. The endoscopist performed the procedure using an adult lateral view duodenoscope. The papilla appeared normal but without any flow of bile. A guide wire could be introduced through the papilla and its correct position inside the still contrasted CBD could be confirmed. In this way the standard and more dangerous unpreventable retrograde injection of the Wirsung duct could be avoided. A paediatric endoscopic balloon catheter Olympus Swift ® (4 mm diameter) was then introduced in the CBD, inflated, and retrieved trough the papilla. Some biliary sludge could be retrieved and immediately bile flowed. This procedure was repeated twice and a gentle dilatation of the papilla was also performed for 2 minutes. The endoscope was then extracted and the laparoscopic procedure ended after clipping of the cystic duct. The post-operative laboratory controls showed the normalization of amylase, lipase and bilirubin the day after. On day two after the procedure a control US showed a normal CBD. The patient was discharged three days after the procedure. Neither recurrence of symptoms, nor abnormality in laboratory tests or US controls occurred up to the last 24 mo follow-up.

DISCUSSION

Acute biliary pancreatitis in the child is rare and the main causes are congenital disorders or malformations of the pancreatic-biliary ductal system. In children, the main indication of diagnostic ERCP with or without endoscopic sphincterotomy (ES) are biliary atresia, intrahepatic cholestasis, anomalous arrangement of the pancreatic-biliary ductal system including the dilatation of the CBD.[3,4,5] In the adult, ES is a part of the treatment of ABP when this is caused by stones or sludge impacted in the papilla.[6,7] Concerning the timing of ERCP +/- ES, early ap-

proach is not more beneficial than conservative treatment in patients without jaundice.[8] In the child the literature concerning therapeutic ERCP with ES for ABP is scarce, especially because the pancreatitis, according to Hsu and Nowak[9,10] is often not "biliary" in the "adult" meaning of the term. In the reported case the pancreatitis was truly "biliary", because related to micro-lithiasis and sludge and also combined with an underlying sphyncter of Oddi dysfunction which can often be related to recurrent pancreatitis also in children.[11] The treatment of ABP in childhood needs a multidisciplinary approach[12] to optimize laparoscopic treatment,[13,14] and intraoperative cholangiography is considered mandatory to minimize unnecessary ERCP.[15] In fact ERCP, combined with ES or not, carries the well known risk of iatrogenic acute pancreatitis because of papilla manipulation, pancreatic intraductal hyperpression and/or chemical damage all factors related to unpreventable retrograde injection of the pancreatic duct.[16,17] When ABP in children is related to gallstones there is a relevant risk of recurrence and therefore the standard treatment is endoscopic treatment sequentially combined days or weeks before or after cholecystectomy.[18,19] The sole case of urgent ERCP-ES in a 9-year-old patient with ABP due to a stone impacted in the papilla was reported recently[10] and was performed under anaesthesiological control of sedation. This patient was discharged after ES, with a planned laparoscopic cholecystectomy and was therefore still at risk of recurrent pancreatitis in the possible event of an incomplete sphincterotomy. There is the well known problem of the optimal timing between ERCP and LC, also considered " The bilateral interface…" between the two procedures[20] but the optimal diagnostic and therapeutic approach in children will often depend on the level of expertise of both the biliary endoscopist and laparoscopist.[19,21,22] All these considerations and the favourable outcome of the case convinced us that simultaneous laparoendoscopic treatment carries great advantages especially in children. The literature shows that comparable good success rate of ERCP between adults and children (98% *vs* 97.5%) with a comparable low incidence of complications (2.5% *vs* 3.4%) are obtainable only by very experienced teams[22] but unfortunately complications can reach up to 33%.[18,21,23] Therefore ERCP in the paediatric population should be minimized because stones often pass spontaneously[19] and also because the only sure way to avoid post-ERCP complications is to avoid ERCP itself.[24] This is certainly possible with the combined laparo-endoscopic approach because ERCP is avoided and ES is limited only to those patients with stones or abnormal cholangiography that cannot be resolved laparoscopically via the cystic duct or when a sphyncter of Oddi dysfunction can not be resolved through a simple dilatation. The balloon papilla dilatation carries comparable effectiveness but with a discordant incidence of relevant complications like pancreatitis that can be related to unnecessary retrograde injection of the pancreatic duct or manipulation, but anyway in children dilatation should be preferred to preserve the papilla.[25,26] The dilatation is also effective in early stone retrieval during the attack of ABP.[27]

Other favourable considerations concerning effectiveness of this combined approach can be found in the literature concerning the standard sequential approach. In a large prospective randomized study[28] the effectiveness of ERCP/ ES to clear CBD stones was only 84%, when performed prior to laparoscopic cholecystectomy and therefore 16% can be ineffective. Unfortunately, these patients often also have to undergo urgent surgery, with exposure to a second X-Ray for the mandatory cholangiography and often the need for a surgical opening of the CBD. This carries the need of relevant surgical skill but carries also an increased risk of complications, often need of T tube or other biliary drain, and prolonged hospitalization[29,30]. In the child both open and laparoscopic surgery and especially suturing of the small duct requires a high degree of surgical expertise to avoid the risk of late stenosis of the CBD. On the other hand, laparoscopic clearance of CBD stones in a large prospective study[28] of adults was impossible in 15% of the cases, despite all being operated on by experienced surgeons, and conversion to open surgery or post-operative ERCP carried a relevant incidence of biliary complications, but the post-operative ERCP can itself be related to risk of ineffectiveness.

A recent analysis of management of suspected common bile duct stones in children[31] shows clearly the poor results and pitfalls of the two common pathways of the sequential treatment with ERCP-ES and laparoscopic cholecystectomy. If performed prior to cholecystectomy a total of 71% of ERCP were unnecessary and 7% failed, if performed after cholecystectomy 50% of ERCP were unnecessary because stones were not found. Despite a reported incidence of 0% complication rate, 9 of 12 ES (75%) were unnecessary based on findings of an intraoperative cholangiography, some days prior. Therefore, a large number of unnecessary invasive procedures, all potentially related to morbidity and mortality, are often ineffective especially because they are out of synch with the evolving pathophysiology of gallstone disease.[19] On the other hand, the simultaneous approach of RV is tailored to a "real time" picture of the CBD and the compliance of the papilla, eliminating all previous and subsequent unnecessary procedures, and also minimizing the factors of ineffectiveness. Concerning ABP, endoscopy and laparoscopy, a recent review[32] pointed out that the sequential approach of ERCP-ES first followed by LC is the least efficient, and despite simultaneous treatment is theoretically the best option, the laparoscopic CBD clearance is considered difficult and time consuming.[32] The main experiences with the simultaneous laparoendoscopic approach[33,34] were always extremely positive, showing a high effectiveness in stone clearance of 97%-100%, a low morbidity and hospital stay comparable to simple laparoscopic cholecystectomy.[35] It is also important to consider that for a child younger than 10 years of age the ERCP usually requires general anaesthesia[5,22] and an additional administration is certainly needed for the successive cholecystectomy. On the other hand, to perform the laparoendoscopic RV only one administration of anaesthesia and related psychological trauma is necessary for the child. The outcome of the case and the analysis of the literature, with a comparison of combined laparoendoscopic Rendez-Vous to the other available options convinced us that considering the technical aspects, the related risk of iatrogenic complications, optimization

of the timing and the effectiveness of the procedure, the laparo-endoscopic Rendez-Vous in these cases is the best option for the child, perhaps also for the adult, the surgeon, the endoscopist and the hospital itself.

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CASE REPORT

Complete pancreatic heterotopia of gallbladder with hypertrophic duct simulating an adenomyoma

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Abstract

The gallbladder is an unusual location of pancreatic heterotopia, defined as the presence of pancreatic tissue lacking anatomical and vascular continuity with the main body of the gland. A 28-year-old man presented with anorexia, nausea and pain in the right upper abdomen. On physical examination, the abdomen was tender to palpation and Murphy sign was positive. The patient underwent a cholecystecomy. This case, in our opinion, is very interesting since it permits to consider a controversial issue in the pathology of the gallbladder. The histological appearance of ductal structure in pancreatic heterotopia resembles the histological picture of both Aschoff-Rokitansky (AR) sinuses and adenomyomas. This finding suggests that these lesions are linked by a common histogenetic origin. We suggest that the finding of an adenomyoma in the gallbladder should prompt an extensive sampling of the organ in order to verify the coexistence of pancreatic rests.

Key words: Pancreatic heterotopia; Gallbladder; Hypertrophic duct; Adenomyoma; Aschoff-Rokitansky sinuses

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INTRODUCTION

The gallbladder is an unusual location of pancreatic heterotopia, defined as the presence of pancreatic tissue lacking anatomical and vascular continuity with the main body of the gland[1]. About 75% of all pancreatic rests are located in the stomach, duodenum and jejunum, the appendix, diverticulum of Meckel and the ampulla of Vater[2] tract, ectopic pancreas has been found in the umbilicus, fallopian tube, mediastinum[2], spleen[3] and omentum[4]. Here, we describe a case of gallbladder pancreatic heterotopia associated with a hypertrophic duct simulating a small intrapancreatic adenomyoma.

CASE REPORT

A 28-year-old man presented with anorexia, nausea and pain in the right upper abdomen. On physical examination, the abdomen was tender to palpation and Murphy sign was positive. The patient underwent surgery, and a cholecystectomy was performed. The gallbladder was 10.5 cm in length and 3.5 cm in width, with a mural thickness of 0.3 cm. No stones were present. An intramural, firm, yellow nodule measuring 0.6 × 0.5 cm was seen in the gallbladder body: it was well circumscribed and showed a central umbilication on the mucosal surface. Light microscopy revealed diffuse proliferation of the surface epithelium, resulting in numerous invaginations into a thickened wall and numerous Aschoff-Rokitansky (AR) sinuses. The muscular layers appeared hypertrophied and possibly hyperplastic, with frequent foci of adenomyomatosis. No inflammatory infiltrate was seen. The epithelial component had typical features of pancreatic tissue. Acini were composed of polygonal cells with basal nuclei and apical granular cytoplasm surrounding a minute lumen. Endocrine islands were occasionally detected. The ductal system consisted of intercalated, intralobular and interlobular ducts: the major duct, surrounded by bundles of smooth muscle, appeared to drain into the gallbladder lumen. Ductal epithelium was surrounded by a smooth muscle component and displayed a typical pancreatic duct-like immunophenotype, characterized by immunoreactivity for cytokeratins 7, 8, 18, 19 and CA19-9. No reactivity against CEA and cytokeratin 20 was found. Acinar cells arranged in a single layer with typical exocrine differentiation were focally positive for α1-antitrypsin, chimotrypsin and cytokeratins 8 and 18. They were negative for CEA, CA19-9 and cytokeratins 7, 19 and 20. The patient, after cholecystectomy, had an uneventful recovery and normal development at follow-up.

DISCUSSION

This case, in our opinion, is very interesting since it

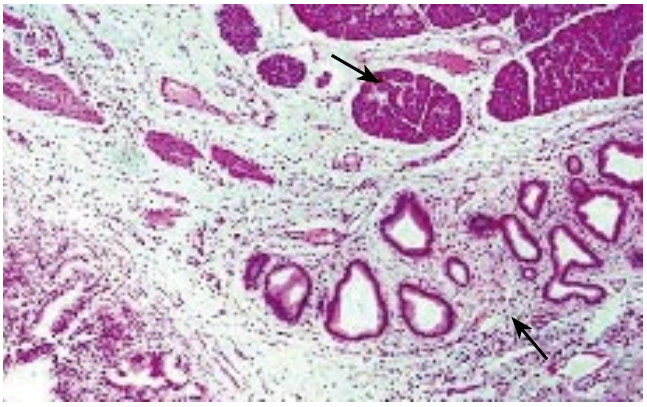

Figure 1 Complete pancreatic ectopia of gallbladder: red arrow indicates acinar components, green arrow indicates ductal structures surrounded by smooth muscle bundles (HE, original magnification, x100).

permits to consider a controversial issue in the pathology of the gallbladder. The histological appearance of ductal structures in pancreatic heterotopia resembles the histological picture of both AR sinuses and adenomyomas. This finding suggests that these lesions are linked by a common histogenetic origin.

In fact, AR sinuses are structures consisting of down growth of the gallbladder mucosa into the wall[5]. They are thought to be the consequence of increased intraluminal pressure, analogous to colonic diverticula[5]. Sometimes, mucosal proliferation is so exuberant as to result in formation of branching sinuses and cystic structures. In many cases, the muscular layer is hypertrophic and hyperplastic, thus resembling focal adenomyomatosis[5]. The latter feature is seen in our case (Figure 1), where the major duct, surrounded by a thickened muscular layer, shows morphological features overlapping those of adenomyomas. Analogously, it is not precisely defined when florid AR sinuses become adenomyomatosis[5].

Likewise, adenomyomas consist of glandular formations lined with cuboidal-to-columnar epithelium and surrounded by bundles of smooth muscle[6,7], similar to the prominent duct seen in our case.

Also heterotopic pancreas, when it includes only ducts, without acinar cells (canalicular or abortive form), shows the same morphological appearance[5].

On the basis of such morphological observations, we believe that AR sinuses, adenomyomas (when focal), adenomyomatosis (when diffuse), and abortive forms of pancreatic heterotopia can be considered variable manifestations of the same pathological process and be grouped into the same nosological category.

Consistent with this hypothesis, several authors have suggested that adenomyomas associated with pancreatic heterotopia found in other organs (stomach and duodenum) should be considered as an abortive variant of pancreatic heterotopia missing an acinar component[7,8]. This hypothesis emphazises the concept of a common pathogenetic origin of these lesions[7,8].

In contrast, Ryan *et al* believed that the adenomyomas of the gallbladder, in spite of histology, arise from diverticular disease of the gallbladder and are not pathogenetically related to heterotopic pancreas[9].

To our knowledge, only 30 cases of ectopic pancreas in the gallbladder have been reported; if the association between adenomyosis and pancreatic rests is true, as we propose, this finding should be more frequently observed. Therefore, we suggest that the finding of an adenomyoma in the gallbladder should prompt an extensive sampling of the organ in order to verify the coexistence of pancreatic rests. Unfortunately, it is not possible to provide immunohistochemical support to our hypothesis, since the gallbladder and the pancreas have the same embryologic origin, and thus there are no specific markers allowing unequivocal differentiation between pancreatic tissue and gallbladder epithelium. Recently, Ko *et al*[10] reported that proliferating duct cells of the pancreas express vimentin. Accordingly, we used immunohistochemistry to detect the expression of vimentin in order to verify their observations, but in our hands duct cells of both normal pancreas and pancreatic adenocarcinoma were negative for this marker. Further studies are thus required, apart from morphology, to support an unequivocal link between adenomyomas and pancreatic heterotopia in the gallbladder.

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CASE REPORT

Fatty liver in H63D homozygotes with hyperferritinemia

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Abstract

To study the clinical correlates of the H63D mu-tation we have analysed the phenotype of H63D homozygotes identified through mutation analysis in a referral laboratory. A total of 366 blood samples referred for *HFE* analysis were screened for C282Y and H63D mutations. Four H63D homozygotes were identified. All had raised serum ferritin but normal transferrin saturation. They were negative for hepatitis B and C and only one patient consumed excess alcohol. In all 4 cases ultrasonography revealed fatty liver. In two patients a liver biopsy was done and showed mild siderosis with an unusual distribution and macrovesicular steatosis. These data confirm the association between fatty liver, hyperferritinemia and increased hepatic iron, but do not clarify whether siderosis was related to steatosis rather than homozygosity for the H63D mutation. Patients with fatty liver may complicate the interpretation of data in population studies of the expression of H63D homozygosity.

Key words: Hyperferritinemia; *HFE* gene; H63D homozygosity; Fatty liver

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INTRODUCTION

Hereditary haemochromatosis (HH) is a common autosomal recessive disorder of iron metabolism with an incidence of up to 1 in 200 and an estimated frequency of carriers of 1 in 10 among people of Northern European descent[1-3]. The disease is characterized by enhanced gastrointestinal absorption of iron to excessive accumulation in tissue, which may result in damage to liver and other target organs[4]. C282Y missense mutation in the *HFE* gene was found to be strongly related to the occurrence of HH[2]. In a UK study, homozygosity for the C282Y mutation was found to account for 91% of HH[5]. A second missense mutation in the *HFE* gene, H63D, is found in around 4% of patients with HH, but its role in iron overload is still debated[2,6-8]. The H63D mutation is variably distributed worldwide. It is more prevalent than the C282Y mutation so that approximately one in five of the European population are H63D heterozygotes[3,9]. Individuals who are compound heterozygous for C282Y and H63D can have iron overload in the range diagnostic of haemochromatosis, although the penetrance of the genotype is low[2,10,11]. Similarly, homozygosity for H63D has been associated with iron overload, ranging from asymptomatic subjects to patients with typical haemochromatosis. As with compound heterozygosity for C282Y/H63D, the penetrance is low and the phenotypic presentation of this genotype varied considerably[11,12]. The aim of this study was to analyse the phenotypic expression of H63D homozygotes identified through the genetic screening of patients referred to our Centre for *HFE* mutation analysis.

366 consecutive blood samples, referred to the Centre for Hepatology at the Royal Free and University College Medical School (UCL), were analysed for *HFE* mutations. Mutation analysis was requested on the basis of biochemical or clinical suspicion of HH, family screening or known diagnosis of haemochromatosis. Samples were obtained after informed written consent, where appropriate. C282Y and H63D mutations were detected by polymerase chain reaction (PCR) amplification of total genomic DNA followed by restriction digestion with RsaI and MboI enzymes respectively, as previously described[5].

Table 1 Clinical, biochemical and serological features of the four H63D homozygous patients

	Case 1	Case 2	Case 3	Case 4
Age (yr)	36	35	44	66
BMI (kg/m^2)	30	24	32.5	31
Blood pressure (mmHg)	140/100	130/80	160/100	150/95
Alcohol intake (g/wk)	8	0	60	0
Blood sugar (mmol/L)	4.2	4.6	5.6	9.5
Total cholesterol (mmol/L)	4.1	4.3	5.5	6
Triglicerides (mmol/L)	1.6	1.5	3.6	2.9
AST (U/L)	46	69	44	15
ALT (U/L)	117	175	112	18
γGT (U/L)	50	45	70	30
Ferritin (μg/L)	454	350	568	423
HCV/HBV serology	neg	neg	neg	neg

Legend: BMI=body mass index; wk=week; neg=negative. Normal ranges: BMI 18-25 kg/m^2; blood pressure max<120mmHg, min<80mmHg (see the seventh report of the Joint National Committee on high blood pressure, NIH publication no 03-5233, December 2003); blood sugar<6mmol/L.

CASE REPORTS

Four males were found to be homozygous for the H63D mutation. Their main features are summarised in Table 1.

Case 1

English male patient aged 36 years with suspected iron overload indicated by a serum ferritin concentration of 454 μg/L (reference range: 39-340). Serum iron, transferrin saturation and total iron binding capacity (TIBC) were normal. He was referred because of abnormal levels of liver enzymes and elevated serum ferritin found during investigation for dyspepsia. He consumed 8 g of alcohol per week. He had a positive family history for obesity and maturity onset diabetes mellitus. There was no family history of haemochromatosis. On examination he was well, overweight and mildly hypertensive (140/100). Abdominal examination was normal. Liver function tests showed: ALT 117 U/L, AST 46 U/L and γGT 50 U/L. Viral markers for hepatitis B and C were negative. Ultrasonography revealed a large liver, with diffuse hyperechogenicity, characteristic of fatty change. Since the liver enzymes and the ferritin remained persistently elevated, a liver biopsy was performed. It showed moderate macrovesicular steatosis (grade 2 on a scale of 0 to 3) with grade 1 siderosis (on a scale of 0 to 4)[13] in periportal hepatocytes and Kupffer cells, with evidence of pericellular fibrosis of zone 3 (stage 1 of steatohepatitis according to Brunt *et al*[14]). The sinusoidal cells had unusual granular siderosis (Figures 1A, 1B, 1C). The hepatic iron concentration was increased being 185 μg/100g dry weight (reference range: 35-136). The patient was seen every three months for 2 years to monitor transaminases and ferritin. On the basis of the result of the liver biopsy, venesection therapy was started, together with dietary restrictions. Over a period of two years, he was treated with seven phlebotomies and approximately 1.3 g of iron were removed. The serum ferritin concentration returned to normal at 95 μg/L as did liver function tests except for minimal elevation of ALT at 45 U/L.

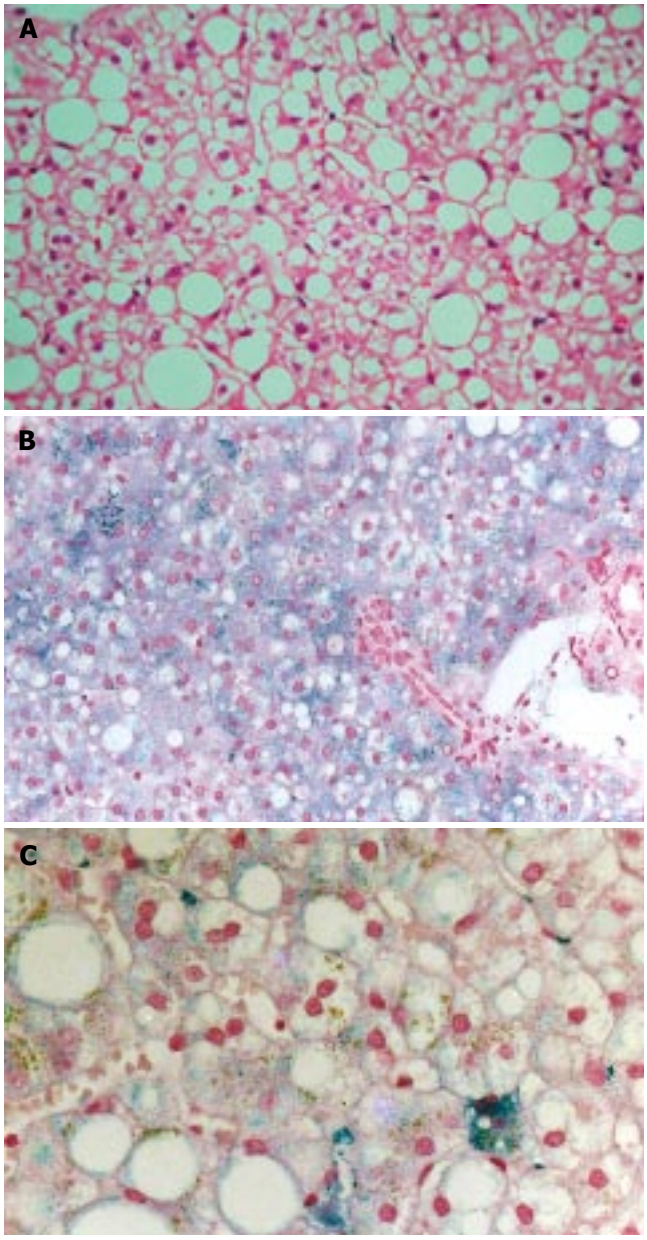

Figure 1 Case 1 liver histology. **A**: Hematoxylin and eosin staining shows grade 2 hepatic steatosis; **B**: Perls' staining shows grade 1 hepatocyte siderosis with predominant periportal distribution; **C**: Higher power of Perls' staining shows clustered Kupffer cell siderosis (right lower field) and also irregular large granular deposits in sinusoidal cells.

Case 2

Male patient aged 35 years from Lebanon with mild abnormality of iron indices. In 1996 he had a routine check-up and was found to have abnormal ALT (175 U/L) and AST (69 U/L), together with a minimally elevated serum ferritin (350 μg/L). Serum iron, TIBC and transferrin saturation were normal, as were the other liver function tests. The family history was negative for haemochromatosis. The patient did not drink alcohol. Viral markers for hepatitis B and C were negative. Between 1989 and 1995 he had been a blood donor giving approximately 2 units (approximately 450 mL each) of blood every year. On examination he had a normal build with gynaecomastia. Blood pressure was normal. Abdominal examination showed a palpable liver

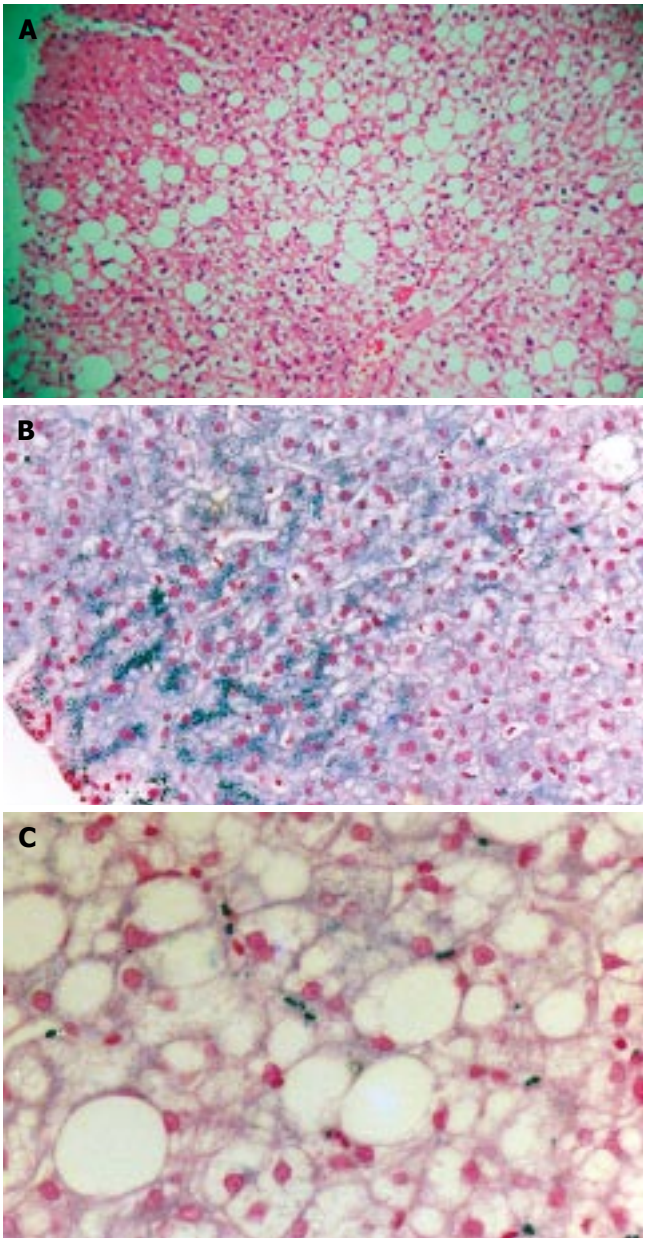

Figure 2 Case 2 liver histology. **A**: Hematoxylin and eosin staining shows grade 2 hepatic steatosis; **B**: Perls' staining shows grade 2 hepatocyte siderosis with predominant periportal distribution; **C**: Higher power shows focal Kupffer cell siderosis and more granular irregular sinusoidal siderosis.

on deep inspiration (2 cm) but no other abnormalities. The alpha-1-antitrypsin level was 1.0 g/L (normal range 1.2-2.6 g/L). Analysis showed him to have phenotype MZ. Ultrasound examination revealed a hyperechogenic liver compatible with hepatic steatosis. A liver biopsy was performed and showed moderate macrovesicular steatosis (grade 2) without fibrosis (stage 0 of steatohepatitis[14]). There was grade 2 siderosis in periportal hepatocytes, and focal Kupffer cell iron. The sinusoidal cell iron had a granular pattern similar to that seen for case 1 (Figures 2A, 2B, 2C). No cholestasis or alpha-1-antitrypsin staining was detected.

The patient was seen every three months for 4 years to monitor transaminases and ferritin, which remained persistently elevated.

Case 3

Irish male patient aged 44 years referred with possible iron overload indicated by a ferritin concentration of 568 μg/L. Serum iron, transferrin saturation and TIBC were normal. He was referred to our clinic in November 1998 to investigate raised levels of liver transaminases: ALT was 112 U/L, AST 44 U/L and γGT 70 U/L. The family history was positive for obesity and maturity onset diabetes mellitus and was negative for haemochromatosis. Physical examination showed a middle aged obese man (103 kg/178 cm; Body Mass Index (BMI) =32.5 kg/m^2). Blood pressure was elevated in the supine position (160/100) and the hypertension was treated with Tenif one per day (atenolol 50 mg, nifedipine 20 mg) and Valsartan (80 mg/day). The patient had also a history of moderate alcohol abuse (alcohol intake: 60 g/week) and elevated triglyceride levels (3.61 mmol/L). Viral markers for hepatitis B and C were negative. Ultrasonography of the abdomen revealed a liver of normal size, but with echoreflectivity consistent with fatty liver. No liver biopsy was done. The patient was seen every three months for 2 years to monitor weight, transaminases and ferritin. He abstained from alcohol. Dietary therapy was started and the patient lost 6 kg in weight. No venesection was performed. During follow-up, liver function tests remained mildly abnormal with an ALT of 84 U/L. The other enzymes fell to normal levels and triglycerides fell to 1.73 mmol/L. Despite abstinence and losing weight, his ferritin remained elevated (482 μg/L). The possibility of iron overload was raised and the patient was genotyped for mutations in the *HFE* gene and found to be homozygous for H63D.

Case 4

English male patient aged 66 years with an elevated serum ferritin concentration (423 μg/L) on a background of type 2 diabetes and peripheral neuropathy. Serum iron, transferrin saturation and TIBC were normal. He was diagnosed as hypertensive in February 1988 and he is currently treated with Enalapril. He also had atrial fibrillation for which he has been treated with warfarin. He was treated for diabetes mellitus with gliclazide, metformin and acarbose. Physical examination showed no features of chronic liver disease and no hepatomegaly. Liver function tests were normal. Viral markers for hepatitis B and C were negative. Abdominal ultrasonography revealed an enlarged and fatty liver. Since his ferritin levels remained in the region of 400 μg/L, the patient was tested for mutations in the *HFE* gene and found to be homozygous for H63D mutation.

DISCUSSION

Four H63D homozygotes were identified by screening 366 blood samples referred for genetic analysis in the *HFE* gene. All the four patients had high serum ferritin. It is noteworthy that in all the cases an abdominal ultrasonography showed fatty liver. In the two cases where liver biopsy was done and histology showed not only mild siderosis of hepatocytes, typical of iron overload of HFE-related haemochromatosis, but also siderosis of sinusoidal cells, with a granular pattern that could be related to nonalco-

holic steatohepatitis (NASH). Both patients had significant macrovesicular steatosis. One patient was treated with dietary restriction and venesection, after which liver function tests and serum ferritin concentration returned to normal. These findings suggest that hepatic steatosis, together with a biological effect of the H63D mutation, could be responsible for the hepatic siderosis in these patients. Recent evidence suggests that H63D homozygosity could lead to iron overload with variable penetrance and phenotype[12]. However, an association with fatty liver has not been previously reported. Another study of more than 10,000 blood donors suggested an effect of H63D homozygosity on iron metabolism[15], although the mean serum iron indices for this genotype were within the normal range, possibly reflecting the fact that blood donors are in general healthy and young (mean age was 38 years for men and 36 years for women). These findings have been reinforced experimentally by the demonstration that transgenic mice homozygous for the H63D mutation have elevated transferrin saturation and hepatic iron concentration compared to wild type mice[16].

H63D homozygosity could thus contribute to iron overload but the phenotypic expression may be influenced by cofactors. Sex, age, diet and modifier genes are likely to influence penetrance of the genotype. In this series all 4 cases were male and this reflects the relative protection of women from iron overload by menstruation and pregnancy. Furthermore, three of the cases had one or more metabolic disorders which are part of the insulin resistance syndrome (IRS)[17]. Case 1 was overweight (BMI>25 kg/m^2) and mildly hypertensive, Case 3 was obese (BMI>30 kg/m^2) and had hypertriglyceridemia and hypertension requiring antihypertensive treatment. Case 4 was obese, he had type 2 diabetes mellitus requiring therapy, hypertrigliceridemia and hypertension under therapy.

Although hyperferritinemia may be associated with hepatic steatosis per se[18,19], in two patients presented here hepatic siderosis with an unusual histological pattern was present. Homozygosity for H63D may have played a role, but the unusual cellular pattern raises the possibility that in some cases of hepatic steatosis there are other changes in cellular iron handling which result in iron accumulation. Alterations in cytokines and hence hepcidin may result in accumulation of iron in sinusoidal cells[20,21]. A role for the mutations in the *HFE* gene in other diseases, however, has been suggested by their over-representation in subjects with NASH and with the dysmetabolic iron overload syndrome (DIOS) characterized by an association between iron overload and insulin resistance[22-24]. The molecular mechanism explaining these associations is not clear. Hepatic steatosis has been recognised as the first of two "hits" in the pathogenesis of NASH, since the presence of oxidisable fat within the liver is enough to trigger lipid peroxidation[25]. However, many patients with fatty liver do not progress to necroinflammation[26]. It has been suggested that the second hit for the development of NASH may be oxidative stress, leading to necroinflammation[27]. Several potential second hits have been suggested. Iron even in relatively low concentrations could synergize with lipid overload and induction of ethanol-inducible cytochrome P450 2E1 (CYP2E1) to increase oxidative stress in hepatocytes[28].

If iron leads to oxidative stress and to progression of non-alcoholic fatty liver to NASH, venesection therapy is theoretically beneficial, and this may have contributed to the normalisation of serum ferritin concentration and improvement of liver function tests in Case 1. In conclusion, our findings confirm a link between fatty liver and mild iron accumulation. Whether homozygosity for H63D contributed to the association is uncertain. Further studies of iron regulatory proteins are needed in hepatic steatosis.

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CASE REPORT

Hepatic encephalopathy with status epileptics: A case report

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Abstract

A 62-year-old male with decompensated liver cirrhosis due to hepatitis C virus developed severe hepatic encephalopathy with status epileptic us. The blood ammonia level on admission was more than twice the normal level. Brain computed tomography and magnetic resonance imaging were normal. In addition, electroencephalogram showed diffuse sharp waves, consistent with hepatic encephalopathy. The status epilepticus was resolved after antiepileptic therapy (phenytoin sodium) and treatment for hepatic encephalopathy (Branched chain amino acids). The blood ammonia level normalized with the clinical improvement and the patient did not have a recurrence of status epilepticus after the end of the antiepileptic treatment. Additionally, the electroencephalogram showed normal findings. Thus, we diagnosed the patient as hepatic encephalopathy with status epilepticus. We consider the status epilepticus of this patient to a rare and interesting finding in hepatic encephalopathy.

Key words: Status epilepticus; Hepatic encephalopathy; Decompensated liver cirrhosis; Electroencephalogram

Tanaka H, Ueda H, Kida Y, Hamagami H, Tsuji T, Ichinose M. Hepatic encephalopathy with status epileptics: A case report. *World J Gastroenterol* 2006; 12(11): 1793-1794

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INTRODUCTION

Hepatic encephalopathy typically presents with alterations in behavior, impairment of consciousness, and alterations in motor tone[1]. Although, according to some reports, the incidence of seizures in patients with hepatic encephalopathy is unknown, reports of epileptic seizures in patients with hepatic encephalopathy during the clinical course are very rare, but not in the terminal stage of liver cirrhosis[2-4]. We present a rare and interesting case of a 62-year-old male with hepatic encephalopathy with status epilepticus accompanying decompensated liver cirrhosis.

CASE REPORT

A 62-year-old patient was diagnosed as having decompensated liver cirrhosis due to hepatitis C virus one year earlier. At that time he reported a ten-year history of mild psychosis but no history of diabetes mellitus, epilepsy, stroke, or brain trauma. He was admitted to our hospital because of generalized tonic-clonic seizures for several hours and loss of consciousness (status epilepticus) in October 2004. He had a history of recurrent episodes of hepatic encephalopathy. The results of further clinical examination, except for signs of decompensated liver cirrhosis, were normal. Laboratory data showed anemia, thrombopenia, increased transaminase, alkaline phosphatase and total bilirubin, and decreased albumin, total cholesterol and BTR (branched chain amino acid/tyrosine ratio). Furthermore, the plasma ammonia (NH_3) level on admission was twice the normal level (186 μmol/L; normal value is 23-76 μmol/L). Chest and abdominal roentgenograms, brain computed tomography (CT) and magnetic resonance imaging (MRI) were also normal, although abdominal echosonography and CT revealed atrophic cirrhotic liver and splenomegaly. Electroencephlogram (EEG) on day 2 after admission showed diffuse sharp waves, consistent with hepatic encephalopathy (Figure 1). According to the history and the results of EEG, in combination with laboratory and radiological data, hepatic encephalopathy with status epilepticus was suspected. Status epilepticus and loss of consciousness were resolved after antiepileptic therapy (phenytoin sodium) and treatment for hepatic encephalopathy (branched chain amino acids; BCAA). The plasma NH_3 level returned to a normal level concurrently with his clinical improvement. He did not have a recurrence of status epilepticus after the end of antiepileptic treatment and his conscious level returned to normal with continuous therapy for hepatic encephalopathy with BCAA. Another EEG on day 33 after admission showed a normal level (Figure 1). Thus, we diagnosed our patient as having hepatic encephalopathy with status epilepticus. In the follow-up, 7 mo later, he was in good condition, in spite of

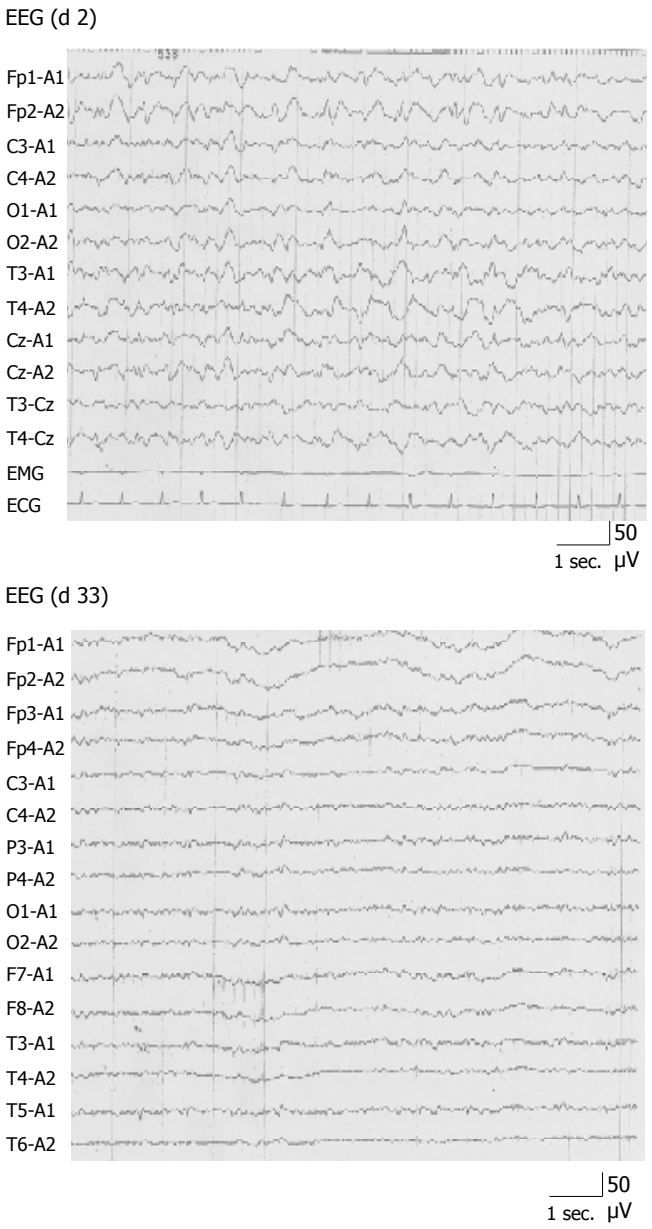

Figure 1 Diffuse EEG activity on day 2 after admission characterized by delta waves mixed with theta waves. Signs compatible with hepatic encephalopathy. The EEG activity on day 33 after admission returned to normal.

the recurrence of hepatic encephalopathy.

DISCUSSION

The present case was diagnosed as hepatic encephalopathy with status epilepticus. Although the patient had a history of mild psychosis, there were no psychological findings except for the loss of consciousness at the time the seizures occurred. In addition, he had a history of recurrent episodes of hepatic encephalopathy. Second, no focal brain lesion could be detected by brain CT and MRI, and the EEG was suggestive of metabolic dysfunction, especially hepatic encephalopathy. Third, the elevated plasma NH_3 level and EEG findings (diffuse sharp waves) on admission and its subsequent normalization with the clinical improvement after the end of antiepileptic treatment strongly suggested hepatic encephalopathy with status epilepticus.

Epileptiform abnormalities in EEG, seizures and status epilepticus may be seen in patients with hepatic encephalopathy, although their incidence is unknown. Ficker DM et al. reported in a retrospective study that the majority of cirrhotic patients with epileptiform changes in EEG either died or deteriorated[3]. However, reports of epileptic seizures in hepatic encephalopathy during the clinical course are very rare[5, 6].

The pathophysiology of seizures in hepatic encephalopathy remains unknown. Various metabolic factors may be suggested. The plasma NH_3 levels are consistently elevated, while other factors, such as short chain free fatty acids, phenols, mercaptanes and false neurotransmitters, have also been implicated. These factors may be responsible for the generation of epileptiform discharges as well as epileptic seizures in patients with hepatic encephalopathy[7, 8].

On the other hand, it has been reported that the focal hyperemia/hypermetabolism may be related either to the seizures or to the encephalopathy itself, based on intracranial ultrasonography and single-photon-emission computed tomography (SPECT)[6].

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CASE REPORT

Left paraduodenal hernia in an adult complicated by ascending colon cancer: A case report

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Abstract

Paraduodenal hernia is the most common internal hernia. The clinical symptoms of paraduodenal hernia may be intermittent and nonspecific. Therefore, it is difficult to diagnose preoperatively. Abdominal computed tomography (CT) scan currently plays an important role in the evaluation and management of paraduodenal hernia before surgical operation. We report one unique case of preoperatively diagnosed left paraduodenal hernia complicated by advanced ascending colon cancer and reviews of Japanese literature.

Key words: Paraduodenal hernia; Internal hernia; Colonic malrotaion

Kurachi K, Nakamura T, Hayashi T, Asai Y, Kashiwabara T, Nakajima A, Suzuki S, Konno H. Left paraduodenal hernia in an adult complicated by ascending colon cancer: A case report. *World J Gastroenterol* 2006; 12(11): 1795-1797

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INTRODUCTION

Paraduonenal hernia is an uncommon cause of small bowel obstruction. It has been reported that the most common type of internal hernia, reported in more than 50% of internal hernia cases. We report a case of left paraduodenal hernia complicated by ascending colon cancer, which was diagnosed correctly with abdominal CT. This case was confirmed at surgical operation and the clinical and imaging findings of these hernias were reviewed in Japanese literature.

CASE REPORT

A 47-year-old woman was admitted with right lower quadrant pain and nausea. An elastic hard mass was palpable in the right lower quadrant abdomen. The body temperature was 36.8℃. Arterial blood pressure and cardiac rate were 96/80 mmHg and 70/min respectively. Laboratory tests detected only an anemia. Plain abdominal X-ray showed air-fluid levels of the small intestine loop in the upper abdomen (Figure 1). Abdominal CT scans with intravenous administration of contrast medium showed ascending colon with enhanced mass and ileum dilatation (Figure 2). Abdominal CT scan also showed an encapsulated small bowel loop with no dilatation in the left upper abdomen and the inferior mesenteric vein (IMV) was located in the anterior of the encapsulated small bowel loop formation (Figure 3). Colonoscopy revealed advanced ascending colon cancer with obstruction. We preoperatively diagnosed as ascending colon cancer with left paraduonenal hernia.

Laparotomy revealed the hernia orifice and hernia sac was located in left mesocolon (Figure 4). IMV was located in the upward of the anterior portion of the hernia sac. Approximately 40 cm of jejunum was within a hernial sac. There were no volvulus or ischemic changes of the jejunum. The patient underwent right hemicolectomy with an end-to-end ileocolonic anastomosis. Anterior hernial sac incision was also performed. The postoperative course was uneventful.

DISCUSSION

Paraduonenal hernia has been reported that the most common type of internal hernia, reported in more than 50% of internal hernia cases[1,2]. The clinical symptoms of paraduodenal hernia may be intermittent and nonspecific. It is difficult to diagnose preoperatively. Therefore, paraduodenal hernia was found incidentally at laparotomy, autopsy or during radiological investigation for unrelated disease[2-5].

Based on the published cases of paraduodenal hernia in Japan, approximately 68% (82/120 cases) were left sided, and 32% (38/120 cases) were right sided. 70% of these cases were man, and 30% cases were woman[6,7]. The mean age of the onset was 39.5 years. These frequencies of

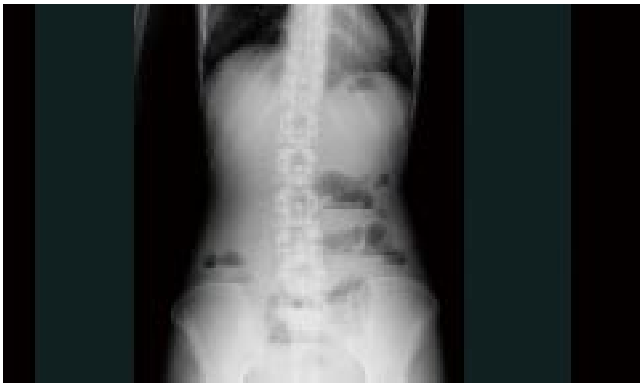

Figure 1 Plain abdominal X-ray showed air-fluid levels of the small intestine loop in the upper abdomen.

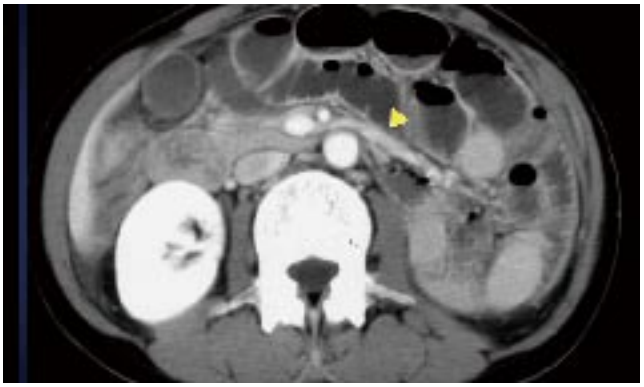

Figure 3 Inferior mesenteric vein (IMV) was located in the anterior of the encapsulated small bowel loop formation (yellow arrow head).

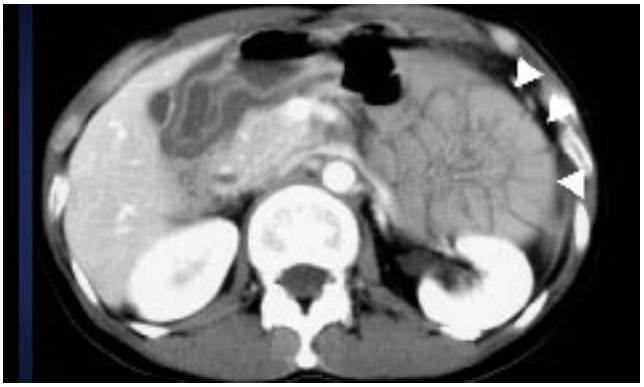

Figure 2 Abdominal CT scan also showed an encapsulated small bowel loop with no dilatation in the left upper abdomen (white arrow head).

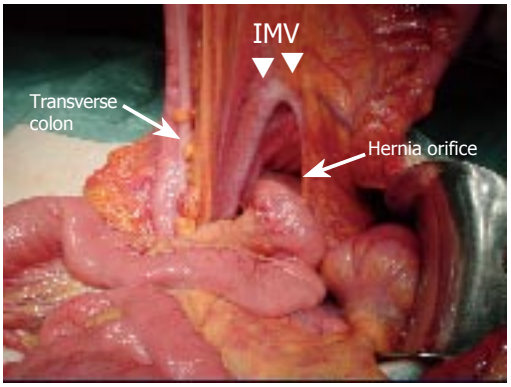

Figure 4 Large orifice of the hernia sac (white arrow) in the transverse mesentery and IMV formation. The small intestine had herniated through hernia orifice.

paraduonenal hernia were almost similar to the incidence in English literature.

Internal hernia complicated by colorectal cancer was reported only 3 cases. In our patient case, the origin of the bowel obstruction was not caused by left paraduonenal hernia but obstructed by advanced ascending colon cancer. Clinical findings of paraduodenal hernias are variable, ranging from mild digestive complaints to acute or chronic symptoms of obstruction, sometime asymptomatic.

Many of the paraduodenal hernia can be diagnosed incidentally at laparotomy, autopsy or during radiological investigation for an unrelated disease. Therefore, diagnosis of asymptomatic paraduodenal hernia has been difficult. On the other hand, recently reported cases of symptomatic paraduodenal hernia, 80% (8/10 cases) were diagnosed before operation[6,7]. Especially, abdominal CT currently plays an important role in the evaluation and management of internal hernias[2,3,8,9].

Enhanced CT is helpful in establishing a preoperative diagnosis of the presence of paraduonenal hernia. The specific findings of paraduonenal hernia by CT scan show clustering of small bowel loops, well-circumscribed edge that corresponds to the hernia sac, stretched and engorged mesenteric vessels. In the case of left paraduodenal hernia, IMV were usually located in the upward and anterior displacement of the hernia sac. Abnormalities in the mesenteric vessels including engorgement, crowding and stretching, and displacement of the main mesenteric trunks to the left are important clues to this diagnosis[1,10].

Paraduodenal hernias are believed to occur due to a congenital defect in the descending mesocolon. The small bowel may occur to invaginate into this space, the fossa of Landzert, which lies to the left of the fourth portion of the duodenum. The herniated small-bowel loops may become trapped within this mesenteric sac.

Treatment for the duodenal hernia is based on reduction of the hernia sac and closure of the defect or incision of the hernia sac[11,12]. Paraduodenal hernia induces an over 50% lifetime threat of incarceration, leading to bowel obstruction and strangulations. Therefore, surgical operation should be performed to avoid these complications. Indeed, 20% cases of internal herinas needed for small intestinal resection caused by bowel necrosis[6,7]. In general, postoperative course was uneventful, recurrence case was rare. Recently, laparoscopic surgery for left paraduodenal hernia without bowel necrosis may be useful surgical method because of its minimal invasiveness and aesthetic advantage[13,14]. In our case, we performed anterior hernia sac incision following the right hemicolectomy. Surgeons should pay attention to the mesenteric vessels located near the hernia sac orifice. The displacement of the main mesenteric trunks may

cause vessel injury during surgical treatment.

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CASE REPORT

Destructive granuloma derived from a liver cyst: A case report

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Abstract

We herein report the case of an idiopathic liver cystic mass which aggressively infiltrated the thoraco-abdominal wall. A 74-year-old woman who had a huge cystic lesion in her right hepatic lobe was transferred to our hospital for further examinations. Imaging studies revealed a simple liver cyst, and the cytological findings of intracystic fluid were negative. She was followed up periodically by computed tomography (CT) scans. Seven years later, she complained of a prominence and dull pain in her right thoraco-abdominal region. CT revealed an enlargement of the cystic lesion and infiltration into the intercostal subcutaneous tissue. We suspected the development of a malignancy inside the liver cyst such as cystadenocarcinoma, and she therefore underwent surgery. A tumor extirpation was performed, including the chest wall, from the 7th to the 10th rib, as well as a right hepatic lobectomy. Pathologically, the lesion consisted of severe inflammatory change with epithelioid cell granuloma and bone destruction without any malignant neoplasm. No specific pathogens were evident based on further histological and molecular examinations. Therefore the lesion was diagnosed to be a destructive granuloma associated with a long-standing hepatic cyst. Since undergoing surgery, the patient has been doing well without any signs of recurrence.

Key words: Destructive granuloma; Liver cyst

Kawashita Y, Kamohara Y, Furui J, Fujita F, Miyamoto S, Takatsuki M, Abe K, Hayashi T, Ohno Y, Kanematsu T. Destructive granuloma derived from a liver cyst: A case report. *World J Gastroenterol* 2006; 12(11): 1798-1801

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INTRODUCTION

A liver cyst is a common lesion that tends to be mostly asymptomatic, however, once it is associated with other events such as bleeding, infection and rupture, it often becomes symptomatic and treatment is thus usually needed. Granulomas can be seen in any part of the body and they reflect a host inflammatory responses elicited by a wide variety of stimuli[1, 2], however, it is uncommon that a granulomatous reaction develops in a liver cyst[3].

We experienced the case of highly destructive, non-malignant, granulomatous lesion that originated from a long-standing hepatic cyst that infiltrated the thoraco-abdominal wall mimicking hepatic cystadenocarcinoma. To the best of our knowledge, this is the first known occurrence of a case of a hepatic granuloma that developed secondary to a liver cyst with a highly invasive capacity.

CASE REPORT

A 73-year-old Japanese woman presented at our hospital with right. hypochondralgia on September 1997. Computed tomography (CT) showed a huge liver cyst measuring 10cm in the right lobe of the liver without any solid component (Figure 1A). Subsequently, 2.4L of serous fluid were percutaneously drained, without any sclerosing agent administered into the cyst. A cytological examination of the aspirated fluid was negative for neoplastic cells. Since her symptom had subsided after the fluid drainage, she was carefully followed-up without any further treatment. On May 2003, she was again referred to us with abdominal distention and pain in the right thoraco-abdominal region. The results of laboratory tests showed slight elevations in the C-reactive protein (CRP) level (0.5mg/dL, normal range < 0.17). Serological tests for hepatitis B and C virus were negative. The serum levels of carcinoembryonic antigen, carbohydrate antigen 19-9 and α-fetoprotein were all within the normal ranges. Other tests including her liver function were within the normal limits. A CT scan showed a cyst in the right. lobe of the liver similar to the one observed 6 years previously. In addition, a calcified lesion was also found around the cyst (Figure 1B). An axial and coronal view of an MRI showed a multilocular cyst with a thick wall and solid component extending into the subcutaneous tissue (Figure 2A and B). MR cholangiopancreatography demonstrated no dilatation in the biliary system, thus indicating that there was most likely no communication with the liver cyst (Figure 2C). These findings closely resembled hepatic cystadenocarcinoma.

After admission, the cystic tumor gradually increased

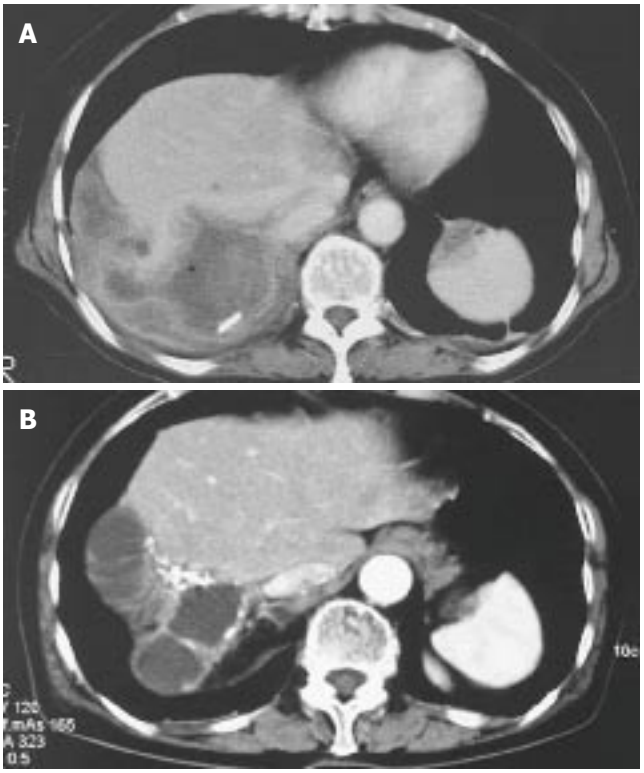

Figure 1 Computed tomography revealed a huge multilocular cyst measuring 10 cm in the right lobe of the liver on September 11th, 1997 (**A**). A similar cyst with a calcified lesion was found in the right lobe of the liver on May 15th, 2003 (**B**).

in size and finally her skin above the invasion site became reddish and swollen (Figure 2D), thus suggesting malignant tumor invasion. After obtaining the patient's informed consent, we performed an *en bloc* resection of the hepatic right lobe including the whole cyst, along with the diaphragm, rib bone, and skin. After completing the resection, the defect of the diaphragm was covered with the great omentum, and the thoraco-abdominal wall defect was reconstructed using a musculo-cutaneous flap including the anterior sheath and the left rectus abdominis muscle which both receive the blood supply from the superior abdominal artery (Figure 3A and B). Macroscopically, the cystic tumor directly invaded the intercostal space through the diaphragm (Figures 4A and 4B). No microorganism was found in the cyst fluid or the resected materials according to the results of microbilological culture examinations. A pathological examination revealed that the bone had been destroyed by the invasion of the granuloma, which was composed of epithelioid cells with necrotic areas (Figure 4C). No malignancy or microorganism was detected in any tissue samples. Because the histology was similar to tuberculosis, Ziehl-Neelsen staining and DNA-PCR using three different primers for a tubercle bacillus were performed with negative results. Hepatic hydatid disease was excluded by the fact that she had no history of living or traveling to the epidemic area such as Hokkaido prefecture in Japan and her blood samples did not show the presence of the antibodies against ehinococcus. From these findings, the cystic tumor was therefore histologically diagnosed to be a hepatic epithelioid granuloma. (Figure 4D)

The patient had an uneventful postoperative course and was discharged on postoperative day 27. She remains asymptomatic until 24 mo postoperatively, without any signs of recurrence.

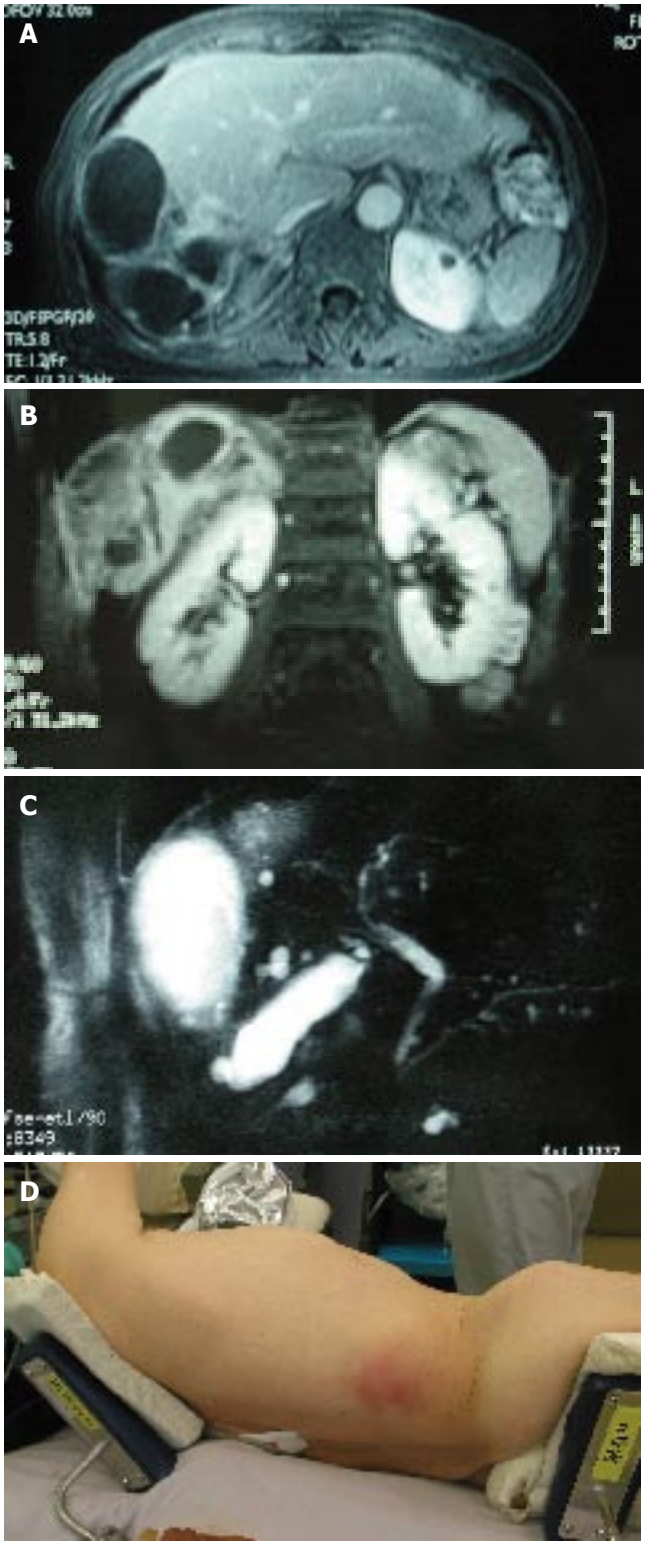

Figure 2 An axial (**A**) and coronal (**B**) view of magnetic resonance imaging (MRI) showed a multilocular cyst with a thick wall with a solid component extending into the subcutaneous tissue. MR cholangio-pancreatography showed no dilatation in the biliary system, and most likely no communication with the liver cyst (**C**). The skin showed red swelling by the subcutaneous extension of the hepatic cyst (**D**).

DISCUSSION

There has been a great progress in the diagnostic modali-

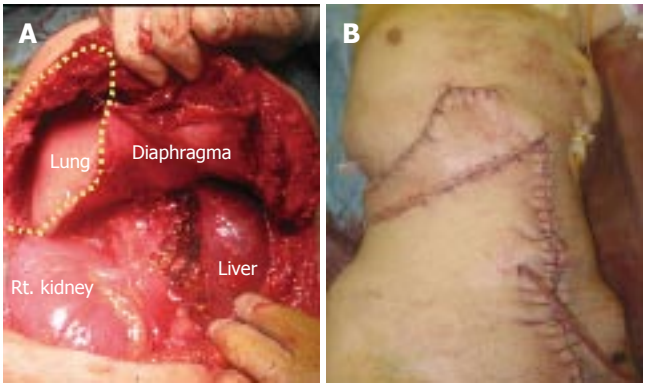

Figure 3 An en bloc resection resulted in a huge defect (**A**, yellow dotted circle). The defect of the diaphragm was covered with the great omentum, and the thoraco abdominal wall defect was reconstructed by a musculo-cutaneous flap including the anterior sheath and the left rectus abdominis muscle which both receive the blood supply comes from the superior abdominal artery (**B**).

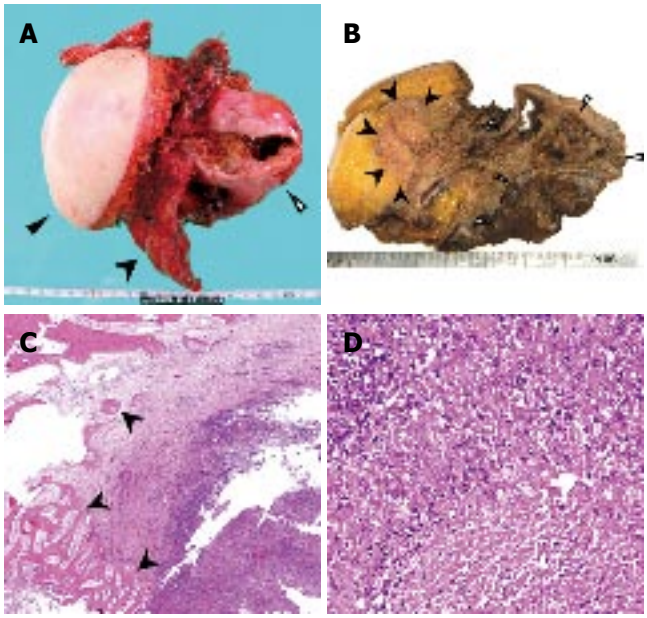

Figure 4 Macroscopically, the cystic mass directly invaded the intercostal space through the diaphragm (**A**. Resected crude specimen, black triangle; skin, black arrow head; ribs, white triangle; cystic tumor; **B**. Cut surface of the specimen, black arrow head; subcutaneous invasion, white triangle; cystic tumor). Microscopically, the bone was destroyed by an invasion of granuloma tissue (**C**). The granuloma was composed of epithelioid cells with necrotic areas (**D**).

ties for hepatic lesions including ultrasonography, CT, magnetic resonance imaging (MRI) and angiography[4]. Cystic lesions of the liver, which occupy a large part of hepatic lesions and are seen in up to 5% of the population, can be classified as developmental, neoplastic, inflammatory, or miscellaneous lesions. If a solid component develops inside the cyst, the possibility of a malignant transformation should be considered[5]. However, even routine imaging procedures such as CT or MRI generally fail to differentiate hepatic granulomas from other neoplasms[2].

Aspiration cytology can help in making a preoperative diagnosis, however, it is sometimes difficult to accurately hit the target tumor under US guidance, and this procedure also carries a risk of potentially causing needle-track and peritoneal seeding if the lesion is a malignant tumor[6, 7].

A wide variety of underlying conditions such as sarcoidosis[8], malignant lymphoma[9], tuberculosis[2], and HCV infection[10,11] can cause hepatic granulomas, with resulting prognostic and therapeutic implications. However, the histological features of such granulomas are not distinctive, while a specific etiological agent often cannot be identified despite serological, immunological microbiological, and radiological investigations, thus often resulting in a diagnosis of "idiopathic" hepatic granulomas. Although the exact etiology and pathogenesis of this aggressively infiltrating granuloma seen in the present case remains unclear, we speculated that an unidentified organism infection possibly occurred via two different routes: (1) the endogenous route, via the blood stream; (2) the exogenous route: a reverse infection through a drainage catheter when aspiration had previously been performed.

A spontaneous regression occurs in some cases of hepatic granulomas, therefore, patients can be treated by simple observation or conservative therapy with anti-inflammatory drugs including antibiotics or steroids in most cases. In the present case, the fact that destroyed and necrotic tissue caused inflammatory reactions suggested that empirical antibiotic therapy would be ineffective, to make matters worse, such conservative treatment could have possibly induced the development of such antibiotic-resistant bacteria as Methicillin-Resistant Staphylococcus Aureus (MRSA). In addition, steroid treatment is generally effective for sarcoidosis, however, it risks exacerbating tuberculosis and an earlier paper described a patient who died from miliary TB after the administration of empirical steroids [12].

Great improvements in operative techniques, perioperative patient management, and patient selection criteria have not made a hepatic resection one of the standards and effective methods for liver cysts[13]. As a result, a hepatectomy is recommended for cystic lesions when a tumor of the liver with a potential malignancy cannot be ruled out[14,15].

In the present case, the clinical course in which a solid component developed during the follow-up of a liver cyst, while infiltrating into the subcutaneous tissue with the destruction of the ribs was highly suggestive of the development of a malignancy, such as cystadenocarcinoma. The pathological diagnosis turned out to be a cancer-free lesion, namely, benign granuloma. Despite the highly invasive capacity of this lesion, the good clinical outcome in this case supports the suitability of a hepatic resection with the total removal of the affected areas as the treatment of choice.

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CASE REPORT

Acute pancreatitis associated with peroral double-balloon enteroscopy: A case report

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Abstract

A 58-year-old Japanese man had tarry stool and severe anemia. Neither upper nor lower gastrointestinal (GI) endoscopy showed any localized lesions. Thus, the source of his GI bleeding was suspected to be in the small intestine, and he underwent peroral double-balloon enteroscopy (DBE) using EN-450T5 (Fujinon-Toshiba ES System Co., Tokyo, Japan). There were no lesions considered to be the source of GI bleeding. After the procedure, the patient began to experience abdominal pain. Laboratory tests revealed hyperamylasemia and abdominal computed tomography revealed an inflammation of the pancreas and the peripancreas. He was thus diagnosed to have acute pancreatitis. Conservative treatments resulted in both clinical and laboratory amelioration. He had no history of alcohol ingestion, gallstone disease or pancreatitis. Magnetic resonance cholangiopancreatography demonstrated no structural alterations and no stones in the pancreatobiliary ductal system. As his abdominal pain started after the procedure, his acute pancreatitis was thus thought to have been related to the peroral DBE. This is the first reported case of acute pancreatitis probably associated with peroral DBE.

Key words: Enteroscopy; Small intestine; Pancreatitis; complication

Honda K, Mizutani T, Nakamura K, Higuchi N, Kanayama K, Sumida Y, Yoshinaga S, Itaba S, Akiho H, Kawabe K, Arita Y, Ito T. Acute pancreatitis associated with peroral double-balloon enteroscopy: A case report. *World J Gastroenterol* 2006; 12(11): 1802-1804

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INTRODUCTION

The small intestine is located quite far from both the mouth and the anus, and thus it has been difficult to be examined by endoscopy until recently. However, capsule endoscopy and double-balloon enteroscopy (DBE), new methods of enteroscopy, have been introduced in recent years which have now made the observation of the entire small intestine possible.

DBE is a novel technique which was developed by Yamamoto *et al*, in Japan[1-3], and is spreading also in other countries. Both a 200-cm enteroscope and a 145-cm overtube which have a soft latex balloon at their tips are included in the system. Both balloons can be inflated or deflated by a balloon-pump controller which thus allows for a shortening of the proximal intestine, thus allowing the enteroscope to be inserted into the deep small intestine. Currently, two different diameter enteroscopes, EN-450P5/20 (Fujinon-Toshiba ES System Co., Tokyo, Japan) with an outer diameter of 8.5 mm (an overtube with an outer diameter of 12.2 mm) and EN-450T5 with an outer diameter of 9.4 mm (an overtube with an outer diameter of 13.2 mm) are available. With a larger accessory channel, EN-450T5 allows the performance of a variety of enteroscopic treatments including argon plasma coagulation/ablation, coagulation/biopsy (hot biopsy) and balloon dilatation. The indications of DBE are for the diagnosis and the treatment of small intestinal diseases. DBE is quite useful for the diagnosis of the origin of obscure gastrointestinal (GI) bleeding in the small intestine and for the endoscopic evaluation and histopathological diagnosis from the biopsy samples of small intestinal lesions such as ulcers, tumors and strictures. It is also useful for the treatment of small intestinal diseases, for example, the management of active GI bleeding and polypectomy of the small intestinal polyps. The technique of DBE is considered to be safe as only few complications related to sedation and original diseases have been reported previously[4,5].

In this paper, we report a case that developed acute pancreatitis after peroral DBE. To our knowledge, this is the first report which describes a case of acute pancreatitis probably associated with peroral DBE.

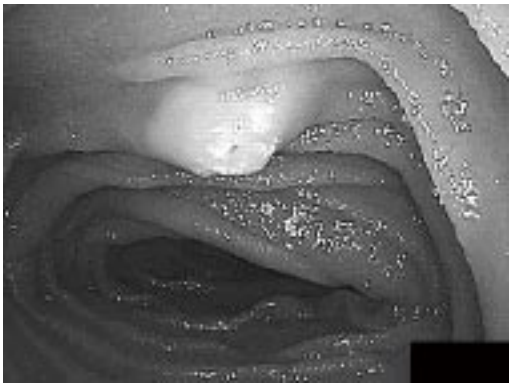

Figure 1 Peroral double-balloon enteroscopy revealed a small yellowish submucosal tumor with white specks on the surface in the upper jejunum.

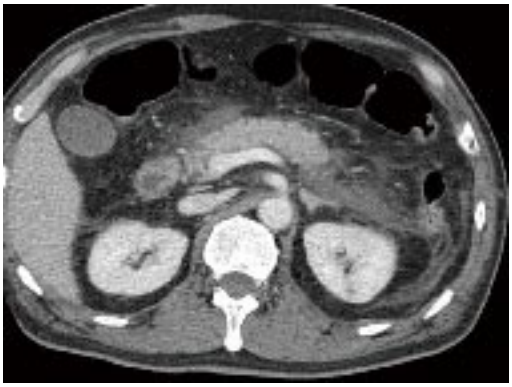

Figure 2 Abdominal CT showed inflammation and swelling of the pancreas, and a blurring of the fat planes in both the peripancreas and the left anterior pararenal space.

CASE REPORT

A 58-year-old Japanese man had tarry stool and syncope and was hospitalized by ambulance during the previous month. Since laboratory tests revealed severe anemia (hemoglobin level: 77 g/L, normal 140-180 g/L), he received red blood cell transfusion. Neither upper nor lower gastrointestinal GI endoscopy showed any localized lesions. Barium meal radiography of the small intestine and computed tomography (CT) also failed to show any lesions. As a result, the source of his GI bleeding was suspected to be a small lesion in the small intestine such as angiodysplasia, small ulcer or erosion, and he was thus referred to our hospital. Thirty days had already passed from the day when he had tarry stool and his hemoglobin level recovered up to 114 g/L. On the day of his admission, written informed consent was obtained from him and he underwent peroral DBE using EN-450T5. The total time of the procedure was 100 min. At the most distal site, the small intestinal mucosa was marked with a tattoo by a submucosal injection of sterilized ink through an injection catheter. There was a small submucosal tumor, which had a yellowish surface with white specks, located in the upper jejunum (Figure 1), which was considered to be a lymphangioma because of its typical image. But it was not considered to be the source of GI bleeding because there was no ulceration or erosion on its surface. After finishing the procedure, the patient began to experience abdominal pain. An abdominal examination revealed moderate epigastric tenderness and silent bowel sounds. His laboratory findings were as follows: white blood cells, 13 100/μL (normal 3 500-9 000 /μL); C-reactive protein, 2.37 mg/dL (normal < 0.10 mg/dL); serum amylase, 702 U/L (normal 50-159 U/L). Abdominal CT revealed an inflammation of the pancreas and the peripancreas (Figure 2). He was thus diagnosed to have acute pancreatitis. The patient was treated by total parenteral nutrition with intravenous rich fluids and gabexate. These treatments resulted in both clinical and laboratory amelioration and he started oral intake on day 16. Since then, his condition was good and he underwent peranal DBE on day 26. No lesions were observed on the anal side of the small intestine although the mark with a tattoo was not detected. It probably disappeared since it has been 26 d from the day of peroral DBE. Hence, his GI bleeding source remained unknown. He was discharged on day 30. His hospitalization was extended for more than 10 d because of pancreatitis after peroral DBE. The grade of his pancreatitis was diagnosed as severe according to the criteria of Cotton *et al*[6].

He had no history of alcohol ingestion, gallstone disease or pancreatitis. Magnetic resonance cholangiopancreatography on day 18 demonstrated no structural alterations and no stones in the pancreatobiliary ductal system. As his abdominal pain started after the procedure, his acute pancreatitis was thus thought to have been related to the peroral DBE.

DISCUSSION

Newly developed methods of enteroscopy, capsule endoscopy and DBE, are quite useful for the detection of the origin of GI bleeding in the small intestine. Capsule endoscopy causes minimum discomfort to patients because the patients are free after loading a recorder and taking a capsule orally. But in Japan, experience with capsule endoscopy is limited as it has not yet been approved for the clinical use at the present time and thus it can be used in only limited facilities as clinical studies. DBE is currently available in Japan. We therefore performed DBE in this case but we could not find a lesion which caused GI bleeding. We might have a greater chance to find a responsible lesion if we could perform DBE when the patient had a active bleeding. We chose EN-450T5 as we considered the possibility of the treatment of a lesion with hemorrhage by argon plasma coagulation and hemostatic clipping. The patient unexpectedly developed acute pancreatitis after the DBE procedure.

Regarding the safety of DBE, May *et al*[4] reported that the only complications observed were related to sedation in 248 enteroscpies (including 153 peroral enteroscopies). Yamamoto *et al*[5] demonstrated that two complications, mainly associated with the original diseases, occurred among 178 procedures (including 89 peroral enteroscopies). One was multiple perforations occurred in a patient with intestinal lymphoma who received chemotherapy and the other was in a patient who was discovered to have Crohn's disease and had postoperative fever and abdomi-

nal pain. In the above two studies, EN-450P5/20 was used and no instances of acute pancreatitis were reported. Up to now, we have performed peroral DBE using EN-450P5/20 on 13 cases (one case reached to the ascending colon), and no previous cases demonstrated pancreatitis. This was the first case in which we performed peroral DBE using EN-450T5. The thicker diameter of EN-450T5 might therefore be associated with the development of pancreatitis.

A few cases developing pancreatitis have been reported as a complication of upper or lower GI endoscopy without ampullary cannulation[7-9] although GI endoscopy is not known to be associated with an increased risk for pancreatitis. The underlying mechanism of pancreatitis in these cases is unclear. In case of upper GI endoscopy, it was possible that trauma to the papilla during duodenal intubation might have led to edema of the papilla and subsequent pancreatitis. In case of colonoscopy, it was possible that over insufflation of the colon might have caused pressure of the pancreas or compression of the colonoscope against the spine might have caused a direct trauma to the pancreas. The mechanisms of developing pancreatitis associated with peroral DBE were thought to be as follows: the one may be a pancreatic duct obstruction due to the direct oppression of the papilla by an overtube and the other may be an increase in the intralumenal pressure of the duodenum by a thick overtube and shortening technique.

Here we reported a case of acute pancreatitis that was thought to be associated with DBE. This case indicates that, when peroral DBE is performed, especially with EN-450T5, we have to keep in mind the possibility of developing pancreatitis. And if a patient complained a strong abdominal pain during and after the procedure, we have to rule out acute pancreatitis by examining serum amylase level and abdominal CT as soon as possible.

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CASE REPORT

Living-related liver transplantation for multiple liver metastases from rectal carcinoid tumor: A case report

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Abstract

A 42-year-old woman was admitted to our hospital because of multiple liver tumors detected by ultrasonography. Colonoscopy revealed submucosal tumor in the rectum, which was considered the primary lesion. Endoscopic mucosal resection followed by histopathological examination revealed that the tumor was carcinoid. The resected margin of the tumor was positive for malignant cells. Two courses to transcatheter arterial chemotherapy for liver metastasis were ineffective. Accordingly, the rectal tumor and metastatic lymph nodes were surgically resected. One month after the operation, she received liver transplantation (left lateral segment and caudate lobe) from her son. No recurrent lesion has been observed at two years after the liver transplantation. Liver transplantation should be considered as a treatment option even in advanced case of carcinoid metastasis to the liver. We also discuss the literature on liver transplantation for metastatic carcinoid tumor.

Key words: Carcinoid; Liver metastasis; Living related liver transplantation

Nakajima Y, Takagi H, Sohara N, Sato K, Kakizaki S, Nomoto K, Suzuki H, Suehiro T, Shimura T, Asao T, Kuwano H, Mori M, Nishikura K. Living-related liver transplantation for multiple liver metastases from rectal carcinoid tumor: A case report.

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INTRODUCTION

Carcinoid tumors are slow-growing neuroendocrine tumors, and often metastasize to the liver. There is no established treatment for liver metastases and the prognosis is poor[1, 2]. Liver transplantation for metastatic neuroendocrine tumor has already been reported worldwide[3-16], but the procedure is rarely performed in Japan[17]. We report here a case of living-related liver transplantation for liver metastases of rectal carcinoid tumor.

CASE REPORT

A 42-year-old woman was admitted to our hospital because of multiple liver tumors detected by ultrasonography. The medical history included bronchial asthma. There was no history of blood transfusion. Physical examination revealed a hard and swollen liver in the upper abdomen. Laboratory tests showed erythrocyte count of $367 \times 10^4/mm^3$ (normal: $400\text{-}500 \times 10^4/mm^3$), hemoglobin 10.5 g/dL (11.8-15.1 g/dL), leukocyte count $8200/mm^3$ ($4000\text{-}9600/mm^3$), platelet count $25.9 \times 10^4/mm^3$ ($16.0\text{-}35.0 \times 10^4/mm^3$), serum albumin 4.2 g/dL (3.9-5.0 g/dL), total bilirubin (T-Bil) 1.0 mg/dL (0.3-1.2 mg/dL), aspartate aminotransferase (AST) 30 IU/L(13-33 IU/L), alanine aminotransferase (ALT) 39 IU/L (6-27 IU/L), alkaline phosphatase (ALP) 311 IU/L (115-359 IU/L), γ-glutamyl transpeptidase (γ -GTP) 137 IU/L (10-47 IU/L), blood urea nitrogen (BUN) 20.0 mg/dL (8.0-20.0 mg/dL), and creatine (Cr) 0.9 mg/dL (0.6-1.0 mg/dL). Hepatitis B surface and hepatitis C virus antibody were negative. Serotonin and 5-HIAA in serum were within the normal range. Carcinoembryonic antigen, CA19-9, alfa-fetoprotein and protein induced by vitamin K antagonist (PIVKA)-II were normal but neuron-specific enolase (NSE) was elevated 46.1 ng/mL (0-10.0 ng/mL).

Abdominal ultrasonography (US) revealed multiple hyperechoic masses in both lobes of the liver (Figure 1A). Abdominal computed tomography (CT) also revealed multiple liver tumors enhanced mildly (Figure 1B). Abdominal angiography showed hepatomegaly and multiple liver tumors supplied by the hepatic artery. Colonoscopy showed

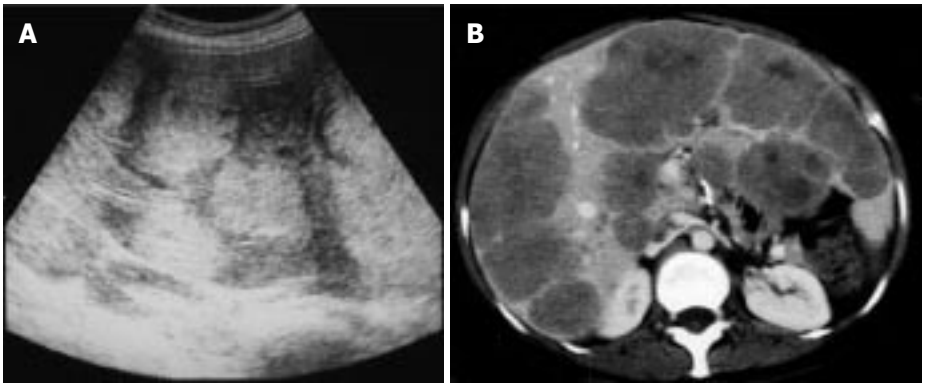

Figure 1 A: Ultrasonography showed multiple hyperechoic masses in the liver; **B**: Dynamic computed tomography showed multiple liver tumors. The surfaces of these tumors showed mild enhancement.

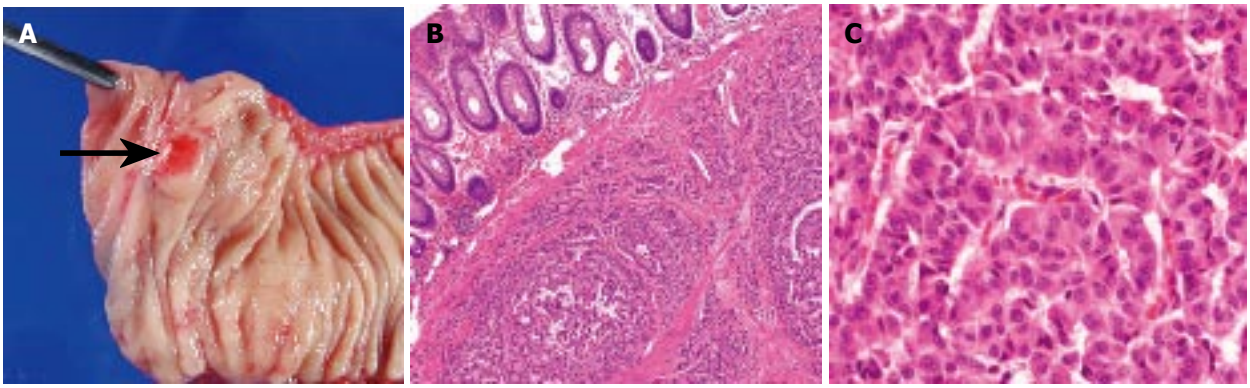

Figure 2 A: Macroscopic findings of the resected rectum. Arrow shows the primary lesion; **B**: The tumor cells showed tubular and alveolar formation, and their nuclei were slightly swelling (**C**). (**B**, **C**: H&E, original magnification, **B**: x 40, **C**: x 400).

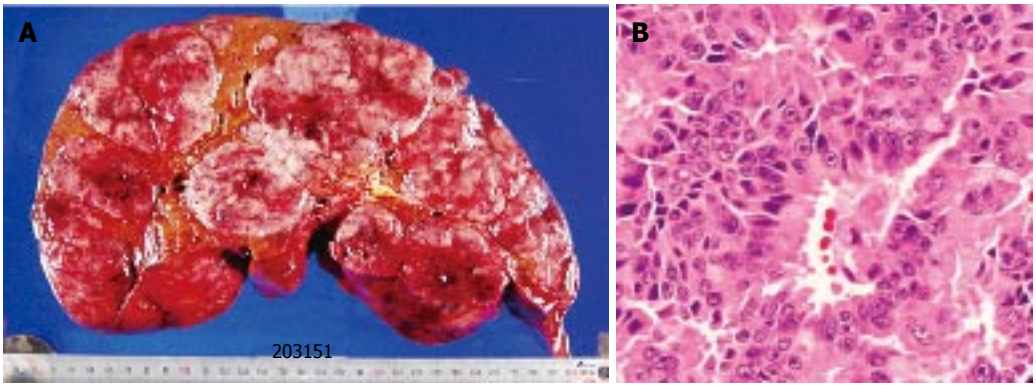

Figure 3 A: The cut surface of the resected specimen showed multiple tumors; **B**: Histopathological findings of the liver tumor were similar to those of the rectal tumor (H&E, original magnification, x 400).

submucosal tumor in the rectum. This tumor appeared as a low echoic mass by endoscopic ultrasonography (EUS). Then we performed endoscopic mucosal resection (EMR) for the submucosal tumor of the rectum, which was histopathologically diagnosed as carcinoid tumor. The resected margin of the tumor was positive for malignant cells. Transcatheter arterial chemotherapy for liver metastasis was applied twice (first: 5-fluorouracil [5-FU] + epirubicin [EPI] + mitomycin C [MMC], second: 5-FU + methotrexate [MTX]), but was ineffective because the liver tumors did not decrease in size and even grow 2 mo after the TACE. Accordingly, the rectal tumor and metastatic lymph nodes were resected surgically. Macroscopically, the rectal tumor was an elevated lesion with a central depression, measuring 58 mm in a diameter (Figure 2A). Histopathological examination showed atypical cells forming tubular and alveolar structures, with slightly swollen nuclei (Figures 2B and 2C). Lymph node metastases and blood vessel invasions were detected. Immunohistochemical examination revealed that most tumor cells were stained with chromogranin A, NSE and synaptophysin. The Ki-67 index was 6.1, but p53 protein was negative.

One month after the operation, there were no recurrence except for the liver. She received a liver transplantation (left lateral segment and caudate lobe) from her son. Standard liver volume (SLV) was 1014.4 g, graft volume (GV) was 450 g, GV/ SLV ratio was 44.4% and graft-to-recipient weight ratio (GR-WR) was 0.98. The volume of the resected liver was 4750 g, and multiple nodules of white and brown colors occupied the whole liver (Figure 3A). The histopathological findings of the liver tumor (Figure 3B) were similar to those of the primary lesion.

Table 1 Literature review of liver transplantation for metastatic neuroendocrine tumors

Reference	Number of patients	Median follow-up (m)	Survival rate (%)			Disease-free survival rate (%)	
			1-yr	3-yr	5-yr	1-yr	5-yr
Rosenau *et al* (2002)[3]	19	38	89	89	80	56	21
Coppa *et al* (2001)[4]	9	39	100	100	70	100	53
Lehnert *et al* (1998)[5]	103	-	68	54	47	60	24
Le Treut *et al* (1997)[6]	31	25	59	47	36	45	17
Florman *et al* (2004)[7]	11	30	73	48	36		
Cahlin *et al* (2003)[8]	10	28	80	80	-	-	-
Olausson *et al* (2002)[9]	9	22	89	89	-	-	-
Ringe *et al* (2001)[10]	5	18	80	80	-	-	-
Pascher *et al* (2000)[11]	4	42	100	75	50	-	-
Frilling *et al* (1998)[12]	4	54	50	50	50	-	-
Lang H *et al* (1997) [13]	12	49.5	83	83	83	-	-
Dousset *et al* (1996)[14]	9	29	33	33	33	-	-
Anthuber *et al* (1996)[15]	4	11	25	0	0	-	-
Routley *et al* (1995)[16]	11	-	82	-	57	-	-
Japan (2005)[17]	6	-	66.7	66.7	-	-	-

No invasion of the portal vein, hepatic vein, and bile duct was noted.

Her clinical course has been good and no recurrence has been demonstrated two years since the liver transplantation.

DISCUSSION

Neuroendocrine tumors have generally been classified by the site of origin. Furthermore, a new histopathological classification was reported by WHO[18]. The WHO classification has been considered by the size of the tumor, the depth of the tumor invasion, angiogenesis, lymphatic invasion, cellular atypia, necrosis, mitoses, Ki-67 index and p53 protein. Based on this classification, neuroendocrine tumors are divided into three types, well-differentiated endocrine tumor (carcinoid), well-differentiated endocrine carcinoma (malignant carcinoid), and poorly-differentiated endocrine carcinoma (small cell carcinoma)[18]. Although the standard therapy for liver metastasis of neuroendocrine origin is surgical resection[19], the prognosis of neuroendocrine tumor with liver metastasis is usually poor[1, 2]. When curative hepatic resection is difficult, transcatheter arterial chemo-embolization (TACE) and intra-arterial chemotherapy are performed and have been reported to be effective[20-23]. Our patient received two courses of intra-arterial chemotherapy but no satisfactory response was observed. Somatostatin analogue and interferon have been used for the treatment of carcinoid tumors[24-27]. These therapies are excellent for improvement of symptoms but the tumor response rate is usually low[24-27].

Liver transplantation has been widely performed in patients with end-stage liver disease and metastatic liver cancers from neuroendocrine tumors[3-6]. The 5-year survival rate of transplant recipients for neuroendocrine tumors metastases to the liver ranges from 0 to 83% (median, 50%) (Table 1). The main cause of death is recurrence of the carcinoid tumors. In Japan, the accumulative living related liver transplantations between 1996 and 2002 are more than 2000. Among these, transplantation was performed in only 6 cases of metastatic neuroendocrine tumors (0.2 %) and the 3-year survival rate is 66.7%[17]. Strictly speaking, the 5-year survival rate of liver transplantation for metastatic carcinoid tumor is 69% but is poor in noncarcinoid neuroendocrine tumors (4-year survival rate, 8%)[6]. Thus, histopathological discrimination is very important to predict the prognosis of neuroendocrine tumors. Our patient had a typical carcinoid tumor which is compatible with well-differentiated neuroendocrine carcinoma, with metastases in the liver and was considered to show good prognosis after transplantation. However, the case was advanced stage with lymph node metastasis, lymphatic and vascular invasion and extensive liver metastasis, and thus was considered a high recurrent risk requiring careful follow-up. Unexpectedly good prognosis of this case could be related to the radical resection of the tumor including primary and metastatic lesion and not classified in poor prognosis such as non-pancreatic primary lesion (rectum) and noncarcinoid apudoma[6]. The prognosis was markedly improved by transplantation and she remains well 2 years and 9 mo after surgery without local recurrence and metastasis. Although much longer follow-up period would provide more meaningful information to elucidate the prognosis of such unusual case, liver transplantation could be life-saving procedure for patients with metastatic neuroendocrine tumor resistant to alternative treatments.

Pelosi *et al*[28] reported that Ki-67 index is a significant predictor of prognosis and survival of patients with pancreatic neuroendocrine tumors. Furthermore, Moyana *et al*[29] reported that MIB-1 and p53 were associated with metastasis of the gastrointestinal carcinoid tumors. Rosenau *et al*[3] pathologically investigated patients who received a liver transplantation for metastatic neuroendocrine tumors. They reported that the survival rate of patients with high Ki-67 index (> 5%) or overexpression of the E-cadherin was low, and suggested that Ki-67 index and E-cadherin expression could be potentially useful prognostic markers after liver transplantation[3]. Our pa-

tient showed moderately positive Ki-67 index (6.1%, rate for carcinoid is around 2-3%) and should be followed as high recurrence risk case.

The Japanese medical insurance system covers liver transplantation for liver cirrhosis and hepatocellular carcinoma (HCC) based on Milan criteria[30]. However, the system does not cover metastatic liver cancer. Liver transplantation is a kind of special treatment for the end-stage liver disease and is also expensive. So, not all the patient in the end-stage liver disease has been covered by medical insurance in Japan. In our patient, distant and lymph node metastases were completely resected, and metastatic neuroendocrine tumors in the liver were removed through hepatectomy and liver transplantation even though the metastasis was far advanced within the liver though localized in the liver. We propose that metastatic neuroendocrine tumors of the liver should be classified as similar to HCC although cases beyond Milan criteria[30], like our case, could be also included in such classification because of its biological low malignant character.

In conclusion, we reported a female patient who underwent successful living liver transplantation for advanced liver metastases of rectal carcinoid tumor. She has been well for the last two postoperative years and remains alive without any recurrence in spite of positivity of poor prognostic parameters. Other parameters, such as oncogene, suppressor gene and cyclin shown in hepatocellular carcinoma[31], apart from those of histopathology and immunohistochemistry, are needed to help in clinical decision making with respect to the indications of transplantation.

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CASE REPORT

Autoimmune pancreatitis: Functional and morphological recovery after steroid therapy

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Abstract

Autoimmune pancreatitis, a recently recognized type of chronic pancreatitis, is not rare in Japan, but reports of it elsewhere are relatively uncommon. We report the first preoperatively diagnosed case of autoimmune pancreatitis in Hungary, which responded well to steroid treatment and provided radiographic and functional evidence of this improvement. A 62-year-old female presented with a 4-month history of recurrent epigastric pain and a 5-kg weight loss. The oral glucose tolerance test (OGTT) indicated diabetes mellitus and the result of the fecal elastase test was abnormal. Ultrasonography (US) and the CT scan demonstrated a diffusely enlarged pancreas, and endoscopic retrograde cholangiopancreatography (ERCP) an irregular main pancreatic duct with long strictures in the head and tail. Autoimmune pancreatitis was diagnosed. The patient was started on 32 mg prednisolone daily. After 4 wk, the OGTT and faecal elastase test results had normalized. The repeated US and CT scan revealed a marked improvement of the diffuse pancreatic swelling, while on repeated ERCP, the main pancreatic duct narrowing was seen to be ameliorated. It is important to be aware of this disease and its diagnosis, because AIP can clinically resemble pancreatobiliary malignancies, or chronic or acute pancreatitis. However, in contrast with chronic pancreatitis, its symptoms and morphologic and laboratory alterations are completely reversed by oral steroid therapy.

Key words: Autoimmune pancreatitis; Steroid therapy; Chronic pancreatitis; Pancreatic endocrine function; Pancreatic exocrine function

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INTRODUCTION

Autoimmune pancreatitis (AIP) is an increasingly recognized type of chronic pancreatitis that is clearly distinct from alcoholic chronic pancreatitis. It is characterized by its special morphology, immunologic features, pathology and steroid responsiveness[1-5]. Since Sarles *et al*[6] first described a case of pancreatitis with hypergammaglobulinaemia more than 40 years ago, several hundred cases have been reported, particularly in the Japanese literature[1-4,7-9]. Its prevalence in other populations is unclear.

We describe here the first preoperatively diagnosed case of AIP in Hungary and discuss the clinical features, diagnosis and treatment of this uncommon disease.

CASE REPORT

A 62-year-old teetotal woman with a 4-month history of recurrent epigastric pain and a 5-kg weight loss was referred to our hospital. There was no personal or family history of pancreatitis. On physical examination, an apple-sized, painless mass was palpated in the epigastrium. With the exception of elevated fasting glucose level, the results of detailed laboratory examinations, including Ca 19-9 antigen, were normal. The oral glucose tolerance test (OGTT) revealed diabetes mellitus. The fecal elastase test result was abnormal (120 μg/g faeces). The antinuclear antibody and rheumatoid factor tests were positive. Abdominal ultrasonography (US) revealed a diffusely enlarged pancreas, and the abdominal CT scan an enlarged, sausage-shaped pancreas (Figures 1A and 1B). On endoscopic retrograde cholangiopancreatography (ERCP), an irregular main pancreatic duct with long strictures in the head and tail was observed (Figure 2A). AIP was diagnosed. The patient was started on 32 mg prednisolone daily and enzyme replacement therapy. After 4 weeks she had become totally asymptomatic. The fasting glucose level, the results of OGTT and the fecal elastase test had normalized, and the repeated US and CT scan demonstrated a marked improvement of the diffuse pancreatic swelling (Figures 1C and 1D). On repeated ERCP, the main pancreatic duct narrowing was seen to be ameliorated (Figure 2B). We ad-

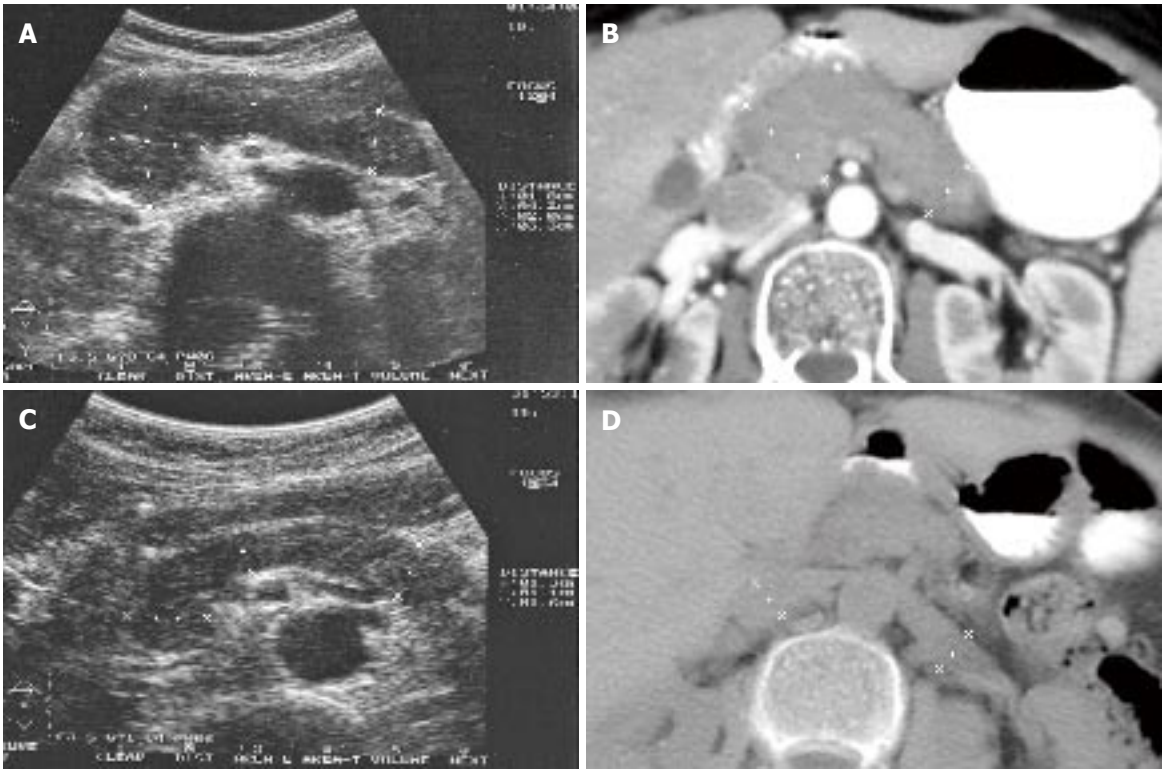

Figure 1 Abdominal ultrasonography (**A**) and CT (**B**) revealed a diffusely enlarged pancreas with sharp margins similar to sausage appearance. Four weeks after the steroid therapy the size of the pancreas became normal (**C** and **D**).

vised tapering and cessation of the steroid.

DISCUSSION

To date, several hundred cases of AIP have been reported, particularly in the Japanese literature[1-5,7-9]. This has led to the proposal of AIP as a distinct clinical entity, and AIP is currently categorized as an established etiology of chronic pancreatitis[10]. The disease has appeared outside Japan only very rarely; few cases have been published in Europe[11-16], most of which were diagnosed postoperatively. It is unlikely that AIP is a disease endemic to Japan. It appears likely that physicians in other countries may overlook this entity due to the lack of their familiarity with it, and regard such patients as cases of ordinary or idiopathic chronic pancreatitis, or pancreatobiliary malignancy.

The diagnosis of AIP is challenging as its symptoms are few and non-specific. Its clinical characteristics are jaundice, abdominal pain, weight loss and diabetes mellitus. Most of the patients are older than 50 years. In consequence of the symptoms and the age of these patients, they are often initially thought to have pancreatic carcinoma, and many of them actually undergo laparotomy[5,17]. AIP may be associated with a number of other autoimmune diseases, such as Sjögren's syndrome, primary sclerosing cholangitis, inflammatory bowel disease, systemic lupus erythematosus or retroperitoneal fibrosis. Laboratory findings suggestive of AIP include hypergammaglobulinaemia, increased levels of total IgG or IgG4, and the presence of autoantibodies such as antinuclear antibody, anti-lactoferrin antibody and anti-CA-II antibody[1-5]. Characteristic radiological features are an enlarged, sausage-shaped pancreas on abdominal US and CT, and narrowing of the main pancreatic duct on ERCP[18]. A common microscopic picture in AIP is periductal lymphoplasmacytic infiltration with pronounced acinar atrophy, marked fibrosis and obliterated phlebitis[19].

The diagnosis of AIP should be based on a combination of the clinical and laboratory findings, pancreatic imaging, and the exclusion of other conditions[4].

The principal treatment is corticosteroid at a recommended starting dose of 30 to 40 mg of prednisolone/day until the symptoms improve; this is followed by a taper of 5 to 10 mg. Steroid treatment usually leads to a rapid improvement in the clinical symptoms and the laboratory and morphological findings. Steroid therapy has also been reported to be beneficial as concerns the pancreatic fibrosis, exocrine and endocrine functions and associated diabetes mellitus[7-9,20]. It has been proposed that the response to steroid therapy should be included in the diagnostic criteria[21]. Steroid therapy may be tried empirically, when AIP is suspected on the basis of the typical pancreatic imaging results alone, if laboratory data and histologic findings are not available or do not fully meet the diagnostic criteria[5, 7] The response to steroid therapy can facilitate confirmation of the diagnosis of AIP.

In our patient, the diagnosis of AIP was indicated by the morphological alterations of the pancreas in US and CT, the narrowing of the main pancreatic duct on ERCP, and the autoimmune abnormalities. Histological findings were not available. The clinical symptoms and the abnormalities observed on US, CT and ERCP responded rapidly to steroid therapy, confirming the diagnosis of AIP.

It is very important to be aware of this disease, because

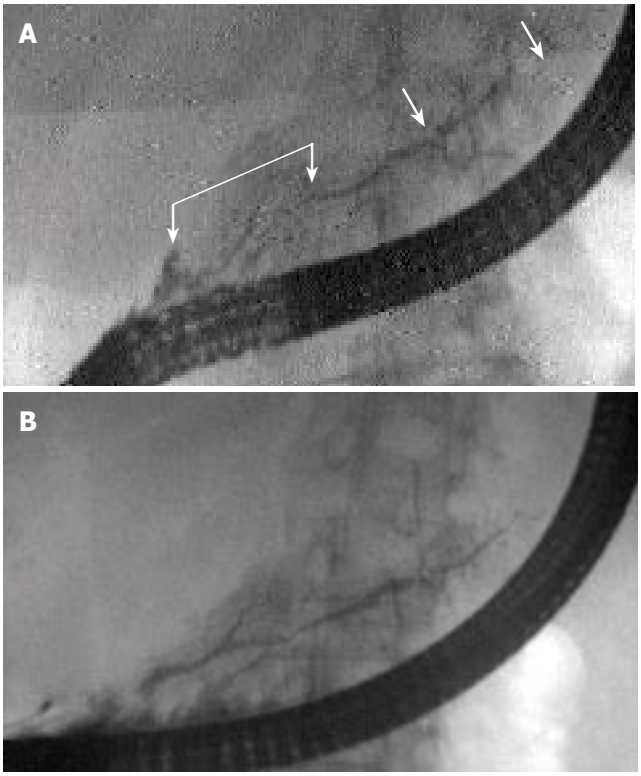

Figure 2 ERCP demonstrated multiple strictures of the Wirsung duct without posttenotic dilation (**A**). Four weeks after the steroid therapy the main pancreatic duct narrowing was seen to be ameliorated (**B**).

AIP may be mistaken for ordinary chronic pancreatitis or pancreatic cancer, leading to pancreatic resection instead of treatment with steroid. As awareness of this disease grows, the reported prevalence of AIP outside Japan may be expected to increase and the possibility of unnecessary surgery may be reduced.

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CASE REPORT

Sporadic somatic mutation of *c-kit* gene in a family with gastrointestinal stromal tumors without cutaneous hyperpigmentation

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Abstract

We described two members in a family with gastrointestinal stromal tumors (GISTs) without cutaneous hyperpigmentation. The patients were father and son who did not have cutaneous hyperpigmentation. Histological examination showed that these tumors were GISTs expressing CD34 and CD117. Tumor DNA extracted from paraffin-embedded specimens revealed somatic mutation with a deletion mutation at different codons in exon 11 of c-kit gene after direct sequencing analysis. No germline mutation was detected in DNA extracted from peripheral leukocytes obtained from the father and son. We propose that GISTs could be caused by sporadic somatic mutation in a family without germline mutation and hyperpigmentation.

Key words: Sporadic GIST; Somatic c-kit mutation

Yeh CN, Chen TW, Jan YY. Sporadic somatic mutation of *c-kit* gene in a family with gastrointestinal stromal tumors without cutaneous hyperpigmentation. *World J Gastroenterol* 2006; 12(11): 1813-1815

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INTRODUCTION

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the human gastrointestinal (GI) tract, representing 0.1 to 3 % of all GI tract tumors[1]. It has been suggested that a mutation in the juxtamembrane (JM) domain of c-kit contributes to the development of GIST[2]. Furthermore, germline deletion or point mutation of the c-kit JM domain has been shown in a family with GIST and cutaneous hyperpigmentation[3,4]. GIST-cutaneous hyperpigmentation disease has been used to describe familial multiple GISTs with associated cutaneous hyperpigmentation[4]. Familial GIST is a rare autosomal dominant genetic disorder associated with kit germline mutations. We now report that GISTs could be caused by sporadic somatic mutation in a family without germline mutation and cutaneous hyperpigmentation.

CASE REPORT

Patient 1

A 79-year-old man was referred to our hospital because of a gastric tumor (Figure 1). He complained of epigastria pain and fullness. There was no cutaneous hyperpigmentation. Gastroduodenal endoscopic examination and endoscopic ultrasonography (EUS) showed a gastric submucosal tumor (SMT) about 1.7 cm in size with small ulcer located at anterior wall, greater curvature side of high body. Proton pump inhibitor therapy was administered for 10 months. The SMT enlarged to 5cm in size demonstrated by upper gastrointestinal (GI) series, endoscopic ultrasonography (EUS), and abdominal computed tomography (CT) (Figure 2A). Wedge resection of the gastric SMT at the anterior wall, greater curvature side of high body with spleen preservation was performed with clear resected margin. Histological and immunohistochemical examination revealed low risk gastrointestinal stromal tumor (GIST) with strong positivity of c-kit and CD34 (Figure 2B). The patient lived free of disease for 9 months after surgery.

Patient 2

A 54-year-old man, the son of the patient 1, was referred to our hospital because of a gastric tumor (Figure 1). He complained of epigastria pain, abdominal fullness, body weight loss 7 to 8 kilograms for one month. There was no cutaneous hyperpigmentation. Gastroduodenal endoscopic examination and EUS showed a huge gastric SMT about 10 cm in size located at greater curvature side from high body to antrum with a deep ulcer and easily touch bleeding. Subsequent upper GI series and abdominal CT revealed a gastric SMT about 13×10 cm in size occupying the whole stomach (Figure 2C). Exploratory laparotomy revealed advanced gastric GIST with major vessel involvement. Biopsy of the gastric SMT and feeding jejunostomy

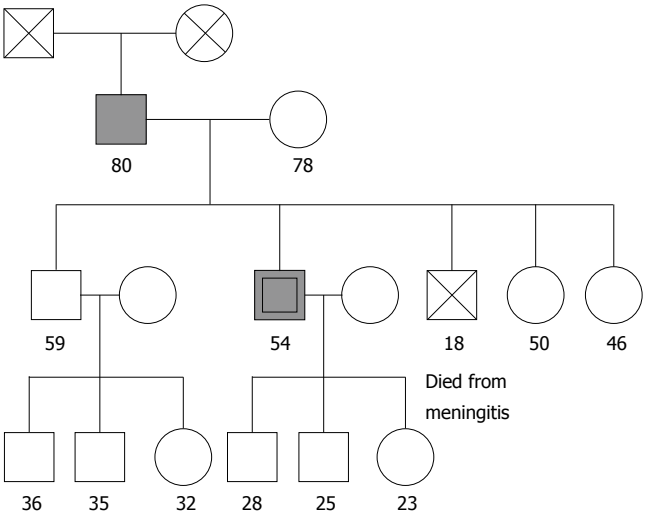

Figure 1 Pedigree of the family with GISTs without cutaneous hyperpigmentation. Hatched symbols indicate family members with GISTs. Double circles denote multiple GISTs. Squares indicate males and circles indicate females.

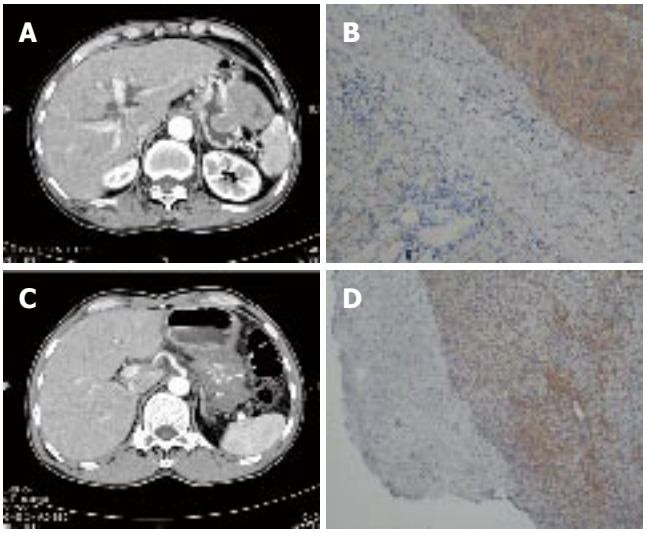

Figure 2 A: Abdominal CT revealed gastric GIST at the anterior wall and greater curvature side of high body of the stomach; **B**: Microscopic findings of the tumor resected. Tumor cells were composed of resicular spindle cells with mild nuclear pleomorphism but no necrosis, expressing strong positive c-kit staining immunohistochemically. (IHC staining, 200X); **C**: Abdominal CT revealed a gastric GIST measuring 13 cm x 10 cm in size occupying the whole stomach. **D**: Microscopic findings of the tumor resected. Tumor comprised proliferation of spindle cells with mild nuclear atypia in the mxyoid stroma but no necrosis, expressing positive c-kit staining. (IHC staining, 200X).

was performed. Histological and immunohistochemical examination revealed advanced high-risk GIST with strong positivity of c-kit and CD34 (Figure 2D). The patient received 400 mg Glivec for 7 months and partial response was illustrated by subsequent abdominal CT. The patient lived with disease for 7 months after surgery.

Genetic examination

To determine whether these GISTs showed a genetic defect, DNA was extracted from paraffin-embedded specimen of tumor tissues. Polymerase chain reaction amplification of genomic DNA for kit and PDGFRA was performed and amplification was analyzed for mutations as previously described[5]. Direct sequencing analysis of DNA from patient 1 showed deletion mutation at codon 560 in exon 11, causing a deletion mutant 560 del V (Figure 3B). While direct sequencing analysis of DNA from patient 2 revealed deletion at codons 557-559 in exon 11, resulting in replacement of WKV by C (Figure 3C). To determine if the mutation is familial, DNA was extracted from peripheral leukocytes obtained from patients 1 and 2. No mutation was detected in exon 11 of c-kit gene (Figure 3A).

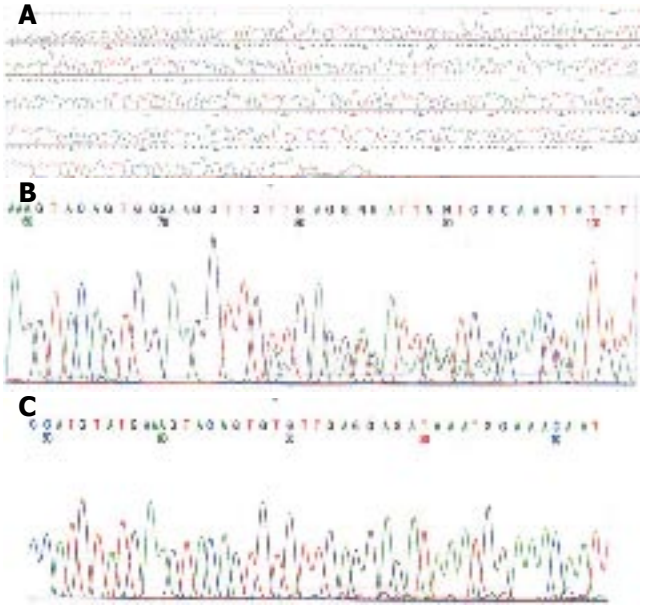

Figure 3 A. Direct sequencing analysis of DNA from peripheral leukocyte obtained from patients 1 and 2 revealed no mutation in exon 11 of c-kit gene; **B**. Direct sequencing analysis of DNA from patient 1 showed deletion mutation at codon 560 in exon 11, causing a deletion mutant 560 del V; **C**. Direct sequencing analysis of DNA from patient 2 revealed deletion at codons 557-559 in exon 11, resulting in replacement of WKV by C.

DISCUSSION

By histological, immunohistochemical examination, and molecular genetic analysis, this study has uncovered sporadic c-kit somatic mutation in a family with GIST without cutaneous hyperpigmentation.

GISTs appear to be related the interstitial cells of Cajal of the mesenteric plexus[7]. These cells are considered as GI pacemaker cells, from the interface between the automatic innervation of the bowel wall and its smooth muscle[8, 9]. GISTs express the cell-surface transmembrane receptor c-kit with a tyrosin kinase activity and kit oncoprotein. There are frequent gain-of-function mutations of c-kit in GISTs. These mutations result in constitutive activation of kit signaling, which leads to uncontrolled cell proliferation and resistance to apoptosis. It has been recently reported that kit activation occurs in all cases of GISTs, regardless of the mutation status of kit. Most GISTs express constitutively activated mutant isoforms of kit kinase or platelet-derived growth factor receptor alpha (PDGFRA), which are potential therapeutic targets for Imatinib mesylate (Glivec).

Gain of function mutations in the JM domain of c-kit contribute to the development of GIST[2]. Because normal kit gene is responsible to normal pigmentation, relationships between GISTs and cutaneous hyperpigmentation have been reported before. GIST-cutaneous hyperpigmentation disease has been used to describe familial multiple GISTs associated with cutaneous hyperpigmentation[3]. Furthermore, germline deletion mutation of the c-kit JM domain has been shown in tumors and normal somatic cells from a family with multiple GISTs who exhibited perineal hyperpigmentation[3]. A single-point germline mutation of c-kit has also been proposed to cause a familial GIST associated with systemic cutaneous hyperpigmentation[4]. A germline PDGRF missense mutation could be a second familial predisposing gene[5].

We described a rare case report regarding two members in a family with GISTs without cutaneous hyperpigmentation. No mutation was detected in DNA extracted from peripheral leukocytes obtained from the father and son. DNA extracted from paraffin-embedded specimens revealed different somatic mutation with a deletion mutation in the exon 11 of c-kit gene after direct sequencing analysis (deletion mutation at codon 560 versus deletion at codons 557-559 in exon 11). So mutation in the tumor is sporadic somatic but not germline. However, the cause of sporadic c-kit mutation in one family is unknown.

In summary, we propose that GISTs could be caused by sporadic somatic mutation in a family without germline mutation and hyperpigmentation.

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Digestive Disease Week
107th Annual of AGA, The American Gastroenterology Association
20–25 May 2006
Loas Angeles Converntion Center, California

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24–25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3–8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26–30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14–16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1–5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4–5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6–7 May 2006
Berlin
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ILTS 12th Annual International Congress
3–6 May 2006
Milan
ILTS
www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006–1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15–18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006–1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6–8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

7th World Congress of the International Hepato-Pancreato-Biliary Association
3–7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

Society of American Gastrointestinal Endoscopic Surgeons
26–29 April 2006
Dallas – TX
www.sages.org

Digestive Disease Week 2006
20–25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25–26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3–7 June 2006
Seatlle - Washington
www.fascrs.org

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isde@sapmea.asn.au
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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	$10\times10\times10cm^3$	10 cm×10 cm×10 cm	
66	N·km	KN·m	moment
67	$x\pm s$	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 *et al.*	
72	EcoRI	*Eco*RI	*Eco* in italic and RI in positive. Restriction endonuclease has its prescript form of writing.
73	Ecoli	*E.coli*	Bacteria and other biologic terms have their specific expression.
74	Hp	*H pylori*	
75	Iga	*Iga*	writing form of genes
76	*igA*	IgA	writing form of proteins
77	-70 kDa	~70 ku	

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REVIEW

Gastrointestinal manifestations in myotonic muscular dystrophy

Massimo Bellini, Sonia Biagi, Cristina Stasi, Francesco Costa, Maria Gloria Mumolo, Angelo Ricchiuti, Santino Marchi

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Abstract

Myotonic dystrophy (MD) is characterized by myotonic phenomena and progressive muscular weakness. Involvement of the gastrointestinal tract is frequent and may occur at any level. The clinical manifestations have previously been attributed to motility disorders caused by smooth muscle damage, but histologic evidence of alterations has been scarce and conflicting. A neural factor has also been hypothesized. In the upper digestive tract, dysphagia, heartburn, regurgitation and dyspepsia are the most common complaints, while in the lower tract, abdominal pain, bloating and changes in bowel habits are often reported. Digestive symptoms may be the first sign of dystrophic disease and may precede the musculo-skeletal features. The impairment of gastrointestinal function may be sometimes so gradual that the patients adapt to it with little awareness of symptoms. In such cases routine endoscopic and ultrasonographic evaluations are not sufficient and targeted techniques (electrogastrography, manometry, electromyography, functional ultrasonography, scintigraphy, *etc*.) are needed. There is a low correlation between the degree of skeletal muscle involvement and the presence and severity of gastrointestinal disturbances whereas a positive correlation with the duration of the skeletal muscle disease has been reported.

The drugs recommended for treating the gastrointestinal complaints such as prokinetic, anti-dyspeptic drugs and laxatives, are mainly aimed at correcting the motility disorders.

Gastrointestinal involvement in MD remains a complex and intriguing condition since many important problems are still unsolved. Further studies concentrating on genetic aspects, early diagnostic techniques and the development of new therapeutic strategies are needed to improve our management of the gastrointestinal manifestations of MD.

INTRODUCTION

Myotonic dystrophy (MD) is an autosomal dominant genetic disorder[1]. It is caused by an unstable trinucleotide repeat expansion containing cytosine-thymidine-guanosine $(CTG)^n$, located in the 3' untranslated region of chromosome 19q13.3. The CTG trinucleotide is repeated in the normal population from 5 to 36 times[2,3], but has been found to be expanded up to 2 000 times in MD patients[4,5]. This amplification is correlated to the severity of the disease. Patients can be divided into four groups based on the number of CTG repeats. Group E1 (50-100 CTG repeats) may have either the minor or the classical form of MD, group E2 (500-1 000 CTG repeats) have the classical form of MD, group E3 (1 000-1500 CTG repeats) have the classical form of MD with onset during childhood, and group E4 (more than 1 500 CTG repeats) are affected with the congenital form of MD[6].

Although MD is primarily characterised by myotonic phenomena and progressive muscular weakness, multi-system involvement is often present, taking the form of cardiac conduction abnormalities, cognitive deficits, cataracts and diabetes, as well as endocrine, sexual and reproductive disturbances[1]. Involvement of the gastrointestinal (GI) tract is frequent, and may occur at any level from the pharynx to the anal sphincter. Dysphagia, heartburn, emesis, regurgitation, coughing while eating and dyspepsia are the most common complaints involving the upper digestive tract, while abdominal pain and bloating, changes in bowel habits (diarrhoea or constipation) and dyschezia are common signs of impairment of the lower digestive tract[7-9]. These clinical manifestations have generally been attributed to motility disorders caused by striated and more rarely, smooth muscle damage[10],

although recent studies suggest that neurological alterations may also play a role[11,12].

There appears to be little correlation between the degree of skeletal muscle involvement and the presence and severity of the gastrointestinal disturbance, but some authors have reported a positive correlation with the duration of the skeletal muscle disease[13, 14]. In some patients the impairment of gastrointestinal function is so gradual that they adapt to it with little or no awareness of any disturbance and only a thorough anamnesis may elicit the recall of possible symptoms[12]. In such patients, routine endoscopic and ultrasonographic evaluations are of little value and only highly sensitive, targeted techniques such as electrogastrography, manometry, electromyography, functional ultrasonography, compartmental scintigraphy, *etc.* may help to reveal the true extent of the involvement.

In this review, the available data on the pathophysiologic mechanisms of GI involvement in myotonic dystrophy, their impact on clinical manifestations of the disease, and the tests that can be used to detect them, are discussed in turn for each segment of the gastrointestinal tract.

PHARYNX AND ESOPHAGUS

Because most complaints by MD patients focus on the pharynx and esophagus, which can be quite easily investigated, these are the manifestations that have been most thoroughly studied thus far. MD patients often report dysphagia, sometimes associated with coughing while eating, chest pain, regurgitation and heartburn. Different studies have reported a prevalence of dysphagia ranging from 25% to 80%[9, 12, 15, 16].This is usually considered by both patients and doctors to be the most serious symptom of MD, probably because it is associated with a high incidence of recurrent pulmonary infection[17].

Manometry is useful to detect motility disturbances, which show in the form of asymmetric contractions of the pharynx and weak contractions of the upper esophageal sphincter (UES)[14]. Studies have reported a reduced basal UES pressure in MD patients compared to controls[11] , although the prevalence and duration of UES relaxation during swallowing are similar between the two groups and more significantly, no difference in the residual pressure during relaxation has been found[11, 12,1 8] .

In the esophageal body significant decreases in the peristaltic amplitude and/or simultaneous waves have often been reported[12, 18]. A study of 14 MD patients showed a marked decrease in the median amplitude of the contractions at every level of the esophagus. A higher percentage of non-transmitted and dropped contraction sequences after swallowing has been observed in patients compared to controls, 43% exhibited complete atony of the esophageal body with a scleroderma-like pattern and no detectable esophageal contractions. In this series, although manometry showed impaired esophageal motor function in 71% of the patients, 50% of these were quite asymptomatic[18].

Involvement of the lower esophageal sphincter (LES) is a somewhat more complex matter. Modolell *et al.*[12] failed to detect a significant difference in basal LES pressure between patients and controls, only reporting some cases of incomplete LES relaxation[12]. In contrast, other studies have found a significantly reduced resting tone, competence, and area vector volume of the LES in MD patients than in controls[9, 18]. These data suggest that some alteration in the smooth muscle may take place, leading to a higher risk of developing gastroesophageal reflux disease (GERD)[18].

Esophageal spasm and segmental dilatation can be seen on x-rays, while radiological and scintigraphic techniques are useful for detecting both a lack of tone and a reduced, ineffective or absent peristalsis[12, 15, 19].

Most authors hold that pharyngo-esophageal myotonia, or problems with post-contraction relaxation, should no longer be considered as a typical feature of esophageal involvement[12, 18]. A myotonic response to pharyngeal contractions has been reported by Siegel *et al*[20], but we now know that this is due to limitations in x-ray technology at the time when the study was conducted.

It has been hypothesized that distal esophageal impairment may be myogenic in origin and abnormalities in both the striated and smooth muscle fibers may play a role in MD. However, the histologic data published to date have failed to demonstrate any alteration in the esophageal smooth muscle fibers[19]. Marked atrophy of the esophageal striated muscle, but only small changes in the smooth muscle were observed in a patient reported by Jéquier *et al*[21]. In another case, using electron microscopy to study the esophagus, Ludatscher *et al*[22] detected mild degenerative changes with disoriented filaments of the smooth muscle.

Smooth muscle damage could also explain the reduced LES tonic resting pressure and the elevated frequency of heartburn observed in MD patients. Unfortunately, most of the reported cases did not include an upper digestive tract endoscopic examination or a 24-h pH-metry to determine the presence and degree of GERD[18] .

Recently some authors have suggested that a combined myopathic - neuropathic etiology could provide a more satisfactory explanation for the pharyngo-esophageal symptoms of MD, but at present this remains speculative [12]. One proposed hypothesis is that decrease in the normal amplitude of the peristaltic waves could reflect the inability of the muscle to contract under normal neural control. On the other hand, uncoordinated motor activity in the presence of a normal amplitude contraction could indicate a lack or defect in neural control[12]. This hypothesis is not supported by the study of Eckardt *et al*[19], who failed to detect any neuropathic alterations in MD patients.

However, there are data to suggest that there is no association between the degree of muscular disability and motor abnormalities of the pharynx and esophagus, the presence and severity of dysphagia, or other esophageal symptoms[12]. For example, manometric findings are not significantly different between symptomatic and asymptomatic patients or in patients with different degrees of striated muscular involvement[9, 12, 18, 19]. In particular, in a study of 18 MD patients Modolell *et al*[12] showed that while severe pharyngo-esophageal abnormalities are present in all patients, symptoms are spontaneously reported by only 30% of them. This figure rose but only to 55%, when the patients were specifically questioned on

this point.

These conflicting reports may be explained by the fact that the tongue and pharyngeal muscles can contract sufficiently to push a bolus through the UES into the esophagus, after which the force of gravity is enough to complete the swallowing process. Moreover, because of the slow evolution of the disease, MD patients may develop compensatory mechanisms and thus gradually adapt to the impairment of their esophageal function[18].

STOMACH AND DUODENUM

MD patients frequently complain of dyspeptic symptoms such as early satiety, nausea, vomiting, and epigastric pain[9]. Radiographic, manometric, ultrasonographic and scintigraphic evidence of decreased peristaltic activity causing the delayed emptying of liquids and solids, visceral dilatation, gastric bezoars and even gastroparesis have been reported[10, 13, 23-25].

Kuiper *et al*[23] have described the case of an MD patient who developed symptoms suggestive of gastric retention. Radiological and endoscopic examination revealed polypoid masses, cytologic washing showed them to be food concretions, probably a consequence of abnormal gastric motility[23]. More recently, scintigraphic studies by Ronnblom *et al*[25] indicate a delayed emptying of the stomach in MD patients with dyspeptic symptoms, demonstrated by a prolonged lag phase, a slower emptying rate, and a prolonged T/2.

Bellini *et al*[13] used ultrasound to evaluate gastric emptying abnormalities and their relationship with the severity and duration of the disease in 11 MD patients without dyspeptic symptoms. They found that MD patients and healthy subjects exhibit comparable fasting and maximal antral areas. The findings that (i) the basal and maximal post-prandial values for the gastric antral area between MD patients and controls were quite similar, and (ii) the time required to reach the maximal post-prandial values did not differ in the two groups, are in agreement with the fact that the patients studied were not dyspeptic[13]. Analogous results have been reported for a group of non-dyspeptic patients affected by systemic sclerosis[26].

Indeed, increased antral dimensions have been reported in patients with functional dyspepsia[27,28], suggesting that such abnormalities may play a role in the pathogenesis of the dyspeptic symptoms in MD. In Bellini's study**[13]**, the final emptying time of the MD patients was longer than that of healthy volunteers and was clearly correlated with the disease duration, but was independent of both the CTG expansion size and the patients' disability class.

It is not surprising that patients without dyspeptic symptoms may have delayed gastric emptying. Indeed the association between delayed gastric emptying and upper GI complaints is still under debate[29]. Other abnormalities in gastric motor function, such as impaired accommodation of the proximal stomach[30, 31], abnormal distribution of the gastric contents[31], gastric dysrythmias[32, 33], and a lack of antro-pyloro-duodenal coordination[34], could play a role in the pathogenesis of dyspeptic symptoms in these patients.

Bellini *et al*[13] have confirmed that a direct relationship between disease severity and the impairment of GI motor functioning remains to be demonstrated, since there is no significant difference in gastric emptying between patients with different degrees of skeletal muscle involvement. These results appear to agree with previous studies, demonstrating that skeletal muscular damage and gastric motor disturbances can progress independently[14]. On the other hand, the direct correlation between final emptying time and disease duration suggests that the impairment of gastric emptying may evolve over time.

The delayed gastric emptying in MD could be explained by the muscular disease itself. Unfortunately no definitive conclusions can be drawn on this point because the histological evidence remains limited. Gastric smooth muscle is only rarely affected[7, 8] and as we have already seen in the esophagus, when damage occurs it appears to be similar although earlier and more severe in nature to that occurring in skeletal muscle with severe fatty infiltration[35] . However, a sufficiently large study focusing on the gastric smooth muscle in MD patients is still lacking. It would be important to determine whether the two types of muscle tissue may be damaged to different degrees in the same subjects.

Recent reports suggest that, as in the pharynx and esophagus, muscle involvement alone is not sufficient to explain the altered digestive function in the stomach and duodenum of MD patients. Impaired nervous conduction as well as altered GI hormone secretions could be involved in the motor abnormalities[25]. For example, changes in the electrical impulses controlling duodenal activity could explain the chronic intestinal pseudo-obstruction occasionally reported in MD. Likewise a reduction in the electrical control of gastric activity could be a possible cause of delayed gastric emptying[25].

An electrogastrographic study carried out by Ronnblom *et al*[36] on 10 patients showed that gastric electric cycling activity manifested as bradygastria, tachygastria and a less stable frequency of the gastric signal are reduced compared with control subjects. The presence of an abnormal EGG in MD patients supports the hypothesis that the disease is a systemic disorder, affecting not only the muscles but also other tissues. Ronnblom *et al*[36] then studied the gut hormone profile in the same group of patients and found that the post-prandial secretion of motilin and glucagone-like peptide-1 (GLP-1) is decreased in MD patients. Since motilin triggers the antral phase III activity of the migrating motor complex and accelerates gastric emptying, reduced motilin secretion could cause delayed gastric emptying. In contrast, low post-prandial GLP-1 secretion is probably a result of delayed gastric emptying, i.e. a consequence rather than a cause of the disease. The patients with the most markedly delayed gastric emptying showed abnormal gastric electric activity and an abnormal post-prandial gut hormone profile. Both factors may contribute to altering gastric functioning**.** These findings indicate a partial dysregulation of the GI endocrine system in MD patients rather than a disorder of the entire digestive endocrine system. This conclusion is supported by an immunohistochemistry study carried out earlier by the same authors on the different types of duodenal endocrine cells in MD patients affected with diarrhoea[37].

They reported that the duodenal endocrine cell area containing the cells that produce serotonin, gastrin/CCK, secretin, GIP and somatostatin, is significantly increased. There are various possible explanations for this finding. The increased cell area may be a primary phenomenon or a consequence (via a feed-back mechanism) of the synthesis of defective and biologically inactive peptides or the result of a reduced receptor sensitivity of the effector cells and a malfunctioning effector organ.

If the increase in the number of endocrine cells is a primary disorder, it could play a role in the gastrointestinal manifestations of MD. However, even if it is merely a secondary phenomenon, a possible link can still be hypothesized, because the intestinal peptides may act on many different organs. Further studies are needed to shed light on this intriguing issue. At present, no correlation has been found between the increase in the duodenal cell area and the severity of the disease[37].

Different drugs, particularly prokinetics, have been proposed to treat the dyspeptic symptoms and motor disturbances in MD patients. In their study of 16 patients with delayed gastric emptying of solids and liquids, Horowitz *et al*[14] showed that oral administration of 10 mg of metoclopramide 3 times per day can improve the gastric emptying of both a solid and a liquid meal, but has no effect on esophageal emptying. In another study the same authors tested the efficacy of cisapride (10 mg, 4 times per day) and reported that it improves both gastric emptying and digestive symptoms such as nausea, vomiting, early satiety, abdominal distension and pain[38]. Metoclopramide also seems to be effective in improving gastric emptying in MD patients with gastroparesis[24]. Its effect may be due to the local release of acetylcholine and/or the hypersensitivity of the smooth muscle cells to this transmitter, and/or a reduction in the inhibitory effect of dopamine on gastric motility[14, 24, 39].

Ronnblom *et al*[25] administered erythromycin (100 mg, 2 times per day, before lunch and dinner) to dyspeptic MD patients over a period of 4 wk. The rationale for the use of erythromycin, which has been proved effective in accelerating gastric emptying in diabetic gastroparesis, lies in its agonistic action on the motilin receptors[10]. No effect on gastric emptying and no immediate improvement in symptoms were observed, although by the end of the 4-wk period a slight improvement in nausea and early satiety and a marked improvement in diarrhoea were noted by some patients. The authors reported that overall the treatment is effective possibly due to the positive effects of the drug on other GI symptoms, such as bacterial overgrowth in the ileum, which causes diarrhea by inhibiting the absorption of bile acid[25].

SMALL AND LARGE INTESTINE

Diarrhea, sometimes accompanied with malabsorption, steatorrhea, and crampy abdominal pain, are frequent complaints in MD patients[9]. Paralytic ileus has also been reported[40, 41]. The pain may be located in any part of the abdomen, with no specific characteristics or eliciting factors. Episodic diarrhoea is the single most common complaint (present in up to 33% of cases). It is often disabling and may have a marked impact on the patient's social life, especially when combined with anal incontinence[9].

Diarrhea and possibly malabsorption, have been attributed to reduced peristaltic activity leading to bacterial overgrowth[10]. Anaerobic bacteria seem to be the principal contaminating agents. Their ability to deconjugate bile acids, thereby causing defective uptake in the terminal ileum, could be the main cause of diarrhea[25, 42]. Norfloxacine, either alone or in combination with cholestyramine, can alleviate this symptom in some patients. Other possible mechanisms of diarrhea, such as the reduced secretion of pancreatic amylase, have been hypothesized but not yet been demonstrated, and further evidence is needed to shed light on this issue[42].

Radiological studies have demonstrated reduced or absent peristaltic and/or segmentary activity with delayed intestinal transit[43-45]. Megacolon, sigmoid volvulus and segmental narrowing due to myotonic contractions have also been reported[43, 46-52].

Megacolon with the accompanying risk of ileus, volvulus and rupture, is a significant complication and must be kept in mind during the management of MD patients who are usually at higher risk of complications during anesthesia and surgical operations than normal subjects. The pathophysiological mechanisms underlying megacolon are not entirely clear, but probably smooth muscle damage is not the only factor involved. An electron microscopy study of an MD patient with megacolon showed that the myenteric plexus is degenerated with a paucity of neurons and fragmented axons. Glial cells are increased in number with vacuoles containing electron-dense material. Swelling of the mitochondria, distension of the endoplasmic reticulum and the accumulation of free ribosomes in the cytoplasm are also observed. The neurons are described as being "fewer, and the argyrophilic ones smaller, with less prominent processes and poor staining quality." The number of nerve fibers exhibiting reactivity to substance P and enkephalin in the *muscolaris externa* is decreased, while normal smooth muscle cells are reported[52].

In concurrence with pregnancy or episodes of gastroenteritis, MD patients have been reported to suffer from recurrent intestinal pseudo-obstruction presenting with nausea, vomiting, abdominal cramps, distension and sometimes, constipation[53, 54]. However, such complications may occur during any stage of the disease and may even precede significant skeletal muscle weakness by 15 years. Recently, an MD patient with pseudo-obstruction and partial malrotation of the intestine has been reported[55], although it should be noted that this association has never been observed before and could represent an isolated case[56].

Manometric studies of the small intestine (jejunal manometry) in MD patients have shown low amplitude contractions of the migrating motor complex during phases 2 and 3, with high frequency activity during phase 2 and retrograde propagation and interruption of the contractions during phase 3. Low amplitude post-prandial contractions and myotonic phenomena have also been reported. A disturbance of the mechanoreceptors that link the circular muscle layer with the enteric nerves and

provide the stimulus for the propagation of migrating motor complex phase 3 activity has been hypothesized to explain these phenomena[57].

Very few manometric studies of the colon have been conducted and the results are conflicting. Some report an absence of the normal rhythmic pressure variation in the descending colon[43], while others have failed to detect any motility abnormalities in the sigmoid colon[58]. In any case, intestinal motility disturbances are detectable in both symptomatic and asymptomatic subjects, raising doubts as to the actual role they play in the pathogenesis of MD. The absence of a correlation between disease severity and the presence of symptoms has also been confirmed in the intestinal tract[57].

Only a small number of histologic studies of the muscular layer of the small intestine are now available. They describe the cells as being swollen, fragmented, partially destroyed, or decreased in size, and replaced by fats, quite similar to the abnormalities reported for the skeletal muscles[35, 59]. Biopsies of the mucosa usually show normal histologic patterns[37, 43, 60, 61], although a case of a patient with intestinal villous atrophy and collagenous sprue has been reported by Woods *et al*[62]. The authors' suggestion that this type of malabsorption may be frequent in MD patients has not yet been confirmed.

RECTOANAL REGION

Although constipation often associated with dyschezia is common, since some patients represent a serious problem, the most burdensome and disabling problem affecting MD patients may be fecal incontinence[9, 63, 64]. Up to 66% of MD patients suffer occasional fecal incontinence, while more than 10% report fecal incontinence one or more times a week. The frequency of urinary incontinence seems to have no difference between MD patients and control subjects[9, 65].

Although the procedure is simple and low in invasiveness, manometric studies of the rectoanal region are not often performed in MD patients. Some studies report a decrease in both the resting pressure (based on the tonic activity of the internal anal sphincter) and the squeezing pressure (exerted by the phasic activity of the external anal sphincter)[65-67]. Others have failed to detect any significant modification in the resting pressure and only a slight, statistically insignificant decrease in the squeezing pressure[11].

Lecointe *et al*[11] found that the amplitude and duration of the recto anal inhibitory reflex (RAIR) in response to rectal distension are markedly decreased in MD patients. This could be explained, however, by a prolonged myotonic contraction of the striated external anal sphincter muscles[67], which could obscure the manometric signal indicating the normal internal anal sphincter relaxation response to rectal distension. Due to this myotonic phenomenon, the duration of the rectoanal contractile reflex (RACR) is longer in patients than in controls[11]. According to Hamel Roy *et al*[68], the amplitude of this myotonic response is decreased by pudendal nerve blockade, suggesting the possibility of at least a partial neural reflex response. Pudendal nerve terminal motor latencies are normal in these patients, confirming the absence of a neurogenic lesion[67, 69]. Finally, it was reported that the maximum tolerable volume at rectal distension is significantly lower in MD patients than in controls[11].

Anorectal motility disturbances are as frequent as pharyngo-esophageal disturbances, the degree of involvement of the upper and distal portions of the gastrointestinal tract may be closely correlated both quantitatively and qualitatively[11]. It has been shown that anorectal motility and esophageal motility are altered in a similar number of patients[11].

Although the prevalence of altered motility is high, symptoms such as anal incontinence, diarrhoea and/or abdominal pain have been found in a small number of patients, suggesting that there is a close relationship between smooth and striated muscle motor function in MD but only in the gastrointestinal tract, where smooth and striated muscle abnormalities may be interdependent[11]. Unfortunately, there are no histologic data to back up this intriguing hypothesis.

Eckardt *et al*[66] studied the external anal sphincter using electromyography and have found myopathic potentials with myotonia. Herbaut *et al*.[65] reported a decreased duration and amplitude of the motor units in the external anal sphincter and the puborectalis muscle of MD patients with fecal incontinence, suggesting that there may be a myopathic component to the pathology. In 25% of these patients, polyphasic high-amplitude motor units were also present, indicating the probable presence of a neuropathic lesion. Taken together, the data suggest that both the striated external anal sphincter and the smooth internal anal sphincter may be altered in MD, even if previous histologic examinations have reported pathological findings only in the striated muscle[66]. More recently, Abercrombie *et al*[67] used electron microscopy to study the anal sphincter in two siblings with MD and found that the external anal sphincter is atrophic in both patients, with marked fibrosis and a high variation in the diameter of the fibres as well as the striated muscle almost entirely substituted by smooth muscle cells deriving from the internal sphincter. These cells are all different in size and electron density separated by large amounts of fibrous connective tissue. Some show features suggestive of dedifferentiation into myofibroblasts. The puborectalis biopsies revealed an analogous decrease in the amount of normal striated fibres with pronounced fibrosis. Some of the fibres were hypertrophic with increased central nucleation and many showed sarcoplasmic masses. Moreover, type 1 fibres were markedly prevalent (99%). In the ano-rectal region, no close correlation between motor abnormalities and overt symptoms has been found, although defecatory behaviour is often severely altered in these patients.

Constipation in MD patients is usually treated with prokinetics, laxatives and enemas[15, 70], while medical treatment with procainamide (300 mg twice a day) has been proposed for fecal incontinence[71]. Surgery to treat fecal incontinence has been attempted, but post-anal repair only temporarily improves the defecatory function[67]. Treating and curing defecatory problems in MD patients remain a difficult challenge. Rehabilitation involving a combination

of volumetric rehabilitation, electroanalytical stimulation, kinesitherapy and biofeedback, can be effective in those patients not suffering from severe damage to the pelvic floor muscle[72,73].

CONCLUSIONS

Since MD is a relatively rare disease, most of the papers published are hampered by the bias arising from the low number of patients studied. Many report just one or two cases, and very few involve series of more than 10 patients. Gastrointestinal involvement is frequently observed in MD patients and digestive complaints may be the first sign of the disease. According to Ronnblom *et al*[9], 25% of patients consider their gastrointestinal problems as the most disabling consequence of MD, 28% have digestive symptoms that may appear up to ten years before the typical musculoskeletal features. During this period the impairment of the digestive functions may be so gradual that patients unconsciously adapt to them by compensatory mechanisms, thus masking the symptoms[18, 74]. They may even develop a higher pain threshold (visceral hypoalgesia) similar to that seen in diabetic gastroparesis[75]. For these reasons, patients often do not undergo a thorough examination of the digestive tract, while at the same time they may be subjected to a broad battery of other examinations because of multi-system involvement. Thus, a careful assessment of the digestive tract may be performed only when the symptoms have become severe, although reliable, non-invasive or minimally invasive techniques such as ultrasonography, scintigraphy, breath test and cutaneous electrogastrography are available[13].

Little correlation has been found between the degree of skeletal muscle damage and gastrointestinal disturbances[12, 14], while there does appear to be a relationship between the severity of GI involvement and the duration of the disease[13]. Nevertheless, the pathophysiologic mechanisms of digestive motor disorders certainly suggest that there is damage to the striated muscles in the upper and lower portions of the gastrointestinal tract. Histologic evidence of smooth muscle alterations is scarce and conflicting[12, 14], although some authors have hypothesized that smooth muscle damage may occur earlier and is more severe than striated muscle changes[10].

Moreover, some authors suggest that a common mechanism may be the generation of the motor abnormalities in both the smooth and striated muscles. For example, Lecointe *et al*[11] studied the esophagus and the rectoanal region of MD patients and found that alteration in the smooth muscle is closely related with that in striated muscle, at least in the digestive tract.

A neural factor could also be involved in the digestive symptoms of MD patients. Neural dysfunctions, such as an alteration in the non-adrenergic, non-cholinergic neuronal control of the GI tract, have been suggested to explain the symptoms and instrumental findings that may be present even in the absence of definite histologic damage. Nitric oxide (NO) can mediate non-adrenergic, non-cholinergic nerve-induced relaxation and hyperpolarization of the smooth muscle in the digestive tract[76]. NO is produced from L-arginine via NO synthase, a key enzyme that is expressed in the myenteric plexus, motor neurons and myenteric fetal interneurons[77-80]. Thus, the NO molecule seems to be common in both the striated and smooth muscles, the inhibition of NO synthase could alter the motor parameters in a manner consistent with the observations of Lecointe *et al*[11]. This "neuronal hypothesis" can explain the absence of histologic abnormalities in the smooth muscle of MD patients. In addition, it could explain the degeneration and decreased number of argyrophilic neurons in the colonic myenteric plexus of an MD patient affected with megacolon[52].

Gastrointestinal involvement in MD remains a complex and interesting condition. Given the limited data available, there are very few certainties and many important questions to be solved[81]. The relationship between myotonic dystrophy, gastrointestinal motility and clinical symptoms needs to be investigated in greater depth. Studies should begin early in their disease course, using non-invasive diagnostic techniques and concentrating on the genetic and histologic aspects of each case. Such efforts can improve our understanding of the pathophysiology of the digestive system involvement in MD and speed up the development of new therapeutic strategies to manage this difficult condition.

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REVIEW

Current concept on the pathogenesis of inflammatory bowel disease-crosstalk between genetic and microbial factors: Pathogenic bacteria and altered bacterial sensing or changes in mucosal integrity take "toll" ?

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Abstract

The pathogenesis of inflammatory bowel disease (IBD) is only partially understood. Various environmental and host (e.g. genetic-, epithelial-, immune and non-immune) factors are involved. It is a multifactorial polygenic disease with probable genetic heterogeneity. Some genes are associated with IBD itself, while others increase the risk of ulcerative colitis (UC) or Crohn's disease (CD) or are associated with disease location and/or behaviour. This review addresses recent advances in the genetics of IBD. The article discusses the current information on the crosstalk between microbial and genetic factors (e.g. NOD2/CARD15, SLC22A46A5 and DLG5). The genetic data acquired in recent years help in understanding the pathogenesis of IBD and can identify a number of potential targets for therapeutic intervention. In the future, genetics may help more accurately diagnose and predict disease course in IBD.

Key words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Pathogenesis; Microbial factors; Genetics; NOD2/CARD15; SLC22A4/A5; DLG5

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INTRODUCTION

There has been a sharp increase in the incidence of inflammatory bowel disease (IBD) in the late 1900s in Western countries as well as in some Eastern parts of Europe and North America[1-3]. Both Crohn's disease (CD) and ulcerative colitis (UC) stem possibly from a common mechanism with an exact etiology that remains obscure[4,5]. Crohn's disease manifests itself as a chronic granulomatous inflammation of the gastrointestinal tract capable of affecting its entire length with the presence of "skip" lesions[6,7]. It preferentially affects the terminal ileum, as was originally described by Crohn *et al*[8]. Ulcerative colitis, on the contrary, presents as a continuous inflammatory lesion affecting the rectum and colon, lacking granulomatous characteristics.

IBD are multifactorial, polygenic diseases with probable genetic heterogeneity. According to this hypothesis, different genetic backgrounds may explain the various clinical patterns of the disease[4,5]. In addition to genetic predisposition, various environmental and host factors (e.g. genetic-, epithelial-, immune and non-immune) play a major role in the pathogenesis of IBD. Extensive heterogeneity is observed in terms of presentation, extraintestinal manifestations and location in CD, while behavior and response to treatment are heterogeneous in both CD and UC[9]. Furthermore, it is now undisputed that enteric bacterial flora play a key role in the pathogenesis of IBD, both in UC and in CD. The exact mechanism by which the intestinal mucosa loses tolerance to its bacterial neighbors remains elusive. The role of host genetic regulation of the innate immune response in the pathogenesis of CD has been brought to sharp focus by the identification of the NOD2 (CARD15) mutations.

The genetic aspect of research is quite focused on numerous chromosomal loci. The environmental contributors are diverse and an "infectious origin" of inflammatory bowel disease has not been confirmed. This review describes the various environmental, immunologic and genetic components leading to the manifestation of inflammatory bowel disease.

MICROBIAL FACTORS: ARE PATHOGENIC BACTERIA AND/OR ALTERED PERCEPTION OF NORMAL FLORA THE KEYS IN THE BREAKDOWN OF TOLERANCE?

The normal intestine encounters a high concentration of foreign antigens, bacteria and food. In the stomach and proximal small intestine, secretion of acid and bile, phasic 'housekeeping' motility patterns hinder colonization. However, the number of bacteria dramatically increases in the distal small intestine to an estimated 10^{10} - 10^{12} bacterial cells/g content in the colon, which contribute to 60% of the faecal mass[10]. More than 400-500 species of bacteria are represented, belonging to 30 genera. Although this antigenic load is separated from the largest complement of lymphocytes in the body (gut associated lymphoid tissue, GALT) by only a single layer of polarised intestinal epithelium, most people do not have an immune response to foreign antigens and interaction between the mucosal immune system and the fecal bacterial mass regulates physiologic bowel functions. The mucosal immune system has evolved to balance the need to respond to pathogens while maintaining active tolerance to commensal bacteria and food antigens. In IBD, this tolerance breaks down and inflammation supervenes driven by the intestinal microbial flora.

A large part of research has traditionally been devoted to finding a causative biological source of any disease. This has also been the case in IBD, but to date there is no compelling evidence of an etiological role for any single pathogenic microorganism.

PATHOGENIC MICROBES

The history of IBD is dotted by cyclic reports on the isolation of specific infectious agents responsible for CD or UC. Several microorganisms, such as *Mycobacterium paratuberculosis, Listeria monocytogenes, Chlamydia trachomatis, Escherichia coli, Cytomegalovirus, Saccharomyces cerevisiae*, have been proposed as having a potential etiologic role.

The suggested etiologic role of *Mycobacterium paratuberculosis* in CD is also controversial. This bacterium is the causative agent of Johne's disease, a chronic granulomatous ileitis in ruminants, which closely resembles CD. *M. paratuberculosis* was initially isolated from CD tissues some 20 years ago[11] and follow-up studies have tried to culture *M. paratuberculosis* for testing specific DNA sequences in intestinal tissues, or measuring serum antibodies against *M. paratuberculosis* with conflicting or inconclusive results[12-14]. Recently, *M. paratuberculosis* has been identified by *in situ* hybridization to the *M. paratuberculosis*-specific IS*900* gene in tissue specimens of Crohn's disease[15] and in 40% of Crohn's disease granulomas isolated from surgical specimens by laser capture microdissection[16]. Others have localized *M. paratuberculosis* by PCR to macrophages and myofibroblasts within the lamina propria[17]. However, the possibility of an association between *M. paratuberculosis* and CD remains inconclusive.

Additionally, *E. coli* bacteria possessing special adhesive properties have been associated with the development of ulcerative colitis.

A fascinating hypothesis was published in the *Lancet* in 2003[18]: an association between refrigeration and CD. Some so called psychrotrophic bacteria are capable of growing slowly at low temperature (known as "cold chain hypothesis"). Common pathogens are *Yersinia enterocolitica*, *Listeria monocytogenes*, and *Clostridium botulinum*[19].

Several studies have demonstrated members of the *Yersinia* species in intestinal mucosal samples of Crohn's disease. The specific pathogens detected are either *Y. enterocolitica* or *Y. pseudotuberculosis*, and sometimes even both[20,21]. There are numerous aspects of yersiniosis which resembles the inflammatory reaction seen in Crohn's disease, making differential diagnoses of these two conditions, including ileitis or ileocolitis, mesenteric adenolymphitis, reactive arthritis and erythema nodosa. Additionally, granulomas may be observed in histological samples[18].

Recent data also demonstrate a role of mucosa-associated and intramucosal bacteria in the pathogenesis of IBD and colorectal cancer. In the study by Martin *et al*[22], mucosa-associated or intramucosal *E.coli* are present in 43% and 29% of CD, and 17% and 9% of controls, respectively, supporting a role of mucosa-adherent bacteria in the pathogenesis of Crohn's disease.

Finally, a viral etiology has also been proposed as the cause of IBD, particularly CD. An early measle infection during the perinatal period notably increases the risk of Crohn's disease[23]. The finding of paramixovirus-like particles in CD endothelial granulomas suggests an association between perinatal measles and predisposition to CD based on some epidemiological and serologic data[24]. However, these preliminary findings are not confirmed by later studies[25]. Importantly, the progressive decline of measle virus infection in the last decades with the concomitant rise of CD during the same period of time speaks against an etiologic role of measles in CD. The hypothesis that measle vaccination rather than measle infection, might be a risk factor for CD, has also been raised, but again results of additional studies fail to confirm this association[26]. In contrast, a role of cytomegalovirus infection is proposed in UC[27].

NON-PATHOGENIC INTESTINAL FLORA

In the last decade, the focus of interest in microbial etiology of IBD has shifted from infectious to commensal agents. Based on fairly solid data, there is a substantial body of evidence that the normal enteric flora plays a key role in the development of IBD[28]. This is even more evident after the discovery of the genetic factors (e.g. NOD2/CARD15, TLR4 and CD14) responsible for alterations in bacterial perception[29]. The beneficial effect of antibiotics in the treatment of CD, and to a lesser extent UC, has been appreciated for years. Diversion of the fecal stream from inflamed bowel loops has also been known to induce symptomatic improvement in CD patients, while re-emergence of inflammation often occurs upon restoration of intestinal continuity[30].

Changes are observed in the normal flora of IBD patients and dysbacteriosis is commonly reported. Based on the clinical picture of IBD, the intestinal flora

might play a role in the initiation and perpetuation of inflammatory reaction. *Fabia et al*[31] demonstrated that the concentration of anaerobic bacteria and *Lactobacillus* are radically decreased in active IBD patients. On the contrary, patients with inactive IBD show no such decrease. *Swidsinski et al*[32] compared over 300 IBD patients with 40 control individuals and found that abnormalities can be observed in the flora using various laboratory techniques. In addition, a much greater number and concentration of bacteria make up the biofilms covering the epithelium involved in IBD. Furthermore, a direct association has also been reported between bacterial concentration and disease severity.

More recently, probiotics (primarily lactic acid bacteria) defined as live microbial feeds that beneficially affects the host by modulating gut microbial balance, have been demonstrated to improve both human IBD and experimental colitis, primarily by preventing relapses, thus adding an important dimension to the role of gut flora in IBD[33,34].

Probably, the most convincing evidence comes from experimental data. In the majority of IBD animal models, intestinal inflammation fails to develop when they are kept in a germfree environment. This critical observation has led to the widely accepted paradigm "no bacteria, no colitis". However, once normal flora is introduced into their environment, the mutant-strain mice quickly develop colitis[35, 36].

It is worth mentioning that bacterial superinfection (most commonly *Clostridium difficile*, but also *Entamoeba histolytica, Campylobacter spp*, etc.) is also able to elicit relapse of IBD. In the study of Mylonaki *et al*[37], 10.5% of all relapses are associated with enteric infections. In another study[38], 20% of all relapses are correlated to *C. difficile* positivity.

The presence of bacterial antigens as the initiating factor in IBD has also been explored. The data support a role of flagellin proteins in activating the innate immune system. Elevated titers of serum anti-flagellin IgG against these proteins could be detected in CD patients[39]. Furthermore, elevated serum IgG2a titers could also be demonstrated in experimental IBD models, similar to results observed in humans. Recently, anti-CBir1 (anti-flagellin) expression has been independently associated with small-bowel, internal-penetrating and fibrostenosing disease features in CD[40].

Finally, the role of bacteria in the pathogenesis of IBD is further supported by the increased intestinal permeability in CD patients. Although it is not an environmental factor *per se*, it has been postulated as an early predisposing factor for the development of this condition. Increased intestinal permeability is also observed in asymptomatic first-degree relatives of CD patients, possibly encompassing another group of individuals who are at an increased risk of developing this form of IBD[41,42]. Documented observations of increased permeability preceding the onset of symptomatic disease are of further interest[43]. In relating this phenomenon to the UC form, electron microscopy has revealed deficiency of those elements comprising the tight junctions necessary for the wholeness of the intestinal epithelium[44]. Recent data reporting an association between genes important in mucosal transport and integrity (OCTN and DLG5) and IBD further support this assumption.

Table 1 Locations of nine major loci showing linkage to inflammatory bowel disease

IBD locus	Chromosome	Identified genes	Disease
IBD1	16q13	NOD2/CARD15	CD
IBD2	12q14	Not known (VDR, STAT6, MMP18, b2-integrin)	UC
IBD3	6p	Not known (HLA, TNF)	IBD
IBD4	14q11-12	Not known (TCR, LTB4 receptor)	CD
IBD5	5q31-33	SLC22A4/A5	CD
IBD6	19p13	Not known (ICAM1, C3, TBXA2)	IBD
IBD7	1p36	Not known (TNF-R family)	IBD
IBD8	16p12	Not known	CD
IBD9	3p26	Not known (CCR5, CCR9,hMLH1)	IBD
	10q23	DLG5	IBD

The phenomenon of decreased bacterial diversity of the intestinal microflora obtained from stool samples in IBD patients has also been suggested, based on culture-dependent microbiological techniques[45]. Recently, a German group has reported the relative lack of the *Bacteroides* group (usually accounting for 50%-90% of the anaerobic faecal microflora) by utilizing 16sDNA-based single strand conformation polymorphism (SSCP) fingerprint, cloning experiments and real time PCR. Moreover, the bacterial profiles are stable, at least for the observed period of six weeks. This is in concordance with previous findings, suggesting that the mucosa-associated microflora contains only a small number of bacteria of the *Bacteroides/Prevotella* group[52]. However, in a recent study comparing the faecal microflora of healthy controls and CD patients, no differences are noted between patients and healthy controls[46]. This lack of significant difference may be partially explained by the differences in mucosal and faecal microflora.

Why an abnormal response to normal endogenous gut bacteria exists in IBD is not clear, but recent data, especially on the genetic background of CD, suggest an association between gut inflammation and abnormal bacterial sensing.

GENETICS AND GENE FUNCTION

Possible genetic loci influencing the presentation of inflammatory bowel disease have already been identified on more than half of all chromosomes, including the X chromosome[47-49]. First, in 1996 Hugot *et al*[50] reported that the pericentromeric region of chromosome 16 (D16S408) is associated with CD renamed as IBD1[50]. This is confirmed by several studies. To this date, results of numerous studies have revealed a total of nine loci associated with specific linkage requirements, subsequently labeled as IBD1-9[51-55] (Table 1). Furthermore, it is clear that some loci correlate with either UC or CD while others are involved in the pathogenesis of both IBD forms[49].

FAMILIAL STUDIES

Evidence of genetic constituents in the induction of inflammatory bowel disease clearly lies within the results of familial studies. Several studies examining the relationship of IBD within families have reported positive findings in 10%-25% of families[56,57]. This finding goes back at least two decades when Farmer and Michener reported 40% concordance for IBD in first-degree relatives. They observed that sibling-sibling involvement occurs in addition to parent-offspring transmission.

Solid evidence of genetic predisposition showed that as many as 50% of monozygotic twins are affected by Crohn's disease whereas ulcerative colitis is seen in approximately 14%[58]. Monozygotic twins are not equally affected but still display greater concordance for IBD than dizygotic twins. The overall risk for IBD is increased by 5-20 times[59], being highest in twins and in siblings. The genetic component is more prominent in CD than in UC.

Finally, genetics certainly play a role in the observed phenotype of IBD, including disease behavior and location[60].

Genetic anticipation is suggested in early studies with a more severe and earlier presentation of the disease in subsequent generation[61], but this has not been replicated in more recent studies[62,63].

GENES INVOLVED IN THE RECOGNITION OF BACTERIA – NOD2/CARD15, NOD1/CARD4, TLR4 AND CD14

NOD2/CARD15

Three independent groups have identified NOD2 CARD15 on chromosome 16 in IBD1 as a candidate gene for CD[29,64,65].

NOD2/CARD15 is an intracellular element responsible for the indirect recognition of bacterial peptidoglycan through the binding of muramyl dipeptide[66,67]. It is a member of the Ced4-APAF1 protein superfamily and is expressed in various cells, including monocytes, dendritic cells, Paneth cells and intestinal epithelial cells[6]. Structurally, NOD2/CARD15 is composed of three segments: the first being composed of two CARD units, the central portion consisting of nucleotide-binding domain (NBD) and finally, a leucine-rich repeat (LRR) region as is found in TLRs[68]. The most intriguing question that remains to be answered concerns the mechanism whereby mutations in the NOD2/CARD15 gene predispose towards the chronic intestinal inflammation characteristics of CD.

At the molecular level, the binding of NOD2/CARD15 to a bacterial motif (muraryl dipeptide-MDP, a component of both Gram negative and positive bacterial cell walls) causes its binding to a second NOD2/CARD15 molecule, thus forming a dimer. Further interaction with other cytosolic proteins leads to the ultimate activation of nuclear factor κB (NF-κB), eliciting pro-inflammatory reactions[68] (Figure 1). Surprisingly, it is still unclear whether NF-κB expression is elevated or depressed in CD due to conflicting observations and studies. *In vitro* experiments demonstrated that the declined activity of this protein indicates a loss-of-function effect[69,70]. Hisamatsu *et al*[71] showed that

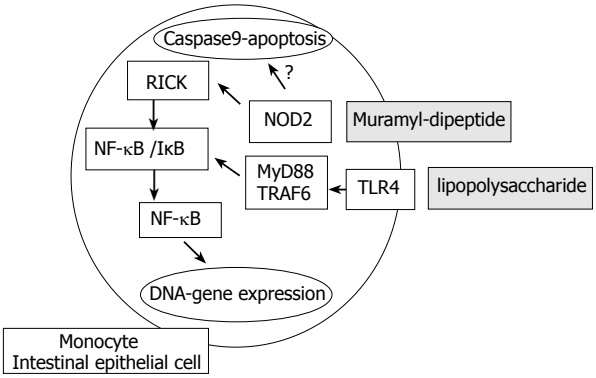

Figure 1 NOD2/TLR4 signaling pathway. NOD2 activation by CARD-CARD dimmer formation results in binding to RICK kinase (RIP-like interacting CLARP kinase). RICK then activates the nuclear factor kB inhibitor (IkB) kinase complex (IKK) via phosphorylation of IKKc. The IKK complex next phosphorylates IkB resulting in nuclear factor kB (NFkB) translocation to the nuclei and transcriptional activation of NFkB responsive genes, such as pro-inflammatory cytokines or defensins. TLR4 induces activation through other molecules (Myd88, IRAK and TRAF6).

CARD15/NOD2 may function as an antibacterial factor in CaCo2 intestinal epithelial cells. Cells stably transfected with a wild-type CARD15/NOD2 gene construct are able to prevent invasion of *Salmonella typhimurium*. This protective effect is lost in cells transfected with gene constructs of mutant CARD15/NOD2. It has also been demonstrated that NOD2/CARD15 expression in intestinal epithelial cells might be upregulated by the proinflammatory cytokine tumor necrosis factor α (TNF-α)[72]. The NF-κB activation is regulated by the LRR region and deletion within this region may theoretically result in its uncontrolled activation. In concordance with this, increased NF-κB activation is observed in the presence of 3020insC mutation by stimulation of MDP[73], which may explain the findings of higher NF-κB tissue levels in samples from IBD patients[74]. Interestingly, mice lacking NOD2 or possessing mutated NOD2 variants do not spontaneously develop Crohn's disease. These findings suggest that NOD2 mutations create an intestinal environment susceptible to IBD[73,75] rather than playing a direct causative role in disease development expression of α- and β-defensins.

Defensins are integral parts of the local intestinal immune system, and are secreted by intestinal epithelium as endogenous antimicrobial proteins. The consequential damage could finally lead to an intensified bacterial invasion and a chronic inflammation of the intestinal mucosa[76]. The possibility exists that NOD2/CARD15 is involved in the regulation of Paneth cell degranulation[77], as NOD2/CARD15 expression is noted in close proximity to the secretory granules of these cells. Kobayashi *et al*[75] and Wehkamp *et al*[78] have shown that carriage of NOD2/CARD15 variants may be associated with reduced α-defensin release from Paneth cells in response to bacterial cell wall components, and the defective defensin release by the Paneth cells could provide the missing link, whereby reduced NOD2/CARD15 activity impairs host defenses against bacteria and underlies persistent intestinal inflammation. Finally, NOD2/CARD15 is involved in the regulation of TLR2 stimuli by peptidoglycans. Defective

NOD2 function results in a pro-inflammatory cytokine bias after stimulation of mononuclear cells with TLR2 stimuli, which may contribute to the overwhelming inflammation in Crohn's disease[79].

Three major mutations have been identified within NOD2/CARD15: one frame shift (3020insC, SNP13) and two missense mutations (R702W-SNP8 and G908R-SNP12).

The presence of NOD2/CARD15 mutations increases the risk for CD by 1.4-4.3-fold in heterozygous patients and 17.6-44.0-fold in homozygous and compound heterozygous patients. Reports on homozygous individuals who are disease-free are available. One such family has been described by van der Linde *et al*[80]. It is estimated that any of these three common mutations involving NOD2/CARD15, is present in heterozygous form in 30-50% of CD patients and 7-20% of controls from North-America and Europe[81-85]. However, various geographical differences are noted. Furthermore, other races display a much lower prevalence of this mutation, sometimes lacking it altogether (e.g. African-American[86], Chinese[87] and Japanese[88]). The prevalence is also lower in other North European countries[89,90].

The association between NOD2/CARD15 and disease phenotype and behavior has also been investigated. The three common mutations are associated with ileal disease and fibrostenosing behavior, but they are relatively less frequent in colonic and fistulous disease[64,66,91,92]. The presence of mutations is not associated with extraintestinal manifestations or the response to infliximab therapy[93]. The mutations are also not associated with UC. In CD, the attributable risk for ileal disease is 40% determined by NOD2/CARD15 and 20% by HLA genes. The numbers are similar for ileo-colonic disease (NOD2/CARD15: 30%, HLA: 40%), while colonic disease is thought to be associated with HLA and other yet unknown genes[91].

It is worth mentioning that NOD2/CARD15 increases the risk for colorectal cancer (CRC). Kurzawski *et al*[94] have found that the presence of SNP13 mutation increases the risk of developing CRC by 2.23-fold in patients with an age >50 years at diagnosis of CRC. This has not been confirmed in a more recent study by Alhopuro *et al*[95].

NOD1/CARD4

A similar protein to NOD2/CARD15 by its structure and function, is located on chromosome 7p. Its particular location is in the midst of a region with strong IBD correlation, being translated into an intracellular bacterial pathogen-associated molecular pattern[96].

Zouali *et al*[97] denounced that NOD1/CARD4 plays a role in IBD following an investigation involving 63 IBD patients. The screening process was conducted on the eleven exons constituting the NOD1/CARD4 gene with the goal of identifying polymorphisms. Indeed, several variants were identified, none of which has proved any association with IBD[97]. On the contrary, McGovern *et al*[96] have located a complex insertion/deletion allele on NOD1, associating it with both an early onset of IBD and extraintestinal manifestations.

Toll-like receptors and CD14

Toll-like receptors (TLRs) expressed in myeloid cells play a major role both in detecting microbes (lipopolysaccharide) and in initiating innate immune responses. Accordingly, a disturbance in its function predisposes to infections with Gram negative bacterial pathogens, possibly influencing the advancement of IBD[98,99]. In contrast, little is known about the expression, distribution and function of TLRs in epithelial cells *per se*. TLR4 is also expressed in the Golgi apparatus of intestinal epithelial cells. Thus, LPS recognition in intestinal epithelial cells may occur in the Golgi apparatus and requires LPS internalization [100]. Recently, it has been suggested that the interaction of LPS with TLR4 / MD2 contributes to the perpetuation of the inflammatory epithelial cell injury via TNFα induced alterations of enterocyte turnover in an autoparacrine/paracrine manner[101]. TLRs may also influence the nature of immune response by skewing T cells toward a Th1 or Th2 profile. Myeloid cells are exquisitely sensitive to TLR ligands and produce significant IL-12p40. They appear to play a key role in the initiation and possibly the Th1/Th2 skewing of inflammatory responses. In this model the inflammation can be normally controlled by myeloid or lymphocyte-derived IL-10 acting through Stat3 in myeloid cells to block further production of IL-12/IL-23 and skewing the responses towards Th1 profile[102].

IBD is characterized by an altered expression pattern of TLRs on the surface of intestinal epithelium and TLR4 expression is up-regulated in patients with CD. In contrast, the expressions of TLR2 and TLR5 are unchanged, while TLR3 that recognizes viral replication is down-regulated[103]. The D299G (Asp299Gly) polymorphism of the TLR4 gene associated with LPS hyporesponsiveness[104] is associated with CD (OR:2.45-2.80) and UC (OR: 2.05)[98,105]. However, other studies have failed to replicate this association[85,106].

Another TLR which binds to and recognizes bacterial DNA, TLR9, may also play an important role in the pathogenesis of IBD. Rachmilewitz *et al*[107] reported that the anti-inflammatory effect of probiotics is transmitted through TLR9 in experimental colitis. More recently, an English group has reported a synergy between TLR9 and NOD2 that is lost in the CD patients carrying NOD2 mutation, indicating impairment in innate immunity[108]. Recently, *Torok et al*[109] reported that the -1237C promoter polymorphism of TLR9 is significantly associated with CD in German patients.

Contradictory data are available on the association between the bacterial receptor CD14 and IBD. Klein *et al*[110] found that the 159 TT genotype is associated with CD but not with UC. In a Japanese study[111], the same genotype is associated with UC (OR: 1.96) but not with CD and also a further study investigating Caucasian patients has failed to demonstrate any association[112].

MUCOSAL INTEGRITY AND TRANSPORT - SLC22A4/OCTN1, SLC22A5/OCTN2 AND DLG5

SLC22A4/OCTN1, SLC22A5/OCTN2

The association between 5q31 and CD is first noted in genome-wide screens (the genes for Th2 cytokines -e.g. IL-3, 4, 5, 9, 13 and IRF1- maps to this region). The

risk haplotype is associated only with a moderate risk for CD (OR: 1.4-1.5). The effect of NOD2/CARD15 is additive while the 5q31 haplotype is an independent risk factor[113-115]. A German study reported that the risk haplotype is also associated with UC while the IBD5 haplotype is not associated with the clinical presentation, nor is it correlated to IBD in Japanese[114]. Based on the available data, IBD5 increases mainly the overall risk for IBD, whereas NOD2/CARD15 mutations are primarily responsible for the determination of phenotype.

One of the most important findings in the genetics of IBD is the identification of OCTN1/SLC22A4 and OCTN2/SLC22A5 genes coding for integral membrane proteins. The function of these proteins is multispecific in bidirectional transmembrane transport of carnitine and organic cations. The SLC22A4 C1672T and SLC22A5 promoter G207C variants increase the risk for CD by 2-2.5-fold when present as a single copy and by 4-fold in homozygous carriers[55,116]. The elevated risk attributed to OCTN TC haplotype and NOD2/CARD15 mutations, is additive with an odds ratio of 7.3-10.5 in double carriers. A more recent Japanese study on SLC22A4/A5 and DLG5 polymorphisms and CD[117], has shown a weak association, concurring with the Ashkenazi population[118], where the frequency of the allele is lower, indicating racial differences. Variant alleles are associated with functional changes. The SLC22A4 variant encodes exchange of a leucine residue on the OCTN1 transmembrane domain for phenylalanine (L503F), a change which reduces carnitine but augments organic cation transporter activity. The SLC22A5 variant impairs heat shock protein-driven promoter transcriptional activation[116]. Carnitine is an essential mediator of fatty acid oxidation, a role subserved by promoting transport of long-chain fatty acids across the mitochondrial membrane. Inhibition of fatty acid oxidation can evoke clinical and pathologic signs of colitis[119], which may explain why impairments in carnitine transport may confer an increased risk for IBD. Another possibility is that the enhanced cation transporter activity of the OCTN1 503F variant may provoke disease by allowing aberrant uptake of toxic substrates.

A possible association between OCTN3 and CD has been reported[120,121]. In contrast, no association has been found between the proton-coupled bivalent metal antiporter located on 2q35 SLC11A1 and IBD[122].

DLG5

The association between DLG5 (Drosophilia Discs Large Homolog 5) at chromosome 10q22-23 is first reported by Stoll *et al*[123]. DLG5 is a member of the membrane-associated guanylate kinase (MAGUK) gene family which encodes cell scaffolding proteins and is also involved in the maintenance of epithelial integrity and regulation of cell growth[124], a role potentially impaired by expression of the CD-associated DLG5 variants causing increased permeability and disease. The impact on the overall risk of developing IBD is much smaller than NOD2/CARD15. The carriage of the 113A allele increases the risk for CD by 1.3-2.1-fold in German, Italian, Canadian patients and is associated with early onset of IBD in Scottish children[123,125,126]. Less common variants have also been identified (C4126A). Interaction between DLG5 and NOD2/CARD15 is also detectable. This however, has not been replicated by the same group in English and Scottish CD patients and by the German group from Munich[127,121].

HLA, CYTOKINE GENES AND OTHER GENETIC FACTORS

HLA genes

Reports on genetic trait of IBD are available, which investigated the genes associated with the immune response. The main region of interest is that of MHC (main histocompatibility complex) genes located on chromosome 6. The human leukocyte antigen (HLA) class II genes are candidates for a role in the pathogenesis of IBD because their products play a central role in the immune response. More than 100 known genes are located in the area, each is highly variable and several polymorphisms are known.

Several studies have addressed the possible association between certain HLA polymorphisms and the risk for IBD. Most of these studies have revealed contradictory results and their findings could not be replicated in different populations. Overall, genes in the HLA region may play a greater role in modifying IBD phenotype rather than in determining overall disease susceptibility[128-130].

The association of UC with HLA genes is stronger than CD[4,130]. In the white Caucasian population, the rare HLA-DRB1*0103 allele is positively associated with UC (OR: 3.42). Others have found that this allele predisposes to extensive disease and is additionally associated with the presence of extraintestinal manifestations[131]. The repeatedly observed association with HLA-DR2 has been confirmed in the cumulative odds ratio of 2.00, while the odds ratio for DRB1*1502 and DR9 is 3.74 and 1.54, respectively. In contrast, the presence of DR4 (OR: 0.54) and DRw6 is preventive for UC[132].

In CD, negative association has been found between DR2 (OR = 0.83) and DR3 (OR = 0.71), while HLA-DR7 and DQ4 seem to be associated with a moderately increased risk (OR = 1.42 and 1.88). The studies on allele DRB3*0301 showed that this allele is positively associated with Crohn's disease (OR = 2.18)[4,31]. Ahmad *et al*[91] investigated 340 polymorphisms in HLA genes and found that the 3 common NOD2/CARD15 alleles, the HLA DRB1*0701 (RR: 1.5) and Cw*802 (RR3.0) are associated with increased risk for CD, while the presence of HLA DRB1*1501 (RR: 0.6) is protective.

Investigating the genotype-phenotype relations, HLA DRB1*0103 allele in both UC and CD is associated with both extensive and severe disease as judged by the need for colectomy[131,133]. However, the low frequency of this allele even in UC patients suggests that this association is unlikely to be clinically useful in predicting disease course. The same allele is associated with late onset, colonic location (38.5% *vs* controls 3.2%) of CD[134], while the presence of HLA-DRB1*04 (OR:1.7) and HLA-DRB1*0701 (OR: 1.9) increased the risk for ileal location[135]. MICA*010 (MHC class I chain-related gene A) and HLA-DRB*0103 are associated with perianal

disease[91].

A number of genetic associations have also been described in extraintestinal manifestations of IBD. Studies comprising both UC and CD patients showed that migratory pauciarticular large-joint arthritis is associated with HLA-DRB1*0103 and B*27 and B*35 class I alleles in linkage disequilibrium. In contrast, chronic small-joint symmetric arthritis is associated with HLA-B*44[136]. Uveitis is correlated to HLA-B*27, B*58 and DRB1*0103, while erythema nodosum to B*15 and TNF-1031C[137]. However, due to the relatively small sample size of these studies, the evidence is lacking to draw a firm conclusion.

Cytokine, multidrug resistance and cell adhesion gene polymorphisms

Interaction between non-pathogenic, commensal bacteria and epithelial cells, M-cells and dendritic cells of the intestinal mucosa is characterized by a cytokine profile of mainly the Th2/Th3 (suppressor) type (IL-4, IL-5, IL-10, TGF-β), which incapacitates the development of an inadequate, progressive, proinflammatory cycle in healthy individuals[138]. Contrary to this, mucosal immune response against pathogenic species is largely mediated by Th1 cells and a specific cytokine milieu (IL-1, TNF-α, interferon-γ). Both ulcerative colitis and Crohn's disease are characterized by an increased expression of "general" proinflammatory cytokines (TNF-α, IL-1, IL-6), which suggests an abnormally intense inflammatory response against commensal bacteria. This loss of immune tolerance to non-pathogenic microbes results in pathologic immune reactivity and a self-supporting inflammatory cascade. In healthy individuals, the tight control of the inflammatory reaction between bowel mucosa and bacterial milieu involves antiinflammatory cytokines (IL-10, TGF-β)[139]. In case of cytokine imbalance, the inflammatory reaction intensifies. However, lack of antiinflammatory cytokines plays only a restricted role in the development and maintenance of IBD, which is suggested by their low therapeutic potential in IBD[140]. In contrast, the widely used chimera or humanized anti-TNF antibody represents one of the most fascinating new therapeutic options in severe and/or penetrating CD cases as well as UC[141-143]. Studies are also underway targeting IL-12[144].

Although the above mechanism is pivotal in the induction of local inflammatory changes from an immunological point of view, lack of reproducibility has challenged many of the other reported genotype-phenotype associations in IBD, such as association between interleukin polymorphisms and IBD.

The association between a low-producing allele, allele 2 of the interleukin (IL)-1RA gene and UC (OR: 1.3), extensive colitis, the need for colectomy[145,146] and development of pouchitis (OR: 3.1) in UC after ileal-pouch anastomosis[147] has been reported in Caucasian population. However, another study investigating 529 IBD patients has failed to confirm these results[148]. IL-10 polymorphisms also lack association with UC. However, the 627A allele is more frequent in patients with left sided colitis[149]. In contrast, the frequency of the high IL-10 producer allele (-1082*G) is decreased in IBD[150].

Bone loss and osteoporosis are well recognized in IBD, but the risk factors have not been clearly identified. Several studies investigated whether genetic markers may predict bone loss and found that variable number of tandem repeats adjacent to IL-6 or within IL-1RA and genes previously implicated in the paracrine stimulation of osteoclasts are associated with bone resorption[151]. A subsequent study from the same group has failed to identify an association with a putatively functional polymorphism in the IL-6 gene. Nemetz *et al*[152] reported that the 511*2 allele of IL-1b is associated with increased *in vitro* production of IL-1b by mononuclear cells in Hungarian CD patients[152].

Furthermore, microsatellite loci of TNF-α are associated with CD[153]. However, only data from a single study suggest that the TNF-308A polymorphism is associated with more intense inflammatory activity and an increased risk of arthritis in CD patients with fistulizing disease[154].

The A6 allele MICA is associated with UC and early onset of disease[155], while the MICA*011 allele increases the risk for UC by almost 2-fold. No association has been found between different polymorphisms of CTLA4, a receptor of activated T cells, which has an inhibitory function in regulating T-cell activation and IBD in different Caucasian and Asian populations[156,157].

Brant *et al*[158] have described a suggestive linkage on chromosome 7q containing the multidrug resistance (MDR)-1 gene, in association with the appearance of UC and CD. This particular gene is a membrane transport protein with several documented human polymorphisms having effects on intestinal absorption and drug pharmacokinetics. The significant mutation designated as Ala-893Ser/Thr is originally identified in knockout mice with spontaneous colitis[159,160]. An additional locus of mutation (C3435T) associated with 50% decreased protein secretion corresponding to UC (OR: 1.6-2.0) especially in extensive colitis (OR: 2.64), showed no manifestation in CD[161,162].

Adhesion molecules mediating cell-cell and cell-extracellular matrix interactions, are pivotal mediators of inflammation in IBD. Catenins and catherins are major contributors of integrity of the intestinal mucosa[163]. Dysregulation in E-cadherin-catenin complex formation leads to decomposition of the mucosal structure characterized by leaky epithelium, as observed in IBD.

Cell surface adhesion molecules conveying leukocyte-endothelial interactions, govern homing of activated inflammatory cells into the bowel mucosa. In order to slow down by rolling along the endothelium, circulating leukocytes in small vessels of the inflamed mucosa interact with adhesion molecules (e.g. CD44) expressed in the endothelium. This is followed by establishing a firm adhesion anchor. Extravasation and migration into the site of inflammation are mediated by integrins (mainly $\alpha_4\beta_2$ and $\alpha_4\beta_7$) and selectins (L-, E- and P-selectin). Vascular endothelium of the inflamed intestine, particularly in CD, is characterized by increased expression of adhesion molecules and integrin ligands, such as E-selectin, ICAM-1, MAdCAM-1, VCAM-1[164,165]. In UC, the soluble E-selectin levels show no preference between active or inactive forms. Also, the sVCAM concentration is far higher in the control group than in the inactive UC patients. Finally, unlike in CD, VEGF levels in UC cases are similar in active, inactive and control subjects[166]. Expression of

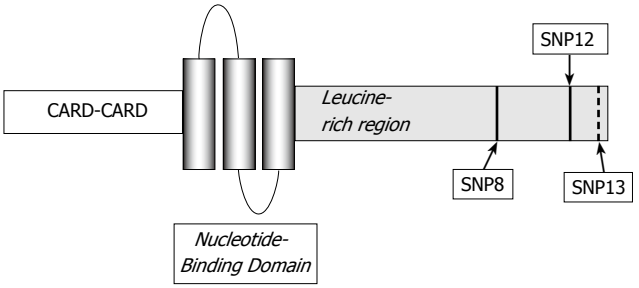

Figure 2 Structure of NOD2/CARD15 protein and locations of the three main mutations.

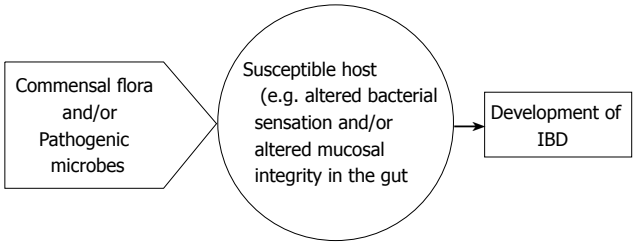

Figure 3 Role of microbial factors and genetics in the pathogenesis of IBD.

certain adhesion molecules (MAdCAM-1, CCL25) is also increased in tissues not primarily involved in the pathological process of IBD (e.g. liver), which might account for the extraintestinal manifestations of the disease[167].

Finally, in Japanese CD patients, the ICAM-1 K469 allele in IBD6 is associated with CD (OR: 2.6) and UC[168]. This association has also been confirmed in Caucasian population[169]. Targeted therapy against certain adhesion molecules (e.g. a4 and ICAM-1) is currently one of the major focuses of pharmaceutical trials in IBD[170,171].

In conclusion, various factors have been implicated in the pathogenesis of IBD, but its mechanism of action is still obscure. Recent data indicate that altered NOD2/CARD15 (or TLR4)-mediated bacterial sensing of normal commensal flora in the gut and mucosal permeability changes may be the key mechanisms (see Figure 2). At present, most efforts are devoted to a better understanding of the genetic changes underlying IBD and to further clarification of how the altered recognition of pathogenic and/or commensal microbial factors by the mucosal immune system leads to inflammation in IBD subjects but not in the general population (Figure 3).

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GASTRIC CANCER

Correlation of Epstein-Barr virus and its encoded proteins with *Helicobacter pylori* and expression of c-met and c-myc in gastric carcinoma

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Abstract

AIM: To investigate the interrelationship of Epstein-Barr virus (EBV) and EBV- encoded proteins with *Helicobacter pylori* (*H pylori*) infection and the expression of c-met and c-myc oncogene proteins in gastric carcinoma, and to explore their role in gastric carcinogenesis.

METHODS: One hundred and eighty-five gastric carcinoma tissues were detected by polymerase chain reaction (PCR)-Southern blot for EBV genome and in situ hybridization (ISH) for EBV-encoded small RNA 1 (EBER1). Gastric carcinoma with positive EBER1 signals was confirmed EBV-associated gastric carcinoma (EBVaGC). The status of *H pylori* infection in 185 gastric carcinomas was assessed by rapid urease test and PCR. The samples with positive PCR and urease test were defined as *H pylori* infection. The expression of c-met and c-myc oncogene proteins in tissues of EBVaGC and matched EBV-negative gastric carcinoma (EBVnGC) were examined by immunohistochemistry. RT-PCR and Southern hybridization were used to detect the expression of nuclear antigens (EBNAs) 1 and 2, latent membrane protein (LMP) 1, early genes BARF1 and BHRF1 in EBVaGC cases.

RESULTS: The positive rate of *H pylori* and EBV in 185 gastric carcinomas was 59.45% (110/185) and 7.03% (13/185) respectively. No difference was found in sex, age, pathological differentiation, clinical stages and lymph node metastasis between *H pylori*-positive and *H pylori*-negative gastric carcinomas. However, the positive rate of *H pylori* infection in the antrum gastric carcinomas was higher than that of cardia and body gastric carcinomas. In our series, age, pathological differentiation, clinical stages, lymph node metastasis and location of cancer were not different between EBVnGC and EBVaGC, while the positive rate of EBV in male patients was significantly higher than that of female patients. The positivity of *H pylori* in EBV-associated and EBV-negative gastric carcinomas was 46.15% (6/13) and 81.40%(104/172) respectively. There was no significant correlation between EBV and *H pylori* infection. The c-met overexpression was significantly higher in the EBVaGC group than in the EBVnGC group. However, c-met and c-myc expression did not show significant difference between the two groups. Transcripts of EBNA1 were detected in all 13 EBVaGCs, while both EBNA2 and LMP1 mRNA were not detected. Six of the 13 cases exhibited BARF1 transcripts and 2 exhibited BHRF1 transcripts.

CONCLUSION: The positivity of *H pylori* in EBVnGCs is higher than that of EBVaGCs, but no significant correlation is found between EBV infection and *H pylori* infection. *H pylori*-positive gastric carcinoma is predominant in antrum location, while EBVaGC has a tendency of predominance in cardia/body location. EBV infection is associated with c-met abnormal expression but not with c-myc protein in EBVaGC. c-met overexpression is not induced by LMP1. BARF1 and BHRF1 may play important roles in the tumorigenesis of EBVaGC through different pathways.

Key words: Epstein-Barr virus; *Helicobacter pylori*; Gastric carcinoma; c-met; c-myc

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INTRODUCTION

Helicobacter pylori (*H pylori*) infection is one of the important environmental risks for gastric carcinoma. In 1994, the

Working Group Meeting of the International Agency for Research on Cancer (IARC) concluded that *H pylori* is a definite carcinogen to gastric carcinoma(GC). Chronic gastritis caused by *H pylori* infection may progress to intestinal metaplasia and even GC[1,2]. The correlation between Epstein-Barr virus (EBV) infection and gastric carcinoma is well known. EBV infection is found in 2%-16% of ordinary gastric adenocarcinoma cases and 80%-100% of gastric lymphoepithelioma-like carcinoma cases[2-5]. However, the pathogenic role of EBV in gastric carcinogenesis remains to be elucidated. Recent studies have shown that the expressions of EBV encoded genes in gastric carcinoma are different from those in Burkitt' s lymphoma and nasopharyngeal carcinoma (NPC), suggesting that the oncogenic mechanism of EBV in gastric carcinoma may be unique[6-8]. The development of gastric carcinoma is a multistep event proceeding from normal to preneoplastic lesions to highly malignant tumors, accompanied by participation of multiple factors and multiple genes. To shed further light on gastric carcinogenesis, we determined the clinicopathologic characteristics and EBV status in 185 patients with gastric carcinoma and correlated with the status of *H pylori* infection, genetic alterations in proto-oncogenes c-myc and c-met.

MATERIALS AND METHODS

Specimens and cases

One hundred and eighty-five surgically resected specimens of gastric carcinoma were collected from the Affiliated Hospital of Qingdao University Medical College, Qingdao Municipal Hospital and Yantai Yuhuangding Hospital. Tumor tissues from each surgical specimen were separately dissected. Partial tissue was used to detect *H pylori* by urease test. DNA was extracted by the standard proteinase K-sodium dodecyl sulfate (SDS) method and purified with phenol-chloroform. Total RNA was extracted with TRIzol reagent (Gibco BRL, Gaithersburg MD, USA) according to the manufacturer's instructions. The tissue sections were used for histopathological diagnosis, *in situ* hybridization (ISH) and immunohistochemical analysis.

Detection of EBV infection

The cases positive for EBV DNA by PCR-Southern blot assay were further confirmed by ISH for EBER1 as previously described[6]. The cases with EBER1 positive signals were classified as EBVaGC group.

Detection of H pylori infection

The resected tissues were used to detect *H pylori* infection by urease test kit. Simultaneously, PCR was used to detect 16 sRNA of *H pylori*. The specific primers were designed as previously described[9]. The sequence of sense primer is 5'-CTGGAGAGACTAAGCCCTCC-3', and that of antisense primer is 5'-ATTACTGACGCTGATTGTGC-3 '. The PCR products were 109 bp. Three microliter DNA was added into a solution containing 200 μmol/L dNTPs, 0.5 μmol/L each primer, 1.5 mmol/L $MgCl_2$ and 1 U Taq DNA polymerase (Promega, USA) in a total volume of 25 μL. PCR was carried out under the following conditions: first denaturation at 94℃ for 5 min; then denaturation for 30 s at 94℃, annealling for 30 s at 55℃, extension for 45 s at 72 ℃ in 35 amplification cycles; and finally extension for 5 min at 72℃. The amplified products were electrophoresed in 2% agarose gel and visualized by ethidium bromide staining and ultraviolet illumination. DNA from the culture of *H pylori* was used as positive control, and that from human leukocyte as negative control.

Immunohistochemistry

Paraffin-embedded sections of tissues from EBVaGC cases and 45 cases of EBVnGC with similar clinicopathological data were immunostained by the standard streptavidin-biotin-peroxidase method. Anti-human mouse polyclonal antibodies against c-met and anti-human mouse monoclonal antibodies 9E10 against c-myc (Santa Cruz Biotechnology Inc) were used as primary antibodies. Phosphate buffered saline (PBS), instead of the primary antibody, was used for negative control sections. The sections of breast carcinoma tissue with highly expressed c-met and c-myc served as the positive controls. The percentage of positively stained tumor cells in each tumor section was evaluated by counting at least 1 000 cells in 10 randomly selected high-power fields. Brown staining for c-myc was located in nuclei, staining for c-met protein was located in both membrane and cytoplasm. The section was considered as expressing the protein if cellular staining ⩾5%, following the methods described previously [10,11]. c-met positivity was divided into three grades: 5%-30%, 30%-50% and >50%. Positive cells >30% (++) was regarded as overexpression.

RT-PCR and Southern hybridization analysis for EBV genes expression

According to the methods previously described[12], RT-PCR and Southern hybridization were used to detect the expression of nuclear antigens (EBNAs) 1 and 2, latent membrane protein (LMP) 1, early genes BARF1 and BHRF1 in EBVaGC cases. cDNAs from EBV-immortalized lymphoblastoid cell lines (LCL) were used as positive controls, and those from EBV-negative Ramos cells as negative controls. The integrity of RNA was checked by parallel amplification of endogenous control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

Statistical analysis

Qualitative data were analyzed by χ^2 test or the Fisher's exact test (two-tail). Quantitative data were expressed as mean±SD and compared between the 2 groups by Student' s *t*-test or *t'*-test. $P<0.05$ was considered statistically significant. Software SAS 6.12 was employed to process the data.

RESULTS

Detection of H pylori infection

H pylori infection in 185 gastric carcinomas was assessed by rapid urease test and PCR. The samples

Table 1 *H pylori* status in relation to clinicopathologic characteristics

	n	*H pylori* (+)	*H pylori* (-)	*P*
Sex				
Male	134	78	56	0.574 (χ^2=0.315)
Female	51	32	19	
Age (yr)				
30-	11	6	5	
40-	29	15	14	
50-	41	23	18	0.779 (χ^2=1.764)
60-	59	38	21	
≥70	45	28	17	
Histological subtype				
LDAC[1]	125	75	50	
MDAC[2]	49	29	20	0.986
WDAC[3]	7	4	3	
Signet ring carcinoma	4	2	2	
Tumor stage				
Ⅰ	27	16	11	
Ⅱ	98	57	41	0.975 (χ^2=0.216)
Ⅲ	41	25	16	
Ⅳ	19	12	7	
Lymph node metastasis				
Present	130	79	51	0.577 (χ^2=0.311)
Absent	55	31	24	
Tumor location				
Cardia[5]	23	8	15	
Body[6]	47	23	24	
Antrum	95	68	27	0.003 (χ^2=13.817)
Multiple	14	8	6	
Remanent carcinoma[4]	6	3	3	

[1]LDAC: Lowly-differentiated adenocarcinoma; [2]MDAC: Moderately-differentiated adenocarcinoma; WDAC: Well-differentiated adenocarcinoma; [4]6 cases of remanent gastric carcinoma were not statistically analyzed; [5]Compared with antrum, q=4.528, P=0.005; [6]Compared with antrum, q=3.681, P=0.010.

Table 2 EBV status in relation to clinicopathologic characteristics

	n	EBV (+)	EBV (-)	*P*
Sex				
Male	134	13	121	0.047 (χ^2=3.940)
Female	51	0	51	
Age (yr)				
30-	11	0	11	
40-	29	3	26	
50-	41	2	39	0.669
60-	59	6	53	
≥70	45	2	43	
Histological subtype				
LDAC[1]	125	11	114	
MDAC[2]	49	1	48	0.141
WDAC[3]	7	0	7	
Signet ring carcinoma	4	1	3	
Tumor stage				
Ⅰ	27	1	26	
Ⅱ	98	5	93	0.259
Ⅲ	41	4	37	
Ⅳ	19	3	16	
Lymph node metastasis				
Present	130	10	120	0.818 (χ^2=0.053)
Absent	55	3	52	
Tumor location				
Cardia[5]	23	1	22	
Body[6]	47	7	40	
Antrum	95	3	92	0.064
Multiple	14	1	13	
remanent carcinoma[4]	6	1	5	

[1]LDAC: Lowly-differentiated adenocarcinoma; [2]MDAC: Moderately-differentiated adenocarcinoma; WDAC: Well-differentiated adenocarcinoma; [4]6 cases of remanent gastric carcinoma were not statistically analyzed.

with positive PCR and urease test were defined as *H pylori*- positive. No difference was found in sex, age, pathological differentiation, clinical stages or lymph node metastasis between *H pylori*-positive and *H pylori*-negative gastric carcinomas. However, the location of tumor was significantly different between the two groups (χ^2=13.817, P=0.003). The positive rate of *H pylori* infection in the antrum gastric carcinomas was higher than that of cardia and body gastric carcinomas (q=4.528, P=0.005; q=3.681, P=0.010) (Table 1).

Detection of EBV infection

The positive rate of EBV in 185 gastic carcinomas was 7.03% (13/185). Age, pathological differentiation, clinical stages, lymph node metastasis and location of cancer were not different between EBV-negative gastric carcinomas (EBVnGC) and EBVaGC (P=0.669, 0.141, 0.259, 0.818, 0.064, respectively), while sex was significantly different between the two groups (χ^2=3.940, P=0.047) (Table 2).

Relationship of EBV and H pylori

The positivity of *H pylori* in EBV-positive and EBV-negative gastric carcinomas was 46.15% (6/13) and 81.40%(104/172) respectively. There was no significant correlation between EBV and *H pylori* infection (χ^2=1.027, P=0.317, r=-0.075) (Table 3).

Immunohistochemistry of c-met and c-myc

Forty-five cases of EBVnGC with similar clinicopathological data were chosen as the control group. No statistical difference was found in age, sex, tumor location, histological subtype, stage, or lymphnode metastasis between the two groups [12]. Immunostaining results of c-met and c-myc are shown in Figure 1. The c-myc and c-met expression was 61.5% (8/13) and 76.9% (10/13), and c-met overexpression was 69.2% (9/13) in EBVaGC group, while they were 55.6 (25/45), 64.4% (29/45) and 37.8% (17/45) respectively in EBVnGC group. The difference in c-met overexpression between EBVaGC and EBVnGC was significant. However, the difference in c-myc and c-met expression between the two groups was not significant (Table 4).

Expression of EBV-associated genes in EBVaGC

We investigated the expression of EBV-associated genes in 13 EBVaGC cases by RT-PCR and

Table 3 Relationship between EBV and *H pylori* in gastric carcinoma

	EBV(+)	EBV(-)	Total
H pylori (+)	6	104	110
H pylori (-)	7	68	75
Total	13	172	185

χ^2 =1.02, P=0.317, r=-0.075

Table 4 c-myc and c-met expression in EBVaGC and EBVnGC

	n	c-myc expression		c-met expression		c-met overexpression	
		+	-	+	-	+	-
EBVaGC	13	8	5	10	3	9	4
EBVnGC	45	25	20	29	16	17	28
χ^2		0.147		0.259		4.035	
P value		0.701		0.611		0.045	

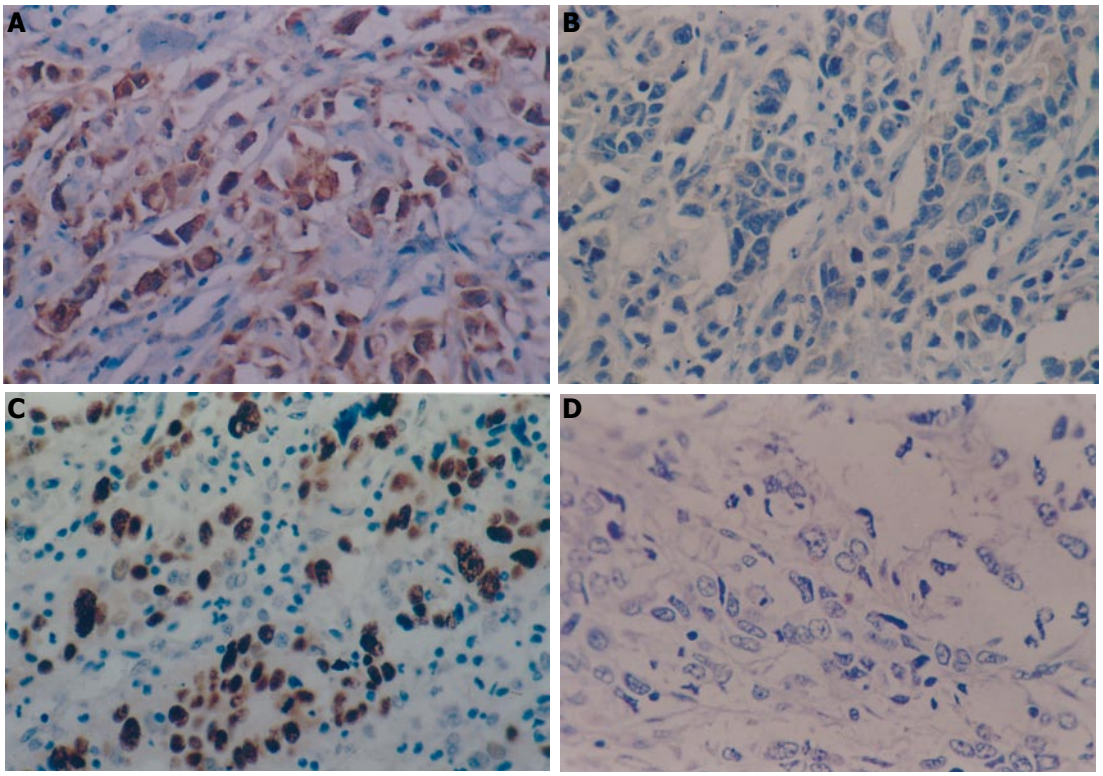

Figure 1 Immunohistochemistry of c-met and c-myc. **A**: Positive expression of c-met; **B**: Negative expression of c-met; **C**: Positive expression of c-myc; **D**: Negative expression of c-myc. (Original magnification × 400).

Southern hybridization analysis (Figure 2). The transcripts of EBNA1 were detected in all 13 cases, while neither EBNA2 nor LMP1 mRNA was detected. Six of the 13 cases exhibited BARF1 transcripts and 2 exhibited BHRF1 transcripts. GAPDH mRNA was amplified to check pertinent RNA extraction. The result showed that the RNA was in integrity.

DISCUSSION

H pylori is believed to be a carcinogen of gastric carcinoma. Recently, it was found that EBV is also linked with the development of partial gastric carcinomas. In this study, we simultaneously detected the status of EBV and *H pylori* infection in gastric carcinomas. No statistical relationship was found between *H pylori* infection rate and sex, age, pathological differentiation, clinical stages and lymph node metastasis. However, the positive rate of *H pylori* infection in the antrum gastric carcinomas was higher than that of cardia and body gastric carcinomas. These results are consistent with those of previous studies[1,13]. In our series, age, pathological differentiation, clinical stages, lymph node metastasis and location of cancer were not different between EBVnGC and EBVaGC, while the positive rate of EBV in male patients was significantly higher than that of female patients. Eleven cases of EBVaGC were low differentiated adenocarcinoma, and 8 cases of EBVaGC were body or cardia cancer. Although no statistical difference was found, it revealed a tendency that included predominance of cardia/body location and low differentiated adenocarcinoma. It remains controversial whether there is a significant clinicopathologic difference between EBVaGC and EBVnGC. Several reports showed that EBVaGC was characterized by male predominance, preferential location in proximal stomach, and a high prevalence of low differentiated adenocarcinoma[14,15]. Wu *et al*[2] found EBV-positive lymphoepithelioma-like carcinoma (LELC) showed less node metastasis and higher survival rate, and tended to be at cardia/body location in contrast to EBV-negative gastric carcinomas, while no significant clinicopathologic difference was found between EBV-positive ordinary gastric carcinoma and EBV-negative gastric carcinoma.

In the present study, no significant correlation between EBV and *H pylori* infection was found in gastric carcinomas. Currently, only a few studies have investigated the effect and interaction of EBV and *H pylori* infection in gastric carcinomas, but no conclusive results have been

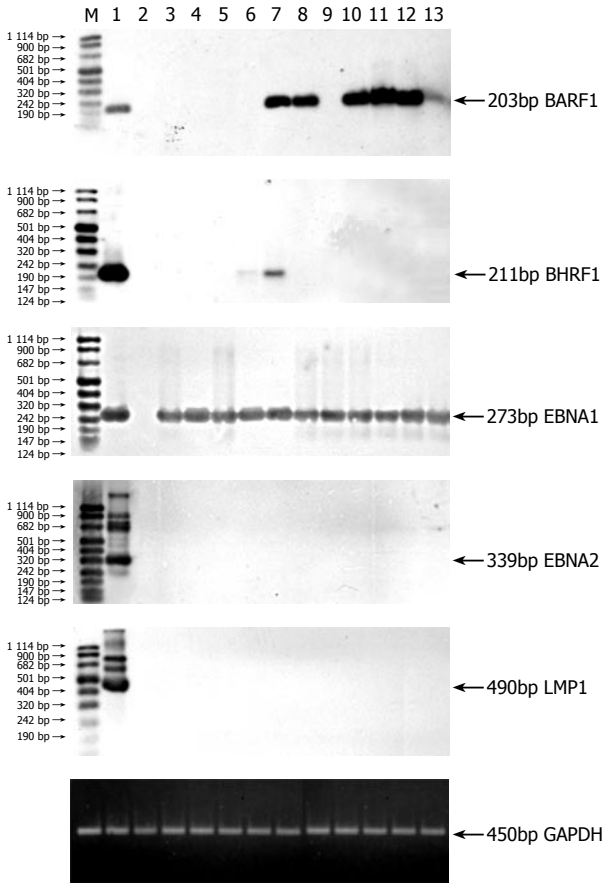

Figure 2 Detection of EBV-associated gene expression by RT-PCR and Southern hybridization in EBVaGC. M: DIG-labeled DNA molecular weight marker VIII (Roche); lane 1: EBV-positive LCL (positive control); lane 2: EBV-negative Ramos cells (negative control); lanes 3-13: EBV-positive gastric carcinoma samples.

reported[2,16-18]. Levine *et al*[16] documented that specific IgG antibody levels against *H pylori* in 39 EBV-negative gastric carcinomas were significantly higher than those of 5 EBV-positive gastric carcinomas. It was thus inferred that an inverse relationship between specific IgG titer against *H pylori* and EBV status existed. In the present study, the positivity of *H pylori* in EBVnGCs was higher than that of EBVaGCs, but no reverse correlation is found between EBV infection and *H pylori* infection. Wu *et al*[2] found EBV was detected in 11 (100%) gastric LELC and 19 (13.7%) of 139 non-LELC. Compared with the EBV-negative gastric carcinomas (68.4%) and EBV-positive non-LELC (68.3%), EBV-positive gastric LELC had a significant lower positive rate of *H pylori* IgG (36.4%). This finding implicates that there is a tendency for gastric LELC, with the high frequency of EBV and predominant proximal location, to have less association with *H pylori* infection than the other two groups, and also suggests that EBV may play a role in *H pylori*-seronegative gastric carcinomas, especially those located at the proximal stomach. Same results were found in other two reports[19,20]. Our result showed that in ordinary non-LELC gastric carcinomas, *H pylori*-positive gastric carcinoma is predominant in antrum location, while EBVaGC has a tendency of predominance in cardia/body location. Several studies found equal *H pylori* infection and distribution of intestinal metaplasia and atrophic gastritis among EBV-positive and -negative gastric carcinomas, indicating that EBV and *H pylori* may play roles together in the pathogenesis of gastric carcinomas. Chronic atrophic gastritis and subsequent intestinal metaplasia caused by *H pylori* infection enhances the susceptibility of EBV to gastric mucosal epithelia, and then EBV facilitated the carcinogenesis of gastric carcinoma[17,18].

Many studies have been focused on the relationship between EBV and the oncogenes or tumor suppressor genes in the carcinogenic mechanism of EBV. c-met protein is a receptor for hepatocyte growth factor (HGF), also known as scatter factor (SF), a tyrosine kinase encoded by the proto-oncogene c-met. HGF/SF-met signaling has been shown to affect a wide range of biological activities in mammalian cells, including cellular proliferation, migration, invasion, morphogenesis, and angiogenesis[21,22]. The oncogene c-myc also has numerous biological activities, such as transformation, immortalization, blockage of cell differentiation and induction of apoptosis[23]. Amplification and abnormal expression of oncogenes of the c-myc and /or c-met are involved in the development of tumor. In this study, EBVaGC had a higher rate of c-met overexpression than EBVnGC, however the difference in c-myc and c-met expression between the two groups was not significant, indicating that EBV infection induces c-met over-expression. The results are inconsistent with previous studies[11,24-30]. The study about the amplification and expression of c-met oncogene and EBV infection in tumor tissues has been little reported, majoring in lymphoproliferative disorder such as Hodgkin's disease (HD). Kijima *et al*[24] found the amplification and overexpression of c-met in EBV-positive and -negative gastric carcinomas did not differ significantly. Teofili *et al*[25] also reported c-met expression was independent of the presence of EBV in HD patients. However, Weimar *et al*[26] found a strong correlation between the expression of the c-met proto-oncogene and EBV infection in patients with HD. Six out of eight EBV-positive samples from HD patients expressed c-met protein, while none of the 10 EBV-negative samples from HD expressed c-met. Furthermore, circulating B lymphocytes,c-met-negative, express c-met after infection by EBV. In the same way, no consistent results were found in the study of c-myc expression in EBV-associated tumor. Several studies showed that EBV facilitates the development of tumor by inducing c-myc and inhibiting p53 expression[27-29]. However, Park *et al*[30] found that significant increase of c-myc gene copy numbers was only found in 12 out of 31 non-Hodgkin's malignant lymphomas (MLs) (38.7%), in which 6 cases were EBV positive and 6 cases were EBV negative, indicating c-myc gene amplification did not correlate with EBV infection. Ishii *et al*[11] found c-myc expression in early stage of EBVaGC was higher than that of EBVnGC, while the c-myc expression in advanced stage of EBVaGC was lower than that of EBVnGC. It was inferred that EBV might cause the host cell to induce c-myc expression and inhibit p53 overexpression in the initial development of the cancers (early stage), but then influence c-myc expression negatively in advanced

stage cancers, making them less likely to have a natural regression via apoptosis. In the present study, most of the samples were advanced stage gastric carcinomas. Apparently, it could not infer whether EBV induces c-myc expression in early stage EBVaGC, but it was certain that EBV does not inhibit c-myc expression in advanced stage EBVaGC.

Some *in vitro* and *in vivo* studies have shown that EBV-encoded genes, such as LMP1, EBNA, BHRF1 and BARF1, can interact with oncogenes and tumor suppressor genes in the carcinogenesis of tumor[28,31,32]. LMP1 are the essential genes for cell transformation. LMP1 can induce c-met expression through the activation of Ets-1 transcription factor *in vitro*, which may contribute in part to the highly metastatic potential of NPC[31]. The expression of c-myc can be induced by LMP1 and EBNA2 *in vitro* and *in vivo*[28, 29, 32, 33]. Our study and other studies failed to detect LMP1 and EBNA2 mRNA in EBVaGC[6-8], suggesting that LMP1 and EBNA2 may not be essential for EBVaGC formation and may not be related with c-met and c-myc expression in EBVaGC. EBV early gene BHRF1 shows partial sequence homologous to the human bcl-2 proto-oncogene, which is involved in inhibiting cell apoptosis. BHRF1 protein can inhibit apoptosis of B lymphocytes and epithelial cells and promote cell growth and transformation[34,35]. BARF1 shares homology with the cellular proto-oncogene c-fms and is able to immortalize epithelial cells and fibroblast cells and B lymphocyte *in vitro*[8,36,37]. Furthermore, it can activate the expression of bcl-2[38]. We demonstrated that 6 of 13 EBVaGC cases exhibited BARF1 mRNA and 2 exhibited BHRF1 mRNA. Zur Hausen *et al*[8] also detected 9 BARF1-positive cases and 2 BHRF1-positive cases in 10 EBV-related gastric adenocarcinomas. Because EBVaGC lacks the expression of LMP1[6-8], BARF1 and BHRF1 might provide an alternative way for the pathogenesis of EBVaGC independent of LMP1. *In vitro* studies showed that BARF1 can activate the expression of c-myc in BARF1 transformed cells, and EBNA1 and c-myc cooperate in lymphomagenesis[38,39]. In our study no correlation was found between c-met and c-myc expression with the expression of BARF1 or BHRF1 in 13 EBVaGCs. So further study is needed to elucidate the relationship of EBV infection and EBV-encoded proteins with expression of c-met and c-myc.

In summary, this study shows that *H pylori* infection is closely linked to the distal location gastric carcinoma, but EBVaGC is predominant proximal location. No correlation exists between EBV infection and *H pylori* infection in the development of gastric carcinomas. EBV infection is associated with c-met abnormal expression but not with c-myc protein in EBVaGC. c-met overexpression is not induced by LMP1. BARF1 and BHRF1 may play important roles in tumorigenesis of EBVaGC through different pathways.

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Anemia and long-term outcome in adjuvant and neoadjuvant radiochemotherapy of stage II and III rectal adenocarcinoma: The Freiburg experience (1989-2002)

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Abstract

AIM: To evaluate the long-term outcome of standard 5-FU based adjuvant or neoadjuvant radiochemotherapy and to identify the predictive factors, especially anemia before and after radiotherapy as well as hemoglobin increase or decrease during radiotherapy.

METHODS: Two hundred and eighty-six patients with Union International Contre Cancer (UICC) stage II and III rectal adenocarcinomas, who underwent resection by conventional surgical techniques (low anterior or abdominoperineal resection), received either postoperative ($n = 233$) or preoperative ($n = 53$) radiochemotherapy from January 1989 until July 2002. Overall survival (OAS), cancer-specific survival (CSS), disease-free survival (DFS), local-relapse-free (LRS) and distant-relapse-free survival (DRS) were evaluated using Kaplan-Meier, Log-rank test and Cox's proportional hazards as statistical methods. Multivariate analysis was used to identify prognostic factors. Median follow-up time was 8 years.

RESULTS: Anemia before radiochemotherapy was an independent prognostic factor for improved DFS (risk ratio 0.76, $P = 0.04$) as well as stage, grading, R status (free radial margins), type of surgery, carcinoembryonic antigen (CEA) levels, and gender. The univariate analysis revealed that anemia was associated with impaired LRS (better local control) but with improved DFS. In contrast, hemoglobin decrease during radiotherapy was an independent risk factor for DFS (risk ratio 1.97, $P = 0.04$). During radiotherapy, only 30.8% of R0-resected patients suffered from hemoglobin decrease compared to 55.6% if R1/2 resection was performed ($P = 0.04$). The 5-year OAS, CSS, DFS, LRS and DRS were 47.0%, 60.0%, 41.4%, 67.2%, and 84.3%, respectively. Significant differences between preoperative and postoperative radiochemotherapy were not found.

CONCLUSION : Anemia before radiochemotherapy and hemoglobin decrease during radiotherapy have no predictive value for the outcome of rectal cancer. Stage, grading, R status (free radial margins), type of surgery, CEA levels, and gender have predictive value for the outcome of rectal cancer.

Key words: Rectal cancer; Adjuvant radiotherapy; Adjuvant radiochemotherapy; Anemia; Prognostic factor

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INTRODUCTION

An improved therapeutic strategy for stage II and III rectal adenocarcinomas is urgently needed because up to 30% of patients still develop recurrent disease after curative surgical resection[1]. Several studies are ongoing aiming at evaluation of new multimodality treatment strategies[2]. However, pelvic radiotherapy *per se* is nowadays undisputed and accepted as standard therapy in all trials concerning locally advanced rectal cancer. The current standard treatment is the result of two independent, multi-institutional, prospective randomized trials more than 12 years ago by the Gastrointestinal Tumor Study Group

(GITSG 7175) in 1985[3] and the North Central Cancer Treatment Group (NCCTG 79-47-51) in 1991[4]. Both studies demonstrated that combined radiotherapy and chemotherapy following surgical resection of stage II and III rectal cancer can improve the overall survival. These results have prompted the National Institute of Health to publish the NIH Consensus recommending postoperative radiochemotherapy for stage II and III tumors.

Anemia has been shown to have impact on the rate of local control or distant metastasis of other tumor entities[5, 6]. To identify such prognostic factors and to evaluate the outcome of preoperative or postoperative radiochemotherapy, we conducted a retrospective study including 286 patients with stage II and III rectal carcinoma treated with radiochemotherapy in the Department of Radiotherapy at the University Hospital Freiburg from 1989 until 2002.

MATERIALS AND METHODS

Patient cohort

The retrospective study enrolled patients with stage II or III rectal cancer who were treated with pre- or postoperative adjuvant radiochemotherapy between January 1989 and July 2002. Pretreatment evaluation included complete blood test, chemistry profile, determination of carcinoembryonic antigen (CEA), chest radiography, liver ultrasonography and computer tomography (CT) of abdomen and pelvis[7]. Tumor location was divided in to the lower third (less than 7 cm from the anal verge), the middle third (less than 12 cm) and the upper third (12 or more than 12 cm) according to Phang *et al*[8]. However, there are other definitions[9], and even the anatomic length of the rectum is unclear[10]. If patients suffered from other diseases leading to a Karnofski lower than 80, these patients were counted as having "other serious disease(s)". Concerning survival a complete set of data were available. But due to the missing data about hemoglobin during radiotherapy, the effect of anemia (haemoglobin <120 g/L in women or <130 g/L in men) was analyzed using a subgroup of 192 patients with complete patient documentation.

Multi-modal therapy

According to the surgical reports from the different hospitals, all patients were treated with standard surgical technique. Total mesorectal excision (TME) was performed in all patients with abdominal-perineal resection (APR) but less consequently and not quality-controlled in patients with low anterior resection (LAR). If proximal and distal surgical margins were microscopically free of tumor, the patients were defined as "radically resected" (R0). According to the patient documentation analyzed, the circumferential margins were not systematically assessed. The scheduled radiotherapy delivered 45 - 56 Gy in 25 - 31 sessions using 6 / 18 MeV linear accelerator. The treatment included two parallel opposing right and left portals (using wedges with 40 or 50 % absorption) and a dorsal field. These three (or four, if external iliac lymph nodes were included) field box arrangements were used, representing the generally approved radiotherapeutic scheme during the retrospective study[11]. The upper margin was fixed 1.5 cm cranial of the promontorium whereas the lower margin was chosen depending on the exact tumor localization. The lower margin including the perineal scar (if the tumor was located less than 8 cm from the anal verge or if abdominoperineal resection was performed), was marked by the tuber ischiadicum (between 8 and 12 cm above the anal verge), or covered by the lower border of the obturator foramen (12 cm above the anal verge). The width of posteroanterior portals covered the pelvic inlet with 2 cm margin. Radiotherapy was administered with cycles 1 and 2 instead of cycles 3 and 4 as recommended by the NIH[12], renewed by the 1996 Patterns of Care Rectal Cancer Committee[13]. In the NCCTG study, cycles 1 and 2 of 5-FU plus semustine were given, followed by pelvic radiotherapy plus chemotherapy.

Depending on their R status, patients were treated with combined modality therapy either according to the NIH recommendations[12] or to the protocol[14] of the Arbeitsgemeinschaft Radioonkologie (ARO) of the German Cancer Society. Patients with no evidence of microscopical residuals of the disease (R0 resection) were treated according to the NIH protocol[12]. Following R1 or R2 resection, patients were treated according to the ARO protocol. When the NIH protocol was used, the concurrent bolus 5-FU was given at a daily dose of 500 mg/m² for 3 d during cycles 1-3, the following three cycles were given for 5 d. When the ARO protocol was used, patients received 350 mg/m² 5-FU iv continuously during 24 h for 14 d. Additionally, bolus of 200 mg/m² leucovorin and 4 mg/m² mitomycin C, was given daily for 1 h. In both protocols radiotherapy and chemotherapy were started simultaneously.

Statistical analysis

The data were analyzed using SAS. The statistical methods included Student's *t*-test, Chi-square test, and Kruskal-Wallis-test. Survival was analyzed using univariate and multivariate methods (step down analysis). Kaplan-Meier curves[15] were used to estimate the distribution of overall survival (OAS), cancer-specific survival (CSS) and disease-free survival (DFS). For analysis, the rates of treatment failure were adjusted, local-relapse-free survival (LRS) and distant-relapse-free survival (DRS) were determined as life table analysis referring to freedom of locoregional relapse and freedom of distant recurrence (metastasis). LRS was defined as the rate of local control. Log-rank test (Cox-Mantel) was used to compare the survival distributions between different patient subgroups[16]. Multivariate analysis and proportional hazard models[17] were used to determine the prognostic factors with significant impact on survival, including hemoglobin effect, grading, staging, adjuvant therapy, surgical method and tumor marker.

RESULTS

Two hundred and eighty-six patients (186 men and 100 women) fulfilled the inclusion criteria and were enrolled in the study (Table 1). Their age ranged from 30 to 84

Table 1 Patient characteristics

	Adjuvant radiochemotherapy *n* (%)	Neoadjuvant radiochemotherapy *n* (%)	All *n* (%)
Gender			
Female	148 (63.5)	38 (71.7)	186 (65.0)
Male	85 (36.5)	15 (28.3)	100 (35.0)
Age			
≤Median age of 62 yr	134 (57.5)	32 (60.4)	166 (58.0)
> Median age of 62 yr	99 (42.5)	21 (39.6)	120 (42.0)
Tumor location			
Upper third	35 (15.1)	0 (0.0)	35 (12.2)
Middle third	100 (42.9)	23 (43.4)	123 (43.0)
Lower third	87 (37.3)	25 (47.2)	112 (39.2)
Not known	11 (4.7)	5 (9.4)	16 (5.6)
Surgical Resection			
Anterior (LAR)	133 (56.8)	13 (24.6)	146 (51.0)
Abdominoperineal (APR)	100 (43.2)	40 (75.4)	140 (49.0)
R status			
R0	197 (84.5)	37 (69.8)	234 (81.8)
R1	17 (7.3)	5 (9.4)	22 (7.7)
R2	4 (1.8)	2 (3.8)	6 (2.1)
not known	15 (6.4)	9 (17.0)	24 (8.4)
Stage			
II	75 (32.2)	17 (32.1)	92 (32.2)
III	158 (67.8)	36 (67.9)	194 (67.8)
N stage			
N0	74 (31.8)	17 (32.1)	91 (31.8)
N1	81 (34.8)	27 (50.9)	108 (37.8)
N2	78 (33.4)	9 (17.0)	87 (30.4)
Grading			
G I	11 (4.7)	9 (17.0)	20 (7.0)
G II	161 (69.1)	29 (54.7)	190 (66.5)
G III	44 (18.9)	9 (17.0)	53 (18.5)
Not known	17 (7.3)	6 (11.3)	23 (8.0)
CEA			
< 3 ng/mL	102 (43.8)	16 (30.2)	118 (41.3)
≥ 3 ng/mL	102 (43.8)	16 (30.2)	118 (41.3)
Not known	29 (12.4)	21 (39.6)	50 (17.4)
RTOG			
RTOG 0	96 (41.2)	30 (56.6)	126 (44.1)
RTOG I	75 (32.2)	14 (26.4)	89 (31.1)
RTOG II	46 (19.7)	9 (17.0)	55 (19.2)
RTOG III	13 (5.6)	0 (0.0)	13 (4.6)
RTOG IV	3 (1.3)	0 (0.0)	3 (1.0)
RTOG V	0 (0.0)	0 (0.0)	0 (0.0)

years (median 62 years). The distribution of stages was as follows: 92 patients (32.2%) were assigned to stage II (lymph node negative) and 194 (67.8%) to stage III (lymph node positive). One hundred and forty-six patients (51.0%) were treated with low anterior resection (LAR), 140 patients (49.0 %) with abdominoperineal resection (APR). According to the surgical reports from different hospitals, all patients were treated with standard surgical technique. Total mesorectal excision (TME) was performed in all patients with APR but less consequently and not quality-controlled in patients after LAR. Two hundred and thirty four patients (81.8 %) were defined as R0 (Table 1). Sixteen patients were known to have oncological diseases in their histories. Other severe diseases referred to cardial ($n = 62$), pulmonary ($n = 5$), hepatic or gastric ($n = 18$), psychiatric diseases ($n = 8$), or diabetes ($n = 6$).

Survival and life table analysis

The calculated overall 5-year survival (OAS) of all stage II and III patients was 47% (Figure 1 and Figure 2), the 10-year survival was 36.3%. The cancer-specific survival (CSS) and disease-free survival (DFS) decreased from 60.0% and 41.4% respectively to 52.0% and 34.9 % between 5 and 10 years (Table 3). Local-relapse-free survival (LRS) was 67.2% (5 years) and 65.7% (10 years), distant-relapse-free survival (DRS) was 84.3% (5 and 10

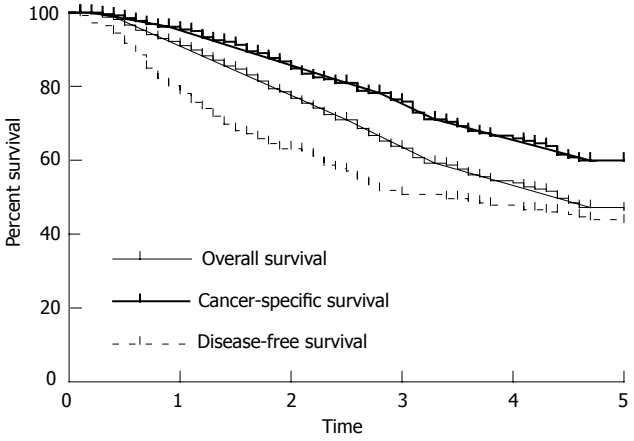

Figure 1 Kaplan-Meier curves of OAS, CSS and DSF.

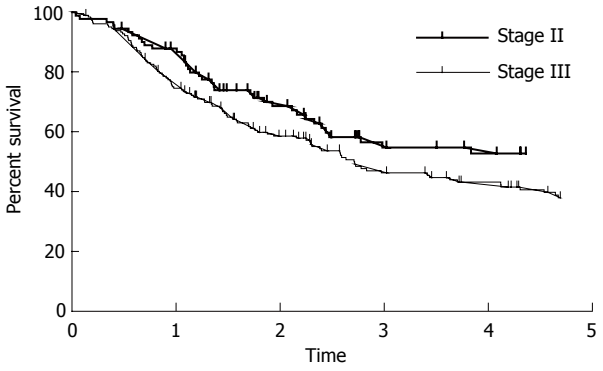

Figure 2 Log-rank test of staging (5-year disease-free survival, $P = 0.015$).

Table 2 Anemia and hemoglobin values during radiotherapy (subgroup analysis of 192 patients)

	Adjuvant radiochemotherapy	Neoadjuvant radiochemotherapy	All
	n (%)	*n* (%)	*n* (%)
Anemia before RT			
No	78 (47.6)	14 (50.0)	92 (47.9)
Yes	86 (52.4)	14(50.0)	100 (52.1)
Anemia after RT			
No	102 (62.2)	8 (28.6)	110 (57.3)
Yes	62 (37.8)	20 (71.4)	82 (42.7)
Hemoglobin during RT			
Hb increase	106 (64.6)	10 (35.7)	116 (60.4)
Hb decrease	58 (35.4)	18 (64.3)	76 (39.6)

years). The mean overall survival time was 4.4 years and the mean disease-free survival time was 2.73 years. The mean follow-up time was 8 years.

Survival and patient characteristics

Complete data about hemoglobin were available from 192 patients. When this subgroup (Table 2) was analyzed, anemia was found in 52.1% of the patients. Of the patients who received adjuvant radiotherapy, 64.6% showed increased hemoglobin during radiotherapy compared to 35.7% of the patients who were treated neoadjuvantly. Patients showed more frequently anemia if they were older than the median age or if R0 resection was performed. Anemia after radiotherapy was less frequently seen if postoperative radiotherapy was given instead of preoperative radiotherapy (37.8% *vs* 71.4%, respectively) or if low anterior resection (LAR) instead of abdominoperineal resection (APR) was performed (36.8% *vs* 51.4%, respectively), while 43.8% of the patients with stage III tumors showed increased hemoglobin during radiotherapy compared to 30.0% of the patients with stage II tumors (Table 7). Furthermore, patients less frequently suffered from increased hemoglobin if they received postoperative radiochemotherapy or their tumors underwent R0-resection. Decrease of hemoglobin was found to be an independent risk factor for DFS with a risk ratio of 1.97 (CI 1.02 - 3.43). Conversely anemia before radiotherapy had a risk ratio of 0.76 (CI 0.57 - 0.99) (Table 6).

Tumor grading was found to be an independent prognostic factor for CSS and DFS (Table 3). The overall 5-year survival rate of patients with stage III tumors was about 10% compared to patients with stage II tumors (50.6% *vs* 40.7%; $P = 0.023$) (Table 3). Patients with stage II tumors showed a 5-year overall survival of 57 % (CSS: 65.8%; DFS: 53.3%) whereas patients with stage III tumors showed a 5-year overall survival of 42.6% (CSS: 57.2 %; DFS: 36.3%). Univariate analysis showed that the N stage was found to be significant for OAS and CSS ($P = 0.06$). The patients' gender was found to be an independent factor for LRS. The 5-year LRS for female was significantly worse than that for males (56.8% *vs* 73.1%). No dependency between tumor location and outcome could be seen (Table 5). Tumor adherence to adjacent structures predicted survival (OAS $P < 0.001$, CSS $P = 0.004$, DFS $P < 0.001$, DRS $P = 0.019$) regardless of the surgical method, but was not significant for LRS ($P = 0.13$).

Survival and treatment characteristics

The overall survival did not vary depending on the radiotherapy treatment type (either pre- or post-operative radiotherapy) (Table 4). Neither gross nor microscopic evidence of disease could be achieved in 37 of 53 (69.8%) preoperatively irradiated patients, and in 197 of 233 (84.5%) of postoperatively irradiated patients. Sphincter-saving surgery could be performed in 24.6% of patients receiving preoperative radiotherapy and in 56.8% of the patients receiving postoperative radiochemotherapy.

Multivariate analysis showed that R status (R0 *vs* R1/2) was an independent prognostic factor for disease-free survival (risk ratio 3.45, CI 1.56 -7.96) whereas surgical method (low anterior or abdominoperineal resection) was an independent prognostic factor for local control, determined as LRS (risk ratio 1.3, CI 1.01-1.60) (Table 4). Chi-square test showed no significant relationship between surgical method and staging ($P = 0.93$) or R status ($P = 0.07$) but a significant relationship between R status

Table 3 Survival rates and results of uni- and multivariate analysis (patient-related variables)

		OAS (%)	CSS (%)	DFS (%)	LRS (%)	DRS (%)
All stages II & III	5 yr / 10 yr:	47.0 / 36.0	60.0 / 52.0	41.4 /34.9	67.2 / 65.7	84.3 / 84.3
	at risk (5 yr / 10 yr):	78 / 34	81 / 36	69 / 34	72 / 46	74 / 34
	median survival:	1602		996		
Gender						
Male	5 yr / 10 yr:	49.2 / 36.3	64.9 / 56.8	24.7 / 12.9	73.1 / 73.1	85.1 / 85.1
Female	5 yr / 10 yr:	43.2 / 37.0	51.7 / 44.3	21.0 / 8.0	56.8 / 53.0	83.2 / 83.2
	uni- / multivariate: *P* =	0.93 / NS	0.16 / NS	0.17 / NS	0.01 / 0.018	0.66 / NS
	multivariate risk ratio:				male: 0.75	
					(CI 0.59 – 0.95)	
Age						
< 62 yr	5 yr / 10 yr:	52.2 / 42.0	62.0 / 55.8	31.1 / 16.0	65.3 / 63.4	85.9 / 85.9
> 62 yr	5 yr / 10 yr:	41.0 / 27.0	57.1 / 42.6	13.6 / 5.1	70.4 / 70.4	80.2 / 80.2
*Median age	uni- / multivariate: *P* =	0.02 / NS	0.22 / 0.005	< 0.01 / NS	0.47 / NS	0.59 / NS
	multivariate risk ratio:		< 61 yr: 0.48			
			(CI 0.28 – 0.80)			
Staging						
II	5 yr / 10 yr:	57.0 / 49.9	65.8 / 62.5	53.3 / 45.5	75.1 / 75.1	85.5 / 85.5
III	5 yr / 10 yr:	42.6 / 30.2	57.2 / 47.4	36.3 / 28.7	63.3 / 61.0	83.8 / 83.8
	uni- / multivariate: *P* =	0.02 / NS	0.09 / 0.009	0.02 / NS	0.06 / (0.07)	0.7 / NS
	multivariate risk ratio:		stage II: 0.45		stage II: 0.79	
			(CI 0.21 – 0.84)		(CI 0.59 – 1.03)	
Grading						
II	5 yr / 10 yr:	50.6 / 39.7	62.0 / 55.1	49.3 / 41.7	73.3 / 70.7	86.8 / 86.8
III	5 yr / 10 yr:	40.7 / 36.6	55.3 / 49.8	23.3 / 23.3	58.8 / 58.8	64.4 / 64.4
	uni- / multivariate: *P* =	0.023 / (0.06)	0.07 / NS	< 0.01 / NS	0.04 / NS	< 0.01 / 0.04
	multivariate risk ratio:	G III: 1.68				G III: 2.87
		(CI 0.97 – 2.78)				(CI 1.08 – 7.01)

Risk ratio with confidence interval (CI) is given if multivariate analysis (step down analysis) revealed a significant result. Five- and 10- year survival rates are given as OAS, CSS, DFS, LRS and DRS.

Table 4 Survival rates and results of uni- and multivariate analysis (treatment-related variables)

		OAS (%)	CSS (%)	DFS (%)	LRS (%)	DRS (%)
Adjuvant therapy						
Postop RCT	5 yr / 10 yr:	47.6 / 35.6	59.0 / 48.4	40.2 / 34.0%	66.1 / 64.1	82.2 / 82.2
Preop RCT	5 yr / 10 yr:	43.9 / 38.0	62.6 / 62.6	45.4 / 39.3	69.8 / 69.8	92.8 / 92.8
	uni- / multivariate: *P* =	0.82 / NS	0.56 / NS	0.61 / NS	0.96 / NS	0.12 / NS
Protocol						
NIH	5 yr / 10 yr:	60.7 / 45.2	72.5 / 59.4	49.8 / 44.2	84.1 / 84.1	73.2 / 73.2
ARO	5 yr / 10 yr:	26.9 / 21.5	45.6 / 36.5	24.5 / 16.3	67.6 / 67.6	64.0 / 64.0
	uni- / multivariate: *P* =	< 0.001 / NS	0.003 / 0.004	0.002 / NS	0.024 / NS	0.57 / NS
	multivariate risk ratio:		ARO: 2.66			
			(CI 1.37 – 5.21)			
Surgery	risk ratio:					
LAR	5 yr / 10 yr:	36.4 / 25.9	49.5 / 41.1	27.8 / 22.7	64.7 / 64.7	81.3 / 81.3
APR	5 yr / 10 yr:	35.2 / 26.0	46.7 / 39.9	27.6 / 22.9	52.4 / 51.1	86.4 / 86.4
	uni- / multivariate: *P* =	0.4 / NS	0.51 / NS	0.27 / NS	0.015 / 0.039	0.6 / NS
	multivariate risk ratio:				LAR: 1.3	
					(CI 1.01 – 1.60)	
R status						
R0	5 yr / 10 yr:	53.2 / 40.3	64.2 / 55.1	45.6 / 39.5	66.2 / 64.5	91.2 / 91.2
R1 or R2	5 yr / 10 yr:	14.2 / 14.2	20.7 / 20.7	17.3 / 17.3	62.9 / 62.9	51.2 / 51.2 5
	uni- / multivariate: *P* =	< 0.001 / NS	0.011 / (0.09)	< 0.001 / 0.026	0.95 / NS	< 0.001 / NS
	multivariate risk ratio:		R1/R2: 1.65	R1/R2: 3.45		
			(CI 0.91 – 3.10)	(1.56 – 7.96)		

Risk ratio with confidence interval (CI) is given if multivariate analysis (step down analysis) revealed a significant result. Five- and 10- year survival rates are given as OAS, CSS, DFS, LRS and DRS.

Table 5 Results of uni- and multivariate analysis (other variables)

	OAS	CSS	DFS	LRS	DRS
N stage					
uni- / multivariate: *P* =	0.009 / NS	0.05 / NS	0.06 / NS	0.09 / NS	0.15 / NS
CEA					
uni- / multivariate: *P* =	< 0.001 / 0.007	< 0.001 / NS	< 0.001 / 0.001	< 0.001 / NS	0.55 / 0.055
multivariate risk ratio:	CEA increase: 1.37 (CI 1.09 – 1.73)		CEA increase: 3.21 (CI 1.61 – 6.82)		CEA decrease: 0.91 (CI 0.76 – 1.00)
CA 19-9					
uni- / multivariate: *P* =	< 0.001 / NS	0.033 / NS	0.22 / NS	0.79 / NS	0.62 / NS
Tumor location					
uni- / multivariate: *P* =	0.34 / NS	0.43 / NS	0.4 / NS	0.83 / NS	0.37 / NS
Adherence to adjacent structures					
uni- / multivariate: *P* =	< 0.001 / NS	0.004 / NS	< 0.001 / NS	0.13 / NS	0.019 / NS
BMI before RT					
uni- / multivariate: *P* =	0.47 / NS	0.55 / NS	0.83 / NS	0.83 / NS	0.15 / NS
Smoking					
uni- / multivariate: *P* =	0.79 / NS	0.49 / NS	0.71 / NS	0.22 / NS	0.31 / NS
Hkt before *vs* after RT					
uni- / multivariate: *P* =	0.047 / NS	0.35 / NS	0.31 / NS	0.87 / NS	0.99 / NS
LDH before *vs* after RT					
uni- / multivariate: *P* =	0.32 / NS	0.69 / NS	0.77 / NS	0.37 / NS	0.21 / NS

Risk ratio with confidence interval (CI) is given if multivariate analysis (step down analysis) revealed a significant result. Five- and 10- year survival rates are given as OAS, CSS, DFS, LRS and DRS

Table 6 Anemia and hemoglobin increase versus decrease during radiotherapy (subgroup analysis of 199 patients with documented hemoglobin values before and after radiotherapy)

		OAS (%)	CSS (%)	DFS (%)	LRS (%)	DRS (%)
Anemia before RCT						
No	5 yr / 10 yr:	44.6 / 31.4	66.4 / 66.4	41.5 / 33.7	76.6 / 76.6	74.9 / 74.9
Yes	5 yr / 10 yr:	49.5 / 47.2	55.4 / 45.6	48.5 / 39.2	61.5 / 59.6	88.1 / 88.1
	uni- / multivariate: *P* =	0.39 / NS	0.14 / NS	0.83 / 0.04	0.04 / NS	0.01 / NS
	multivariate risk ratio:			anemia present: 0.76 (CI 0.57 – 0.99)		
Anemia after RCT						
No	5 yr / 10 yr:	47.5 / 34.0	57.4 / 48.4	42.5 / 34.7	87.6 / 87.6	62.7 / 60.7
Yes	5 yr / 10 yr:	47.0 / 41.5	64.7 / 59.3	39.6 / 35.9	80.0 / 80.0	73.0 / 73.0
	uni- / multivariate: *P* =	0.76 / NS	0.56 / NS	0.83 / NS	0.12 / NS	0.06 / NS
	multivariate risk ratio:					
Hemoglobin (Hb) increase or decrease during RT						
Hb increase	5 yr / 10 yr:	67.7 / 59.3	58.8 / 51.4	45.2 / 45.2	83.8 / 83.8	62.8 / 62.8
Hb decrease	5 yr / 10 yr:	35.3 / 35.3	38.7 / 33.9	36.2 / 36.2	73.9 / 73.9	75.9 / 75.9
	uni- / multivariate: *P* =	< 0.01 / NS	< 0.01 / NS	0.08 / 0.04	0.38 / NS	0.79 / NS
	multivariate risk ratio:			Hb decrease: 1.97 (CI 1.02 – 3.43)		

Survival rates and results of the uni- and multivariate analysis as well as risk ratio with confidence interval (CI) are given if multivariate analysis (step down analysis) revealed a significant result. Five- and 10-year survival rates are given as OAS, CSS, DFS, LRS and DRS

and grading ($P = 0.01$). The chemotherapy protocol was proved to be an independent prognostic factor (risk ratio 2.66, CI 1.37-5.21), showing a higher 5-year cancer-specific survival rate for patients treated with NIH protocol (72.5%) compared to ARO protocol (45.6%). Elevated CEA (> 3 ng/mL) levels were proved to be an independent prognostic factor for OAS and DFS (Table 5). The risk ratios were 1.37 (CI 1.09- 1.73) for OAS and 3.21 (CI 1.61 - 6.82) for DFS, respectively. According to our patient documentation, 126 patients (44%) had no documented

Table 7 Anemia and patient characteristics.

	Hb decrease during RT *n* (%)	*P* value
Age		
⩽ 62[1]yr	42 (42.9)	0.31
> 62[1]yr	32 (35.6)	
Stage		
II	18 (30.0)	0.07
III	56 (43.8)	
Other serious diagnoses		
No	38 (35.2)	0.17
Yes	36 (45.0)	
BMI		
⩽ 24.2[2]	44 (50.0)	0.03
> 24.2[2]	18 (26.5)	
Radiochemotherapy		
Postoperative	56 (35.0)	0.03
Preoperative	18 (64.3)	
Surgical Resection		
LAR	42 (38.2)	0.96
APR	28 (37.8)	
R status		
R 0	24 (30.8)	0.04
R 1/2	10 (55.6)	
Side effects		
RTOG 0	8 (80.0)	0.27
RTOG I / II	62 (38.8)	
RTOG III / IV	4 (22.2)	

Results of chi-square test. [1]Median age, [2]median BMI (body mass index).

side effects (Table 1), while 89 (31.1%), 55 (19.2%), 13 (4.5%), and 3 (0.7%) patients had RTOG grade I, II, III or IV side effects, respectively. Diarrhoea, dysuria and skin reaction were the leading problems whereas other side effects were rarely reported.

DISCUSSION

Concerning the outcome of rectal cancer, patients with younger age, lower stage, tumor grading GII or better, no elevated tumor markers, and a higher distance from the anal verge may fare better[8, 18, 19]. There is also some evidence that R0 resection, no adherence to adjacent structures, and lower anterior resection (LAR), performed with quality-controlled total mesorectal excision (TME), are beneficial factors for the local control whereas positive lymph nodes are risk factors mainly for distant metastases. The impact of radiotherapy is still debated. Moreover, valid data from other tumor entities suggest that anemia may have predictive value for local relapse or distant recurrence[5, 6]. At the Department of Radiotherapy, University Hospital Freiburg from 1989 to 2002, 286 patients with stage II or III cancer were treated and qualified for our retrospective study analyzing patient- or treatment-related variables and their impact on treatment outcome. This study, however, has some limitations mainly due to lack of data about the quality of surgery and incomplete patient documentation. Therefore, the extent and quality of total mesorectal excision could not be assessed. The impact of anemia on 192 patients with complete data about hemoglobin during radiochemotherapy was analyzed.

The overall 5-year survival rate (47.0%) and disease-free survival rate (41.1%) were lower than those of other large randomized trials[3, 4]. It is known that the outcome of therapeutic interventions obtained in studies usually exceed "the reality" of population-based reports of cancer treatment[21]. The rate of distant recurrence (16.7%) was slightly lower in our patient cohort than that reported in literature (Table 4), which may be due to the modified sequence of radiochemotherapy. This scheme of early radiochemotherapy can achieve significantly improved rates of local recurrence and distant metastasis[22], suggesting that early radiotherapy as performed in our study is more effective concerning local control and consequently more effective concerning distant outcome.

The Mayo study and other studies are influenced by a local relapse rate of about 30 %[3, 4]. These studies could reflect the situation before the "era" of trained and quality-controlled total mesorectal excision. Similarly, we found that a local recurrence rate of 32.8% (local-relapse-free survival of 67.2%) is consistent with current literature[23]. After conventional and non-standardized surgery, local recurrence was 15%-55%[24-26]. However, it was reported that the 5-year local control rate was higher than 90 %, even without chemotherapy or any adjuvant treatment[27-29].

Tumor adherence to the adjacent structures can predict local recurrence and survival[30-32]. Consistent with these data, the Freiburg patient cohort including 286 cases showed that all survival rates (OAS, CSS, DFS) were associated with tumor adherence. This may be due to the effect of sample size or support the contention that out-spread of the gross tumor should be estimated as an important indicator for systemic tumor progression leading to worse outcome. According to this, it has been recently shown that even among N2 patients (4 or more positive lymph nodes), T stage influences overall 5-year survival[22]. Furthermore, T and N classifications can predict survival, but only N stage is correlated with local recurrence[30]. On the contrary, in our retrospective study N stage was significant for overall survival ($P=0.009$) and cancer-specific survival ($P=0.05$), whereas a trend for local recurrence ($P=0.09$) and disease-free survival ($P=0.06$) could be seen.

When an average radiation dose of 50 Gy is used for preoperative or postoperative radiochemotherapy, patients classified as N0 have better results than N positive ones[33]. Similarly, analysis of eligible patients has confirmed the effect of N stage[19]. Notably our data suggest that N positive patients are even more substantially at risk for local failure rather than for distant metastasis. Apparently, spread-out to lymph nodes indicates a more aggressive nature of local tumor growth. Concerning treatment intervention, these results support the hypothesis that sterilization of tumor cells in lymph nodes could be achieved in many patients, preventing them from distant metastasis. As a consequence, this contradicts to any efforts to minimize radiation fields. Only if adequately included in the radiation field, the tumor cells in regional

lymph nodes can be killed.

In the present study, when anemia and hemoglobin were analyzed, conflicting results were obtained from the multivariate analysis. When hemoglobin values decreased during radiotherapy it meant to be a risk factor for patients, possibly indicating bad performance status, prolonged convalescence, side effects of therapy or progressive disease (Table 7). But anemia had a risk ratio of 0.76 for DFS (Table 6), suggesting that rectal cancer patients with anemia before radiotherapy have better results than patients without anemia. Considering the adjuvant setting at a time interval of 3 - 5 wk between surgery and radiochemotherapy, anemia can be regarded as a consequence of a more radical surgical intervention. This hypothesis is supported by the analysis of 192 patients with complete data about hemoglobin during radiochemotherapy: 60.0% of patients after R0-resection had anemia compared to 55.6% of patients after R1/2-resection. After radiotherapy only 42.5% patients after R0-resection still had anemia compared to 44.4% patients after R1/2-resection. Only 30.8% patients after R0-resection suffered from hemoglobin decrease during radiotherapy compared to 55.6 % patients after the R1/2-resection, suggesting that R status is an independent prognostic factor for disease-free survival. According to our data, anemia before radiochemotherapy or hemoglobin decrease during radiotherapy does not imply any predictive value for the outcome of rectal cancer. It should be considered as a readout of age, stage, type of surgical resection, or performance status. Taken together, if there is any hemoglobin effect on the outcome of rectal cancer, it is completely biased by the extent of surgical intervention, which is one of the most important predictors for the outcome of rectal cancer.

Multivariate analysis of our data revealed that tumor grading was the most important prognostic factor for CSS and DFS. In a retrospective study of 214 patients with primary rectal carcinoma, Luna-Perez *et al*[33] have identified well-differentiated cancer as a prognostic factor for achieving local control. Martijn *et al*[31] also found that tumor grading has a prognostic value, which is consistent with our study. We found that there was no significant difference in survival rate regarding tumors in the lower, middle or upper third. Thus, the hypothesis that tumor location influences response rate or survival is not supported by our data. Phang *et al*[8] reported that survival is affected by tumor distance from the anus. Lower distance significantly can worsen survival and distant recurrence rate but not the rate of local recurrence. Finally, an independent detrimental influence on local recurrence has been proved by a retrospective study of 197 patients, using conventional resection technique[32].

By analyzing the influence of the resection type, we revealed a better control rate of local relapse (34%) if the patients were treated by low anterior resection (LAR) compared to abdominoperineal resection (APR). Out data are consistent with a study of Stocchi *et al*[30] showing a better rate for LAR (28%) but not with a study of Kuru *et al*[32] who identified anterior resection as a negative independent prognostic factor for local control. However, Stocchi *et al*[30] stated that the overall rate of local recurrence (16%) is unexpectedly low in their study, considering the high-risk patient cohort and the data from literature. Unlike this, our data (32.8 % local recurrence in five years) could obviously reflect the "surgical reality today"[34] or, more accurately, the surgical results in the era (Table 6) before comprehensive surgical quality control of TME[36].

In the Freiburg cohort male gender was found to be an independent prognostic factor for local recurrences. No difference was found in OAS, CSS, DFS and DRS. It was reported that DFS and local recurrence do not differ significantly in gender, but differ significantly in OAS with a risk ratio 1.2 ($P=0.03$) for men. Furthermore, increased grade III or IV toxicity in females has been described. As in our study, no interaction between sex and age was seen. Therefore sex as an indicator for higher age can be ruled out. Studies evaluating psycho-oncological aspects have identified gender-related factors which influence cancer treatment and outcome[38]. However, if sex difference has any significant impact on outcome, this effect seems to be inconsistent and may be influenced by local differences in lifestyle or environmental factors.

Among the patients-related factors, age is known to be crucial, because administration of adjuvant chemotherapy to patients aged over 70 years remains a difficult choice for the clinicians with respect to the expected benefit versus toxicity. Sargant *et al*[39] and Popescu *et al*[40] showed that old patients (with good performance status and renal and hepatic function) share the same benefit from adjuvant chemotherapy as younger patients without increasing toxicity. CEA and CA 19-9 levels are reliable tumor markers for rectal cancer[18, 41, 42]. In our study, the prognostic value of CEA could be shown by uni- and multivariate analysis. Additionally, the increase of CEA during radiotherapy indicated a decreased survival rate. Our results are consistent with that of Behbehani *et al*[43], who demonstrated that patients with preoperatively elevated CEA levels have a 2-year DFS of 23% whereas patients without CEA elevation have a 2-year DFS of 71%.

Our retrospective review of the Freiburg patient cohort did not detect any difference between preoperative and postoperative radiochemotherapy. Cancer-specific survival was 62.6% and 59.0%, respectively. Our data correspond to Rinkus *et al*[34] who reviewed 292 patients showing no significant difference in survival between preoperative versus postoperative radiotherapy (combined with chemotherapy in 66% and 48% of the cases, respectively).

It was reported that preoperative radiation can significantly decrease local failure rate. The 5-year survival curve does not differ significantly between both strategies. Until now the German trial (Protocol CAO/ARO/AIO 94) is the only prospective study with patients randomized to conventional preoperative and postoperative radiochemotherapy[20]. This study showed that preoperative radiochemotherapy could improve local control and reduce toxicity but overall survival was not different between pre- and postoperative radiochemotherapy. However, our retrospective, non-randomized data might be biased as preoperative radiochemotherapy was mainly given if the tumor was inoperable. Therefore these patients were in more

advanced tumor stages. Supposing an advantage of preoperative radiochemotherapy, as shown by Sauer *et al*[20], this may explain why preoperative and postoperative radiochemotherapy seem to be equivalent according to our data.

In conclusion, new therapeutic strategies are urgently needed, preferably based on a better understanding of beneficial or disadvantageous factors determining the outcome of rectal cancer. Tumor stage, grading, R status (free radial margins), type of surgery, CEA levels, and gender are predictive factor for the outcome of rectal cancer. However, anemia before radiochemotherapy or hemoglobin decrease during radiotherapy does not imply any predictive value for the outcome of rectal cancer. It should be considered as an indicator for higher age, stage, type of surgical resection, or performance status. Any hemoglobin effect of radiotherapy on outcome of rectal cancer is completely biased by the extent of surgical intervention.

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Glycine-extended gastrin activates two independent tyrosine-kinases in upstream of p85/p110 phosphatidylinositol 3-kinase in human colonic tumour cells

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Abstract

AIM: To investigate whether Src, JAK2 and phosphatidylinositol 3-kinase (PI3K) pathways are involved in the proliferation of human colonic tumour cells induced by glycine-extended gastrin (G-gly), the precursor of the mature amidated gastrin and to elucidate the molecular interaction between these three kinases in response to this peptide.

METHODS: Using the human colonic tumour cell line HCT116 as a model, we first measured the activation of PI3K, p60-Src and JAK2 in response to G-gly by *in vitro* kinase assays. Then we investigated the involvement of these kinases in G-gly-induced cell proliferation by MTT test.

RESULTS: G-gly stimulation induced p60-Src, JAK2 and PI3K activation in HCT116. The different pathways were involved in proliferation of human colon cancer cells induced by G-gly. Furthermore, we found that both Src and JAK2 were necessary to PI3K regulation by this peptide. However, we did not find any cross-talk between the two tyrosine kinases.

CONCLUSION: Our results suggest that the p60-Src/PI3K and JAK2/PI3K pathways act independently to mediate G-gly proliferative effect on human colonic tumour cells.

Key words: Colon cancer; Src; JAK2; Phosphatidylinositol 3-kinase; Glycine-extended gastrin

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INTRODUCTION

Hyperproliferation of the colonic mucosa is associated with an increased risk of tumour development, and corresponds to an early stage in the adenoma-carcinoma sequence. It is now well established that glycine-extended gastrin (G-gly), the precursor of the mature amidated gastrin, plays an important role in colonic mucosa hyperproliferation. Proliferative effects of G-gly were first described in a pancreatic tumour cell line, AR4-2J[1]. Afterwards, numerous studies have confirmed its mitogenic effects, especially on colonic mucosa, and *in vitro* studies have shown that G-gly is a growth factor for non transformed cell lines from colon origin or human colon cancer cells[2-5]. Trophic effects of G-gly have also been confirmed *in vivo* and MTI/G-gly transgenic mice overexpressing G-gly, as well as gastrin-deficient mice perfused with this peptide, display hyperproliferation of the colonic mucosa[6]. Furthermore, perfusion of G-gly into rats results in proliferation of colonic mucosal cells forming aberrant crypt foci and increases the sensitivity to azoxymethane, a colon carcinogen[7].

The p85/p110 PI3K is a lipid kinase composed of two constitutively associated subunits: p85, the regulatory subunit; and p110, the catalytic subunit. Upon stimulation, PI3K phosphorylates the D3 position of phosphoinositides leading to second messengers, namely phosphatidylinositol 3, 4 biphosphate and phosphatidylinositol 3, 4, 5 triphosphate[8]. The PI3K pathway is involved in the regulation of many cellular processes including prolifera-

tion and survival. During the last years, many studies have shown the implication of the PI3K in colon carcinogenesis. In particular, the PI3K has been found to play an important role in colon cancer development and progression by promoting cell growth and allowing cells to escape apoptosis[9]. Activation of this pathway is also involved in the progression of human colon adenocarcinoma[10].

Different groups including ours, have demonstrated the involvement of the phosphatidylinositol 3-kinase in G-gly functions. We have reported the activation of lipid kinase in response to this peptide in a pancreatic tumoral cell line, AR4-2J[11]. Afterwards, Hollande *et al*[12] have described the involvement of the phosphatidylinositol 3-kinase in adhesion and migration of gastric epithelial cells regulated by G-gly. More recently, we described the overexpression of the regulatory subunit p85 as well as an overactivation of the downstream effector Akt, in the hyperproliferative colonic mucosal epithelium of MTI/G-Gly mice[13]. Moreover, in the same study, we have demonstrated the involvement of the PI3K pathway as well as Src and JAK2 pathways in the proliferation of isolated normal murine colonic epithelial cells in response to G-gly.

Our aim here was to investigate whether these three kinases are involved in G-gly-induced proliferation of human colonic tumour cells and to elucidate the molecular interaction between Src, JAK2 and PI3K in response to the peptide. In HCT116, a human colon tumour cell line, we first measured the activation of these kinases by G-gly as well as their involvement in G-gly-induced cells proliferation. The results indicate that both p60-Src and JAK2 are necessary to PI3K regulation by the peptide. However, we did not observe any cross-talk between the two tyrosine kinases, suggesting that the p60-Src/PI3K and JAK2/PI3K pathways act independently.

MATERIALS AND METHODS

Materials

Polyclonal anti-JAK2 antibody was purchased from Upstate Biotechnology Inc. Monoclonal anti-p60-Src antibody was obtained from Oncogene Science. Rabbit polyclonal antibody specific to p85, the regulatory subunit of the phosphatidylinositol 3-kinase, was kindly provided by Drs. Y. Le Marchand-Brustel and J. F. Tanti (Nice, France). DFO, LY 294002, PP2 and AG490 were from Calbiochem. [γ-32P] ATP (7000 Ci/mmol) was from ICN. Phosphatidylinositol was purchased from Sigma.

Cell culture

HCT116, human colonic tumour cells, were grown in DMEM containing 4.5g/L glucose-glutamax, supplemented with 10% fetal calf serum at 37 ℃ in a 50mL/L CO_2 atmosphere.

Proliferation assay

Approximately 75 000 cells/well were plated into 96-well plates. Forty-eight hours after plating, cells were treated for 48 h with G-gly (10 pmol/L) with or without inhibitors (10μmol/L). MTT colorimetric assay (MTT, Sigma) was used to measure proliferation as previously described[13].

Immunoprecipitation and Western-blot analysis

HCT116 cells were serum-starved for 18 h before peptide addition. After stimulation, the cells were lysed, and the soluble fractions containing identical levels of proteins were immunoprecipitated and analyzed by Western-blot with the indicated antibodies as described previously[14]. Band intensity was analyzed using the image analyzer Biocom (France).

Src kinase assay

Src kinase activities were determined on anti-p60-Src-immunoprecipitates as previously described[15]. Briefly, kinase assay was carried out at 37 ℃ for 10 min in kinase buffer (12.5 mmol/L MnCl2, 1.25 mmol/L DTT, 25 mmol/L Hepes, pH 7.4) containing 3.75 μmol/L ATP, 10 μCi/point ATP[γ-32P]. Proteins were separated by SDS–PAGE and the gel was autoradiographied. The gels were then treated with 1N KOH at 50 ℃ for 1h and autoradiographied. Band intensity was analyzed using the image analyzer Biocom (France).

JAK2 kinase assay

JAK2 kinase activities were determined on anti-JAK2-immunoprecipitates as previously described[14]. Briefly, kinase assay was carried out at 37 ℃ for 10 min in kinase buffer (10 mmol/L MnCl2, 5 mmol/L MgCl2, 0.1 mmol/L Vn2+, 10 mmol/L Tris, pH 7.4) containing 3.75 μmol/L ATP, 10 μCi/point ATP[γ-32P]. Proteins were separated by SDS–PAGE and the gel was autoradiographed. Band intensity was analyzed using the image analyzer Biocom (France).

Phosphatidylinositol 3-kinase assay

Cell lysates were immunoprecipitated with the indicated antibodies. PI3K assay was performed on immunoprecipitates as described previously[16]. Briefly, phosphatidylinositol used as an exogenous substrate, was prepared in 5 mmol/L HEPES and then sonicated for 15 min at 4 ℃. The samples were incubated for 15 min at room temperature with phosphatidylinositol (0.2 mg/mL) and 50 μmol/L ATP[γ-^{32}P] (10 μCi/point). The reaction was stopped by adding HCl 4N and lipids were extracted using agitation in chloroforme/methanol (1/1) solution for 45 s. After centrifugation, the upper phase containing the lipids was analyzed. The phospholipids were separated by thin layer chromatography and analyzed by autoradiography. Spot intensity was analyzed using the image analyzer Biocom (France). When indicated, the cells were pretreated for 1h with 1μmol/L desferrioxamine (DFO).

Statistical analysis

Statistical analysis was carried out by Student's *t* test using GraphPad Prism.

RESULTS

Glycine-extended gastrin activated phosphatidylinositol 3-kinase

We first tested whether the p85/p110 phosphatidylinositol 3-kinase (PI3K) was activated in HCT116 cells after G-gly stimulation. Cells were treated with the peptide for varying

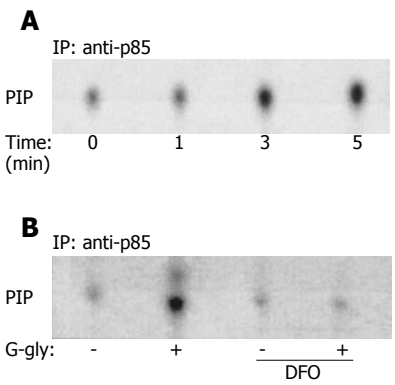

Figure 1 G-gly-induced PI3K (**A**) and G-gly (**B**) activation. HCT116 cells were stimulated with G-gly (10 pmol/L) for the indicated time. PI3K activity was determined in an immune complex kinase assay as described in Methods. The specificity of G-gly activation was accessed by pretreating the cells with ferric chelator DFO (1μmol/L). Representative autoradiograms from at least three separate experiments are shown.

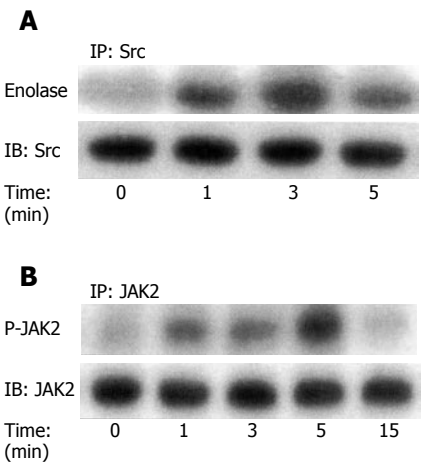

Figure 2 G-gly-induced p60-Src (**A**) and JAK2 (**B**) activation. HCT116 cells were stimulated with G-gly (10 pmol/L) for the indicated time. p60-Src and JAK2 activities were determined in an immune complex kinase assay as described in Methods. Immunoprecipitated proteins were also analysed by Western-blot using the anti-p60-Src and anti-JAK2 antibody respectively. Representative autoradiogram from at least three separate experiments is shown.

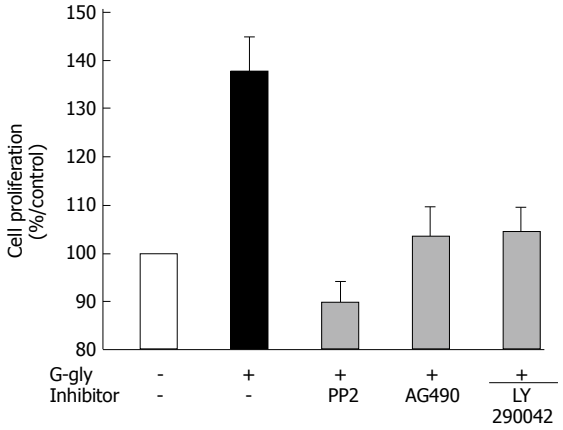

Figure 3 Role of PI3K, Src or JAK2 pathways in human colonic tumour cell proliferation induced by G-gly. Serum-starved HCT116 cells were treated with G-gly for 48h in the presence or absence of the indicated inhibitor (10 μmol/L). The proliferation was determined as described in Methods. Each point represents data from triplicate and is representative of 5 independent experiments. Data are presented as means ± SE.

lengths of time. Lipid kinase assay was performed *in vitro* on anti-p85 immunoprecipitates using inositol phosphate as an exogenous substrate. Lipids were then separated by thin layer chromatography. Increase in phosphatidylinositol phosphorylation was detected within 3-5 min after treatment with G-gly. This stimulation similar to our previous observation in pancreatic tumour cells[11], indicated that PI3K activation was also an early event of G-gly signalling in colon cancer cells. The ferric ion chelator, desferrioxamine (DFO) could inhibit both the binding of G-gly to its receptor and biological activity[17]. To test the specificity of the activation by G-gly, we performed PI3K assays after pretreatment of the cells with DF0 for 1h. DFO could completely inhibit PI3K activation in response to the peptide (Figure 1).

Glycine-extended gastrin activated tyrosine kinases p60-Src and JAK2

To investigate whether glycine-extended gastrin could regulate p60-Src activation, serum-starved HCT116 cells were treated with G-gly for various times and lysed. Tyrosine kinase assays were performed in anti-p60-Src immunoprecipitates using enolase as an exogenous substrate. Our results indicated that p60-src was very rapidly and transiently activated in HCT116 cells in response to G-gly. The activation of p60-Src was detected 1 min after peptide addition. The stimulation was maximal at 3 min and decreased after 5 min (Figure 2A).

To test whether JAK2 was activated by G-gly, lysates from cells stimulated for various times were immunoprecipitated with anti-JAK2 antibodies and *in vitro* kinase assays were performed on immunoprecipitates as described in "Methods". We observed a rapid and transient increase in JAK2 autophosphorylation in response to G-gly stimulation. The maximal activation obtained within 5 min after peptide addition decreased toward the basal level at 15 min (Figure 2B).

Involvement of JAK2, Src and phosphatidylinositol 3-kinase pathways in glycine-extended gastrin-induced proliferation

In order to identify the role of PI3K, Src and JAK2 pathways in the proliferation of human colonic tumour cells induced by G-gly, we measured HCT116 proliferation in the presence or absence of specific inhibitors for each pathway, LY290042, PP2 and AG490 respectively. Stimulation of HCT116 cells for 48h by G-gly induced a significant increase of cell proliferation (mean ± SE; 1.38 ± 0.07; $P<0.05$; $n=5$) (Figure 3). Treatment of the cells with PP2, AG 490 or LY 290042 completely inhibited G-gly-induced HCT116 proliferation while inhibitors alone did not affect basal cell proliferation (data not shown). The result indicated that these three pathways could mediate G-gly proliferative effects not only on normal colonic epithelial cells but also on human colonic tumoral cells.

Involvement of both p60-Src and JAK2 upstream of PI3K activation in response to G-gly

In order to determine whether p60-Src was involved in PI3K activation by G-gly, we first tested whether PI3K activity was detected in association with p60-Src. After peptide treatment for various times, PI3K assays were carried out on anti-p60-Src immunoprecipitates. In response to G-gly, we observed an increased PI-kinase activity co-precipitated with maximal p60-Src after 5 min of peptide stimulation (Figure 4A). To test the PI3K specificity, we

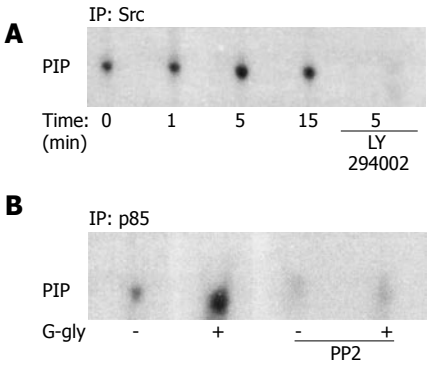

Figure 4 Involvement of p60-Src (**A**) and PI3K (**B**) activation in response to G-gly. HCT116 cells pretreated with or without 10 μmol/L of LY290042 and anti-p85 antibody were stimulated (+) or not (-) with glycine-extended gastrin (10 pmol/L) for the indicated time. After immunoprecipitation of cell lysates with anti-p60-Src and anti-p85 antibody, precipitates were assayed for PIK activity using inositol phosphate as an exogenous substrate. Lipids were separated by thin layer chromatography and autoradiographied. The result shown is representative from five and three separate experiments.

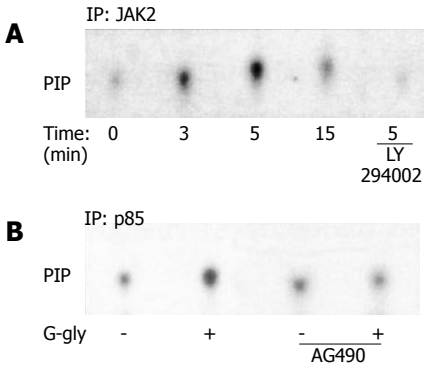

Figure 5 Involvement of PI3K (**A**) and JAK2 (**B**) activation in response to G-gly. HCT116 cells pretreated with or without 10μmol/L of LY290042 and were 10μmol/L of AG 40, stimulated (+) or not (-) with glycine-extended gastrin G-gly (10 pmol/L) for the indicated time. After immunoprecipitation of cell lysates with anti-JAK2 and anti-p85 antibody, precipitates were assayed for PIK activity using inositol phosphate as an exogenous substrate. Lipids were separated by thin layer chromatography and autoradiographied. The result shown is representative from five separate experiments.

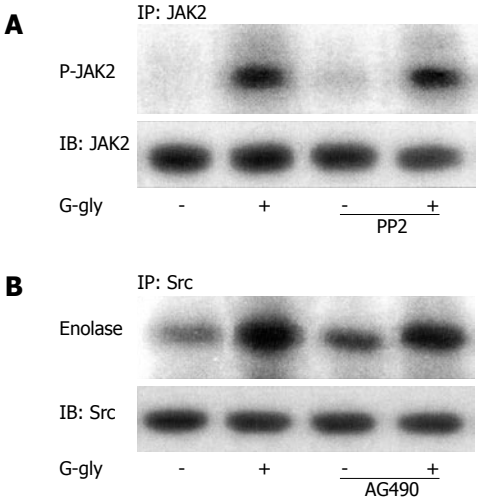

Figure 6 No involvement of p60-Src (**A**) and JAK2 (**B**) activation in response to G-gly. Cells pretreated with or without PP2 and AG 490 (10 μmol/L), were stimulated (+) or not (-) by G-gly (10 pmol/L) for 5 min. JAK2 and p60-Src kinase activities were determined by immune complex kinase assay using anti-JAK2 and anti-p60-Src antibodies as described in Materials and Methods. Proteins were separated by SDS-PAGE and JAK2 autophosphorylation was detected by autoradiography. A representative autoradiogram from at least three separate experiments is shown.

added the PI3K inhibitor, LY 294002, in the *in vitro* lipid kinase assay. Under these conditions, G-gly induced-PI-kinase activity was completely blocked. These results demonstrated that glycine-extended gastrin was associated with p60-Src and PI3K. In order to confirm that an Src family member could act upstream of the PI3K pathway activated by G-gly, we pretreated the cells for 1h with PP2 before G-gly stimulation. PI3K activation was inhibited in PP2 pretreated cells, indicating the role of Src in PI3K activation (Figure 4B).

We then examined the role of JAK2 in PI3K activation by G-gly. After peptide treatment for the time indicated, the cells were lysed and PI3K assays were performed on anti-JAK2 immunoprecipitates. Increased PI-kinase activity was associated with JAK2, detectable at 1 min and maximal after 5 min of peptide stimulation (Figure 5A). The inhibition of PI-kinase activity observed when LY 294002 was added in the *in vitro* lipid kinase assay indicated that glycine-extended gastrin was associated with JAK2 and PI3K. To confirm the involvement of JAK2 upstream of the G-gly-induced PI3K pathway, the cells were pretreated for 1h with AG 490 prior to G-gly addition. The PI3K activation in response to G-gly was inhibited in the presence of JAK2 inhibitor, indicating the role of this tyrosine kinase upstream in PI3K activation (Figure 5B).

No cross-talk between p60-Src and JAK2 pathways in response to G-gly

To investigate the possible association between p60-Src and JAK2, solubilized proteins were immunoprecipitated with an anti-JAK2 antibody and the precipitates were analyzed by immunoblot using an anti-p60-Src antibody. No p60-Src protein associated with JAK2 was detected in control cells or in cells stimulated with G-gly, whereas comparable amounts of JAK2 proteins were detected in immunoprecipitates (data not shown).

Independently of any association, we studied whether Src-family kinases were involved in JAK2 activation by G-gly. We tested the effect of PP2 on JAK2 activity. Cells were pretreated for 1h with PP2 5 min before stimulation with G-gly. JAK2 kinase assay was performed on JAK2 immunoprecipitates. PP2 did not inhibit G-gly-induced JAK2 activation, indicating that Src kinases were not involved in upstream of JAK2 activation by G-gly (Figure 6A). Similarly, using AG490, we tested a possible role of JAK2 upstream in p60-Src activation. The presence of AG490 in the incubation medium did not significantly change p60-Src activation by G-gly (Figure 6B). Indeed, in this human colon tumoral cell line, activation of the two tyrosine-kinases, p60-Src and JAK2, in response to G-gly was entirely independent.

DISCUSSION

The p85/p110 phosphatidylinositol 3-kinase (PI3K) is a

lipid kinase which plays an important role in human colon cancer. Its specific inhibitor, LY 294002, has been shown to inhibit cell growth and to induce apoptosis. Treatment of human colon cancer cell lines *in vitro* with LY 294002 or transplanted *in vivo* abolishes tumour cell growth and leads to apoptosis[9]. In addition, Philp *et al*[18] have identified somatic mutations in the gene of the p85 subunit leading to a constitutive active form of PI3K. These mutations are present in primary human colon tumours and cancer cells, suggesting that PI3K is involved in human colonic tumorigenesis

Non-amidated gastrin precursors, including G-gly, are produced by colorectal cancers and exert growth factor effects on these tissues[19, 20]. They are expressed in 80% - 90% of colorectal tumours and polyps in human beings[21-24]. Identification of signalling pathways involved in colonic mucosa hyperproliferation is important for the understanding of tumour processes. In a previous study we have identified that Src, JAK2 and PI3K pathways are overactivated in the colonic epithelium of mice overexpressing G-gly and involved in G-gly-induced proliferation of normal colonic epithelial cells isolated from control mice[13].

In the current study, we used the human colon tumour cell line, HCT116, to investigate whether these three kinases interact to regulate the growth of human tumoral colon cells. The results indicate that both p60-Src and JAK2 are necessary to PI3K regulation by the peptide. Theses results are in accordance with previous reports showing that JAK2 are involved upstream of the PI3K pathway of other cellular models. Pretreatment of human neutrophils with the JAK2 inhibitor, AG 490 could abolish the stimulation of the p85/p110 PI3K in response of the granulocyte-macrophage colony-stimulating factor. In addition, Attoub *et al*[25] have shown that leptin can induce a transient elevation of the PI3K lipid products in JAK2 immunoprecipitates prepared from MDCK cells. Similarly, Src family kinases are involved upstream of the PI3K/AKT pathway[26, 27]. Previous studies suggest that the IRS proteins could serve as scaffolding intermediates between tyrosine kinases and PI3K[28]. This mechanism is likely involved in PI3K activation by G-gly. In HCT116 cells we observed a basal association between JAK2 and IRS1 and the tyrosine phosphorylation of IRS-1 in response to G-gly (data not shown). In addition, we have previously reported that tyrosine phosphorylation of IRS-1 in pancreatic tumour cells, leading to the rapid recruitment of the regulatory subunit of PI3K, might represent a common mechanism for PI3K activation by different factors including insulin and G-gly[11]. Obviously, we cannot exclude other hypotheses such as interaction between the SH2 domain of p85 and phospho-tyrosine of p60-Src or JAK2. Different studies have previously described the involvement of Src family kinases upstream of JAK2 activation. For example, cell transformation by the oncogene v-Src[29] or the overexpression of certain members of the Src family-kinases as Lck[30] can lead to a constitutive activation of JAK2. However, we did not find any cross-talk between the two tyrosine kinases, suggesting that the p60-Src/PI3K and JAK2/PI3K pathways act independently. The involvement of two different mechanisms upstream of the lipid kinase might be a way to amplify the PI3K signal. However, in our study, the level of PI3K activity associated with JAK2 or p60-Src was not different from that observed in anti-p85 immunoprecipitates. As for many other signalling molecules, it seems that the cellular localization of PI3K is important for the function of the enzyme. Therefore the recruitment of PI3K to different cell compartments by the two independent tyrosine-kinases allows the enzyme to play a complementary role. The finding that p60-Src and JAK2 do not associate in response to G-gly stimulation, demonstrates that the p60-Src/PI3K and JAK2/PI3K pathways act independently and indicates that they might be involved in different cell compartments. The analysis of the cellular localization of phosphatidylinositol 3-kinase, might be crucial to understand the role of this signalling molecule in different biological effects of G-gly.

Our previous studies indicate that gastrin precursors contribute to the initiation phases of colon carcinogenesis by upregulating the signalling pathways involved in cellular proliferation and cell survival. The results of this study demonstrate that G-gly is also able to regulate proliferation of tumoral colonic cells and support the idea that gastrin precursors are not only involved in the initiation steps of the colon tumour process but also involved in the later stages of tumour progression after genetic alterations, by conferring a growth advantage to the cells *via* the constitutive activation of numerous signalling pathways.

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Expression of cytokeratins in *Helicobacter pylori* –associated chronic gastritis of adult patients infected with *cagA*+ strains: An immunohistochemical study

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Abstract

AIM: To investigate the expression of different cytokeratins (CKs) in gastric epithelium of adult patients with chronic gastritis infected with *Helicobacter pylori* (*H pylori*) *cagA*+ strains.

METHODS: The expression of CK 7, 8, 18, 19 and 20 was studied immunohistochemically in antral gastric biopsies of 84 patients. All the CKs were immunostained in *cagA*+*H pylori* gastritis (57 cases), non-*H pylori* gastritis (17 cases) and normal gastric mucosa (10 cases).

RESULTS: In *cagA*+ *H pylori* gastritis, CK8 was expressed comparably to the normal antral mucosa from surface epithelium to deep glands. Distribution of CK18 and CK 19 was unchanged, i.e. transmucosal, but intensity of the expression was different in foveolar region in comparison to normal gastric mucosa. Cytokeratin 18 immunoreactivity was significantly higher in the foveolar epithelium of *H pylori*-positive gastritis compared to both *H pylori*-negative gastritis and controls. On the contrary, decrease in CK19 immunoreactivity occurred in foveolar epithelium of *H pylori*-positive gastritis. In both normal and inflamed antral mucosa without *H pylori* infection, CK20 was expressed strongly/moderately and homogenously in surface epithelium and upper foveolar region, but in *H pylori* -induced gastritis significant decrease of expression in foveolar region was noted. Generally, in both normal antral mucosa and *H pylori*-negative gastritis, expression of CK7 was not observed, while in about half *cagA*+ *H pylori*-infected patients, moderate focal CK7 immunoreactivity of the neck and coiled gland areas was registered, especially in areas with more severe inflammatory infiltrate.

CONCLUSION: Alterations in expression of CK 7, 18, 19 and 20 together with normal expression of CK8 occur in antral mucosa of *H pylori*-associated chronic gastritis in adult patients infected with *cagA*+ strains. Alterations in different cytokeratins expression might contribute to weakening of epithelial tight junctions observed in *H pylori*-infected gastric mucosa.

Key words: cytokeratin 7; cytokeratin 8; cytokeratin18; cytokeratin19; cytokeratin20; *Helicobacter pylori*; Gastritis; *CagA*

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INTRODUCTION

Cytokeratins (CKs), a family of important cytoskeleton structural proteins, have specific spatial and temporal dynamic locations along the epithelial axis of the gastrointestinal tract (GIT), and their expression is linked to the degree of epithelial differentiation[1-4]. Cytokeratins 7, 8 (intermediate-sized and basic), 18 and 19 (smallest in size and acidic) are exclusively expressed in nearly all simple epithelia, pseudostratified respiratory epithelium and

transitional epithelium. CK8 and CK18 pair together and have a similar distribution, while CK19 can be detected in a broad range of epithelial tissues, including many simple epithelia, diverse stratified epithelia, and cultured keratinocytes. CK20 (intermediate sized and acidic) is expressed in gastric foveolar epithelium, intestinal villi and crypt epithelium, cutaneous and oral Merkel cells[4]. Various changes in CK expression and distribution profile have been noted in inflammatory[5], preneoplastic[6-10] and neoplastic[2,11-13] disorders along GIT, including gastric mucosa. Structural changes in the gastric epithelium of adult and pediatric *H pylori*-infected patients with chronic gastritis have been recently demonstrated using cytokeratin immunohistochemistry[14-19].

Results of previous studies postulated that only a subset of individuals infected with *H pylori* develop severe gastritis and/or metaplasia, peptic ulcer or gastric cancer [14,20]. Bacterial strain, environmental and host factors can converge in the gastroduodenal milieu and control the final outcome of *H pylori* infection. However, to the best of our knowledge, relationship between *cagA*+ *H pylori* and changes in CK expression in the gastric epithelium has not yet been studied in patients with chronic gastritis. Since we have previously demonstrated high seroprevalence of antibodies to *cagA* in *H pylori*-infected patients in Serbia and Montenegro[21], this study aimed to identify and describe immunohistochemical pattern of antral CK expression in *H pylori* –associated chronic gastritis of adult patients infected with *cagA*+ strains.

MATERIALS AND METHODS

Subjects

We conducted an outpatient-based prospective study at the Clinic for Gastroenterology and Hepatology (Clinical Center of Serbia, University of Belgrade). All patients gave informed consent for participation in the study and the study protocol was approved by the local Ethics Committee. Adult patients with dyspeptic symptoms and histological signs of gastritis entered the study. Dyspepsia was defined as upper abdominal or retrosternal pain, discomfort, nausea, vomiting or other symptoms referable to the upper abdominal tract lasting for at least one month[22]. Exclusion criteria were in concordance with the recommendations from European *H pylori* Study Group[20].

Ninety-one patients entered the study. After histological examination, we excluded 17 patients (15 with and 2 without *H pylori* infection) due to the presence of intestinal metaplasia (IM) in the antral mucosa. Since specific IM- related changes in CK expression were reported, we aimed to identify changes related exclusively to the presence of *H pylori* infection. Therefore, data from 74 patients (57 *H pylori*-positive and 17 *H pylori*-negative) with chronic gastritis were analyzed. Control group (CG) consisted of 10 asymptomatic healthy volunteers with no histological changes in the gastric mucosa (mean age 32± 11 years, 3 males, 7 females). In the *H pylori*-positive group mean age was 44±13 years (26 males, 31 females), while in the non-infected group mean age was 47±17 years (5 males, 12 females). Esophagogastroduodenoscopy with biopsies from antral and corpus mucosa was performed in all patients and blood was taken for serology and immunoblot.

H pylori infection was diagnosed by rapid urease test (RUT), histology, immunohistochemistry and serology. A patient was defined as *H pylori* positive if histology and at least one of the other applied diagnostic methods were positive.

Routine endoscopy and H pylori status

Each patient underwent upper endoscopy and testing for the presence of *H pylori* by RUT and histology. Six biopsies were taken, 3 from the antrum and 3 from the corpus (2 for histology and 1 for RUT). Biopsies selected for histological examination were stained with hematoxylin-eosin, alcian blue pH 2.5/periodic acid Schiff (AB/PAS) and high iron diamine/alcian blue pH2.5 (HID/PAS). Both Giemsa and immunohistochemical stainings (polyclonal antibody to *H pylori*, dilution 1:10, DAKO A/S, Glostrup, Denmark) were applied for the detection of *H pylori*.

H pylori serology and immunoblot assay

Blood samples were taken from all patients after endoscopic examination and sera were separated by centrifugation and stored at -20 °C until analyzed. The presence of anti-*cagA* antibodies was detected using the *Helicobacter pylori* Vira blot test kit™ (Viramed Biotech AG, Lich, Germany). The concentration of anti-*H pylori* IgG antibodies was analyzed using Pyloriset EIA-G III™ (Orion Diagnostica, Finland)[23]. Both tests were performed according to the manufacturer's instructions.

Immunohistochemistry

Immunohistochemical reactions were performed on consecutive sections of one selected antral biopsy of each patient to detect cytokeratins 7, 8, 18, 19 and 20. Only well-oriented biopsies allowing assessment of full mucosal thickness, were selected for immunohistochemical study. The sections were stained with monoclonal antibodies to CK7 (dilution 1:25, DAKO A/S, Glostrup, Denmark), CK8 (dilution 1:20, DAKO Carpinteria, CA, USA), CK18 (dilution 1:25, DAKO A/S, Glostrup, Denmark), CK19 (dilution 1:50, DAKO A/S, Glostrup, Denmark) and CK20 (dilution 1:25, DAKO A/S, Glostrup, Denmark). Immunohistochemical staining was performed according to the manufacturer's instructions using streptavidin-biotin/HRP detection system (DAKO LSAB+/HRP kit, Glostrup, Denmark), followed by counterstaining with hematoxylin. For negative controls, no staining was detectable when the pre-immune serum was used instead of primary antibodies. In addition, human fetal esophagus of the 13th gestation week was used as positive control for evaluation of CK7 immunoreactivity.

Histological and immunohistochemical evaluation

Mucosal biopsies were evaluated by an experienced pathologist blinded to clinical and endoscopic data. In addition, an experienced gastrointestinal pathologist and a cytologist independently evaluated immunohistochemistry. Differences in immunohistochemical evaluation were resolved by re-examination and consensus. Chronic gastritis was diagnosed and graded according to the

Table 1 Antral histology according to Sydney classification of gastritis in patients with and without *Helicobacter pylori* infection

Antral mucosa (Sydney classification)	*H pylori* + (*n* = 57)	*H pylori*- (*n* = 17)	*P*
Inflammatory infiltrate			
0	0	1	0.000
1	19	14	
2	31	2	
3	7	0	
Activity of inflammation			
0	2	9	0.000
1	39	6	
2	15	2	
3	1	0	
Atrophy			
0	45	13	NS
1	12	4	
H pylori-colonization density			
0	0	17	
1	19	0	
2	25	0	
3	13	0	

H pylori+: *Helicobacter pylori* positive patients; *H pylori* -: *Helicobacter pylori* negative patients; NS: $P > 0.05$.

updated Sydney system[24]. Biopsies showing intestinal metaplasia classified according to previous proposals [13] were not included in this study. Mucosal distribution (surface epithelium, foveolar region, glandular necks and deep glands) and intensity of cytoplasm staining (without staining, weak or moderate/strong staining) were registered together with the expression pattern (focal or diffuse) for each analyzed CK, while an additional semi-quantitative assessment of percentages of immunoreactive cells in each mucosal compartment was performed and graded for CK7 in 3 groups (<10%, 10-20%, and >20% immunoreactive cells).

Statistical analysis

Results were analyzed using non-parametric tests, Kruskall-Wallis, chi-square or Fisher's exact test for independent samples. $P < 0.05$ was considered statistically significant.

RESULTS

Results of our study showed that presence of intestinal metaplasia could be detected in 18.6% (17/91) of dyspeptic patients with histological signs of gastritis (15 with and 2 without *H pylori* infection). These patients were excluded from further analysis, since we aimed to investigate the influence of *H pylori* infection on normal gastric epithelium. Out of the remaining 74 patients, 57 (77%) were infected with *H pylori,* while infection was not found in 17. All infected patients harbored *cagA*+ bacterial strain in the gastric mucosa.

Histology evaluation

Histological examination of antral and corpus mucosa in *H pylori*-infected individuals revealed signs of antral gastritis in 5 (9%), while pangastritis was diagnosed in the remaining 52 (91%) patients. In the *H pylori* negative group antral gastritis was diagnosed in 7 (42%) patients and histological signs of pangastritis were found in 10 (58%). Histological changes in the gastric mucosa were graded using Sydney classification both for antral (Table 1) and corpus mucosa (data not shown).

In the antrum of *H pylori*-infected individuals, inflammatory infiltrate density and activity of inflammation were higher than in the uninfected group ($P < 0.001$ for both histological parameters). Presence of atrophic changes was not different between the two groups, while moderate density of *H pylori*-colonization was most frequent in infected patients.

Immunohistochemical evaluation of CK expression and distribution

Cytokeratin 8 was identified immunohistochemically in antral mucosa of both *H pylori* positive and negative patients with gastritis and controls (Table 2). Diffuse immunoreactivity to CK8 was the predominant expression pattern in surface epithelium, foveolae and glandular neck (moderate/strong immunoreactivty was observed in 90% of controls, 70.6% of *H pylori* negative and 63.1% of *H pylori*+ gastritis and weak in only 11.8% with and 12.3% without *H pylori* infection). On the other hand, deeper glandular structures did not express CK8 in about 10-30% of cases. No significant difference was found in any of the examined antral mucosa regions between the three groups (Figure 1).

Normal antral mucosa was in general immunostained transmucosal (from the surface to the gland region) when antibodies to CK18 and CK19 were applied (Tables 3 and 4), while in foveolar region inconsistent CK18 immunoreactivity with the expression rate of only 10% was observed. In the foveolar epithelium of *H pylori*+ patients more intense diffuse ($P < 0.05$) and focal ($P < 0.01$) CK18 immunoreactivity was detected when compared to *H pylori*-negative gastritis and controls (Figure 2). As opposed to these results, lower antral CK19 patchy immunoreactivity was noted in foveolar epithelium of *H pylori*+ gastritis compared to *H pylori*-negative gastritis and controls ($P < 0.05$) (Figure 3).

All examined antral biopsies showed positive immunostaining of CK20 (Table 5) restricted to the surface and upper foveolar epithelium. In the surface epithelium strong and homogenous CK20 immunoreactivity was predominant (moderate/strong diffuse immunoreactivity was observed in 90% of controls, 70.6% of *H pylori* negative and 64.9% of *H pylori*+ gastritis, as opposed to the patchy staining observed in 10%, 29.4% and 35.1% of individuals, respectively). No significant differences were noted between controls and patients with gastritis irrespective of the presence of *H pylori* infection. In the antral mucosa of the majority of *H pylori* negative patients with gastritis and controls CK20 was expressed strongly/moderately in all foveolar cells while in *H pylori* positive gastritis patients a significantly higher percentage of patients had focal expression of CK20 (Figure 4). Further analysis revealed different CK20 expressions in upper foveolar region related to the presence of gastritis but not

Table 2 Expression of CK8 in antral mucosa of patients with gastritis (*H pylori* positive and negative) compared to the control group

Cytokeratin 8 expression	Antral mucosa epithelium						
	H pylori+G		*H pylori*-G		CG		*P*
	n	%	*n*	%	*n*	%	
	Surface epithelium, foveolae and glandular necks						
Diffuse immunoreactivity	43	75.4	14	82.4	9	90	NS
Focal immunoreactivity	14	24.6	3	17.6	1	10	NS
Without staining	0	0	0	0	0	0	NS
n (%)	57	100	17	100	10	100	
	Deep glands						
Diffuse immunoreactivity	24	42.1	11	64.7	5	50	NS
Focal immunoreactivity	15	26.3	2	11.8	4	40	NS
Without staining	18	31.6	4	23.5	1	10	NS
n (%)	57	100	17	100	10	100	

CG: control group; *H pylori*+G: *H pylori* positive patients with gastritis; *H pylori*-G -: *H pylori* negative patients with gastritis; NS: $P > 0.05$

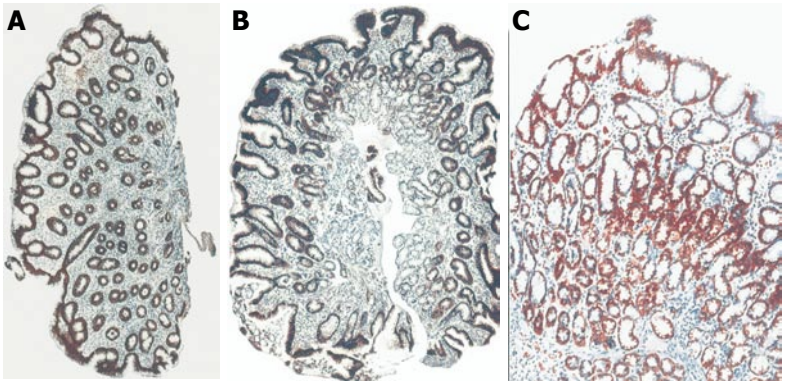

Figure 1 CK8 immunoreactivity in antral mucosa of patients with cagA+ *H pylori* chronic gastritis (**A**), non-*H pylori* chronic gastritis (**B**) and control subjects with normal gastric mucosa (**C**) with differences of CK8 immunoreactivity in antral mucosa. Original magnification: x10 (**A**,**C**); x20 (**B**).

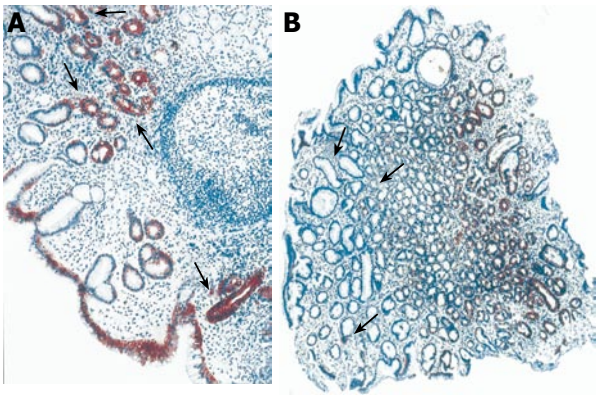

Figure 2 CK18 immunoreactivity in antral mucosa of patients with cagA+ *H pylori* chronic gastritis (**A**) and control subjects without gastritis (**B**). Original magnification: x10. Cytokeratin 18 immunoreactivity was significantly higher in the foveolar epithelium of *H pylori*-positive gastritis compared with *H pylori*-negative gastritis.

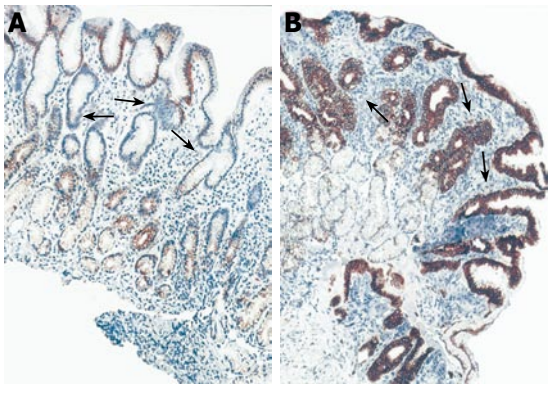

Figure 3 CK19 immunoreactivity in antral mucosa of patients with cagA+ *H pylori* chronic gastritis (**A**) and non-*H pylori* chronic gastritis (**B**). Decrease in CK19 immunoreactivity was found in foveolar epithelium of antral mucosa of *H pylori*-positive gastritis compared to non-*H pylori* chronic gastritis. Original magnification: x20 (**A**, **B**)

H pylori infection. Namely, patchy staining pattern was detected in 44% of *H pylori*+ and 29% of *H pylori* negative gastritis patients and only 10% of controls ($P < 0.05$).

The main findings of CK7 immunoreactivity are listed in Table 6. In both controls and *H pylori*-negative gastritis patients almost no immunoreactivity of CK7 was found in the antral mucosa, with the exception of some inconsistent and faint CK7 immunoreactivity observed in single cells of glandular necks and deep glands in about 2/3 of cases (Figure 5). However, moderate focal CK7 immunoreactivity in the same area was more frequently observed in *H pylori*-positive gastritis compared to both *H pylori*-negative gastritis and control group ($P < 0.01$). Namely, about half in *H pylori*-infected patients (28/55,

Table 3 Expression of CK18 in antral mucosa of patients with gastritis (*H pylori* positive and negative) compared to the control group

Cytokeratin 18 expression	Antral mucosa epithelium						
	H pylori+G		*H pylori*-G		CG		*P*
	n	%	*n*	%	*n*	%	
				Surface epithelium			
Diffuse immunoreactivity	32	56.1	14	82.3	5	50.0	NS
Focal immunoreactivity	24	42.1	2	11.8	3	30.0	NS
Without staining	1	**1.7**	1	**5.9**	2	**20.0**	**0.043**
n (%)	57	100	17	100	10	100	
				Foveolar epithelium			
Diffuse immunoreactivity	**31**	**54.4**	**8**	**47.1**	**1**	**10**	**0.035**
Moderate/Strong	30	52.7	8	47.1	1	10	**0.045**
Weak	1	1.7	0	0	0	0	NS
Focal immunoreactivity	**20**	**35.1**	**1**	**5.8**	**0**	**0**	**0.008**
Moderate/Strong	**5**	8.8	0	0	0	0	NS
Weak	15	26.3	1	5.8	0	0	0.045
Without staining	**6**	**10.5**	**8**	**47.1**	**9**	**90**	**<0.001**
***n* (%)**	**57**	**100**	**17**	**100**	**10**	**100**	
				Glandular necks			
Diffuse immunoreactivity	36	63.2	13	76.5	6	60	NS
Focal immunoreactivity	18	**31.6**	2	11.8	2	20	NS
Without staining	3	5.2	1	5.9	2	20	NS
n (%)	57	100	17	100	10	100	
				Deep glands			
Diffuse immunoreactivity	**15**	**26.3**	**8**	**47.0**	**4**	**40**	NS
Moderate/Strong	15	26.3	5	29.4	3	30	NS
Weak	0	**0**	3	**17.6**	1	**10**	**0.008**
Focal immunoreactivity	**18**	**31.6**	**3**	**17.7**	**4**	**40**	NS
Without staining	**24**	**42.1**	**6**	**10.3**	**2**	**20**	NS
***n* (%)**	**57**	**100**	**17**	**100**	**10**	**100**	

CG:control group; *H pylori*+G -:*Helicobacter pylori* positive patients with gastritis; *H pylori*-G: *Helicobacter pylori* negative patients with gastritis; NS: $P > 0.05$

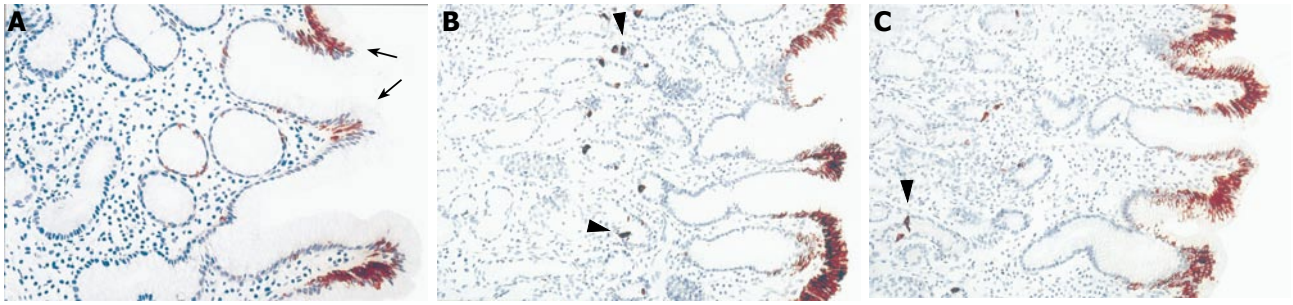

Figure 4 CK20 immunoreactivity in antral mucosa of patients with cagA+ *H pylori* chronic gastritis (**A**), non-*H pylori* chronic gastritis (**B**) and control subjects with normal gastric mucosa (**C**). In the cagA+ *H pylori* chronic gastritis, CK20 expression was decreased in upper foveolar epithelium in comparison to non-*H pylori* chronic gastritis and control subjects with normal gastric mucosa. Numerous CK20 strong immunoreactive endocrine cells could be observed (►) in the glandular epithelium. Original magnification: x20 (A-C)

50.1%) moderate focal immunoreactivity of CK7 in more than 10% of cells in neck and coiled gland areas was registered, especially in individuals with more severe inflammatory infiltrate (Figure 6).

DISCUSSION

H pylori-induced proinflammatory cytokine expression and inflammation affects various gastric cell populations controlling gastric acid secretion[25], including somatostatin-producing D cells[26], gastrin-producing G cells[27], and parietal cells[28]. In addition, in the course of *H pylori*-induced gastritis, acute foveolitis of the pit proliferative zone with characteristic morphological change of epithelial cells, which is often described as the malgun (clear) cell change[29] occurs. It is postulated that malgun cell change in the course of *H pylori* gastritis reflects epithelial genomic damage and repair processes. In addition, various structural changes of mucosal epithelial cells in *H pylori* gastritis have been reported in both adult[14-16, 19] and pediatric patients[18].

Strain-specific *H pylori* gene, *cagA*, has been recognized as a marker of strains that confer increased risk for peptic

Table 4 Expression of CK19 in antral mucosa of patients with gastritis (*H pylori* positive and negative) compared to the control group

Cytokeratin 19 expression	Antral mucosa epithelium						*P*
	H pylori+G		*H pylori*-G		CG		
	n	%	*n*	%	*n*	%	
Surface epithelium							
Expression in all cells	34	60.6	12	70.6	5	55.6	NS
Patchy staining	21	37.4	5	29.4	4	44.4	
Without staining	1	2.0	0	0	0	0	
***n* (%)**	56	100	17	100	9	100	
Foveolar epithelium							
Expression in all cells	**39**	**69.7**	**9**	**53.0**	**5**	**55.6**	NS
Patchy staining	**7**	**12.5**	**5**	**29.4**	**4**	**44.4**	**0.027**
Moderate/Strong	2	3.6	2	11.8	2	22.2	
Weak	5	8.9	3	17.6	2	22.2	
Without staining	**10**	**17.8**	**3**	**17.6**	**0**	**0**	NS
***n* (%)**	**56**	**100**	**17**	**100**	**9**	**100**	
Glandular neck							
Expression in all cells	37	58.1	9	53	6	66.7	NS
Patchy staining	10	17.8	6	25.2	3	33.3	
Without staining	9	16.1	2	11.8	0	0	
***n* (%)**	56	100	17	100	9	100	
Deep gland							
Expression in all cells	22	39.2	6	35.2	3	33.3	NS
Patchy staining	10	17.8	5	29.5	2	22.2	
Without staining	24	43.0	6	35.3	4	44.5	
***n* (%)**	56	100	17	100	9	100	

CG: control group; *H pylori*+G: *Helicobacter pylori* positive patients with gastritis; *H pylori*-G: *Helicobacter pylori* negative patients with gastritis; NS: $P > 0.05$.

Table 5 Expression of CK20 in antral mucosa of patients with gastritis (*H pylori* positive and negative) compared to the control group

Cytokeratin 20 expression	Antral mucosa epithelium						*P*
	H pylori+G		*H pylori*-G		CG		
	n	%	*n*	%	*n*	%	
Surface epithelium							
Expression in all cells	37	64.9	12	70.6	9	90	
Patchy staining	20	35.1	5	29.4	1	10	NS
Without staining	0	0	0	0	0	0	
n (%)	57	100	17	100	10	100	
Upper foveolar region							
Expression in all cells	32	56.1	12	70.6	9	90	NS
Patchy staining	25	43.9	5	29.4	1	10	
Moderate/Strong	20	35.1	4	23.5	1	10	
Weak	5	8.8	1	5.9	0	0	0.044
Without staining	0	0	0	0	0	0	
n (%)	57	100	17	100	10	100	

CG:control group; *H pylori*+G: *Helicobacter pylori* positive patients with gastritis; *H pylori*-G: *Helicobacter pylori* negative patients with gastritis; NS: $P > 0.05$.

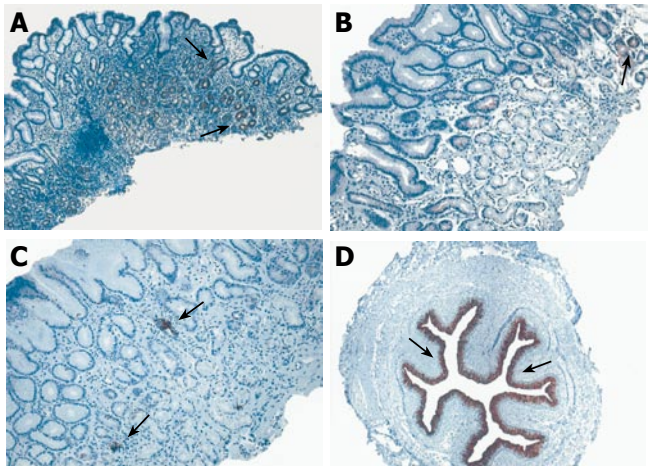

Figure 5 CK7 immunoreactivity in antral mucosa of patients with cagA+ *H pylori* chronic gastritis (**A**), non-*H pylori* chronic gastritis (**B**), control subjects with normal gastric mucosa (**C**), and control specimen of human fetal esophagus (**D**). Moderate focal CK7 positivity (→) in the pits and the glands of antral mucosa in patients with cagA+ *H pylori* chronic gastritis could be observed. CK7 positive cells covering a single gland (→) of antral mucosa could be found in patients with non-*H pylori* chronic gastritis. Normal antral mucosa with a few single cells or cell clusters (→) were strongly decorated with antibody to CK7. Strong CK7 immunoreactivity was displayed in the stratified columnar epithelium (→) of human fetal esophagus of the 13th gestation week. Original magnification: x10 (**A-D**).

ulcer disease and gastric cancer[25]. Since the seroprevalence of *cagA*+ *H pylori* strains was high in chronic gastritis patients of Serbia and Montenegro[21] and there is no evidence that bacterial strain is related to alterations in cytokeratin expression in gastric epithelium, we aimed in the current study to evaluate the changes in distribution and expression pattern of different CKs in the course of *cagA*+ *H pylori* -associated chronic gastritis in adult patients.

In normal antral gastric mucosa, results of our study revealed broad panmucosal (from the surface to the gland) expressions of CKs8,18 and 19, while CK20 immunoreactivity was strong and homogenous in the tip

Table 6 Expression of CK7 in antral mucosa of patients with gastritis (*H pylori* positive and negative) compared to the control group

Cytokeratin 7 expression	Antral mucosa epithelium						*P*
	H pylori+G		*H pylori*-G		CG		
	n	%	*n*	%	*n*	%	
	Surface and foveolar epithelium						
Weak focal immunoreactivity	0	0	0	0	0	0	NS
Moderate focal immunoreactivity	5	9.1	0	0	0	0	NS
<10% cells	0	0	0	0	0	0	
10-20% cells	3	5.5	0	0	0	0	
>20% cells	2	3.6	0	0	0	0	
Without staining	50	90.9	16	100	10	100	NS
n (%)	55	100	16	100	10	100	
	Glandular necks and deep glands						
Weak focal immunoreactivity	6	10.9	1	6.3	3	30.0	
<10% cells	3	5.5	1	6.3	3	30.0	0.01
10-20% cells	2	3.6	0	0	0	0	NS
>20% cells	1	1.8	0	0	0	0	NS
Moderate focal immunoreactivity	38	69.1	10	62.5	7	70.0	
<10% cells	10	18.3	10	62.5	7	70.0	0.002
10-20% cells	14	25.4	0	0	0	0	0.002
>20% cells	14	25.4	0	0	0	0	0.002
Without staining	11	20.0	5	31.2	0	0	
n (%)	55	100	16	100	10	100	

CG: control group; *H pylori*+G: *Helicobacter pylori* positive patients with gastritis; *H pylori*-G: *Helicobacter pylori* negative patients with gastritis; NS: $P > 0.05$.

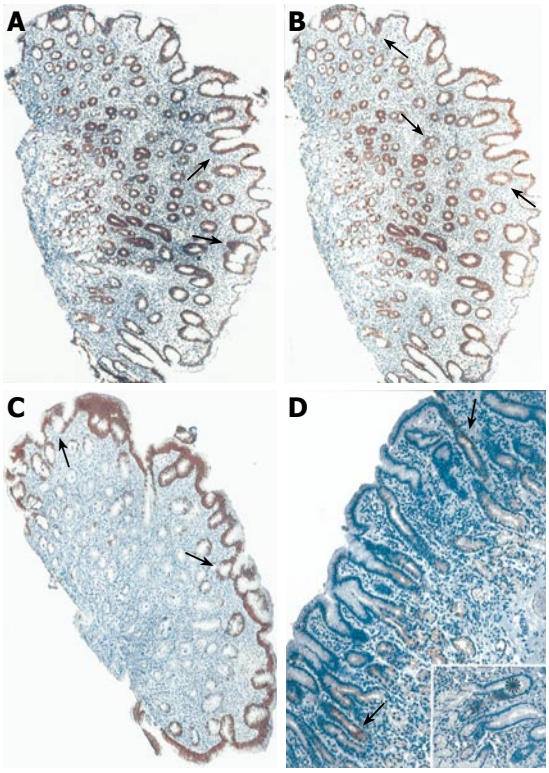

Figure 6 Typical expression pattern of CK18 (**A**), CK19 (**B**), CK20 (**C**) and CK7 (**D**) in antral mucosa of patients with cagA+ *H pylori* chronic gastritis. Strong diffuse cytokeratin 18, moderate focal CK19 and CK20 (**C**) immunoreactivities were demonstrated in upper foveolar epithelium (→) of the antral mucosa. Moderate focal CK7 positivity (→) was revealed in the pits and the glands of antral mucosa. CK7 immunoreactivity was prominent (*) in some cell clusters of the foveolar epithelium (insert). Original magnification: x10 (**A**-**C**), insert x20.

and upper portion cells of foveolae. As opposed to other examined cytokeratins, CK7 was mostly undetectable. Similar results were obtained for gastritis patients without *H pylori* infection and overall these findings are in line with previous reports[1,2,14,15,17].

Several immunohistochemical studies in the past few years demonstrated that altered gastric cytokeratin expression is closely related with *H pylori* infection in adult patients[1,2,14-17,19]. However only two studies by Schwerer *et al*[15] and Louwers *et al*[17] have investigated multiple CKs simultaneously. To the best of our knowledge, all other studies are focused on particular CK. None of these studies however have provided data concerning bacterial strain.

According to previous observations, CK7 is present in fetal but largely absent in normal adult and is transiently *de novo* expressed in metaplastic and neoplastic epithelial cells[2,11]. Our results suggest that CK7, largely absent in normal adult antral mucosa, is expressed in *H pylori* chronic gastritis patients, which is in line with reports in adults describing slight[17] and markedly increased CK7 expression in *H pylori*-associated chronic gastritis in children[18]. As opposed to these findings, a study by Schwerer and Baczako[14] when investigating CK7 expression in normal foveolar epithelium found that *H pylori* can induce gastritis and intestinal metaplasia with CK7 immunoreactivity detected only in intestinal metaplasia.

Study by Kirchner *et al*[2] using animal experimental model of gastritis-cancer sequence in Mongolian gerbils revealed signs of mild gastritis 2 mo after *H pylori* infection together with CK7 expression in epithelial cells of basal glands followed by loss of specific differentiation and changes to duct-like appearance, while after 6, 12 and 24 mo moderate to severe gastritis with loss of differentiated gastric glands and switch to CK7 positive duct-like structures in large mucosal segments was observed. These results may provide evidence that non-neoplastic stomach with non-atrophic *H pylori* gastritis constantly exhibits low score of CK7 positive cells in antrum and corpus, thus supporting our findings. Furthermore, there is evidence that CK7 expression in metaplastic and neoplastic stomach is related to dedifferentiated epithelial cells that can phenotypically be linked to fetal cells at the beginning of gastric pit development[2]. The dedifferentiated cells exhibit low proliferation and beta-catenin accumulation similar to stem cells. Therefore, observations of Kirchner *et al*[2] imply that metaplasia, gastric intraepithelial neoplasia, early gastric cancer and dedifferentiated epithelial cells defined by CK7 expression are related with each other in *H pylori* induced gastritis. Based on the above stated findings, we speculate that CK7 *de novo* expression in gastric mucosa of patients infected with *cagA*+ *H pylori* strains represents the proliferative/regenerative cells rather than pure dedifferentiated cells because CK7 positive flat duct-like

structures have not been identified.

Our results did not reveal any significant difference in CK8 expression between patients with *cagA*+ *H pylori*-induced gastritis and normal mucosa. These findings are supported by results of other authors[15,17], but not by study of Baek *et al*[19] that described under-expressed CK8 in the gastric mucosa of *H pylori* infected-individuals as a result of oxidative stress-induced cytoskeleton damage.

Higher expression of CK 18 in foveolar epithelium together with a decrease in CK19 expression was noted in patients with *cagA*+ *H pylori*-induced gastritis. These findings differ from previous studies, since two independent studies have revealed unchanged CK18 and CK19 expression in gastric mucosa of *H pylori*-infected patients[15,17]. Normal stomach expresses less CK19 in the upper mucosal compartment in comparison to other mucosal compartments[1]. CK19 expression is thought to be inversely related to cell proliferation, strong CK19 expression implying weak proliferation and vice versa[1]. Intestinal metaplasia of the stomach however exhibits more intense CK19-immunoreactivity than gastric cancer tissue[1]. If CK19 immunoreactivity is negatively correlated with cell proliferation and differentiation in fetal, normal and pathologically transformed adult gastric mucosa, our results may suggest good differentiation and enhanced proliferation of upper foveolar cells in patients with *cagA*+ *H pylori*-induced gastritis.

Botta *et al*[3] studied CK20 immunoreactivity in fetal and neonatal human gut, including stomach and demonstrated that CK20 expression is progressive increased during gestation, suggesting that the degree of CK20 positivity is related to the epithelial maturation stage in gastric mucosa. CK20 expression in adults is restricted to the surface foveolar epithelium and is not detectable in gastric pit or glandular region. Previous investigations revealed that CK20 expression is significantly lower in foveolar epithelium of *H pylori*-induced chronic gastritis[15-17] supporting our results, while available studies suggest that this is a reversible change and CK20 expression is normalized within 6 mo after eradication of *H pylori* infection[15].

Taken together, all these findings imply alterations in epithelial cell maturation in the course of *H pylori*-induced chronic gastritis.

Our results suggest that bacterial strain is of importance in inducing alterations of CK expression. It is well known that *cagA* present in 50-60% of all strains is a part of *H pylori* genome termed *cag* pathogenicity island (*cag*PAI) and that proteins encoded by *cag*PAI are responsible for both NFkB and MAPK activation in gastric epithelial cells. It has also been demonstrated that infection with *cagA*+ strains is more likely to result in peptic ulceration, atrophic gastritis and gastric carcinoma[25]. Therefore, presence of *cag*PAI in *H pylori* genome might play a role in signal transduction leading to *H pylori*-induced host gene expression, thus regulating inflammation, proliferation and carcinogenesis. In addition, bacterial strain-related differences in host gene expression have been reported, implying that protein expression profile including CKs, depends on bacterial strains and is related to the presence of *cagA*+ *H pylori* strains. Amieva *et al*.[31] reported that *cagA* appears to target *H pylori* host cell intercellular junctions and to disrupt junction-mediated functions. Since predominant genotype of *H pylori* in Serbia and Montenegro has been reported to be the *cagA*+ genotype[21], it is very important to further investigate the presence and reversibility of different epithelial alterations induced by different *H pylori* strains.

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BASIC RESEARCH

Influence of gastric inhibitory polypeptide on pentagastrin-stimulated gastric acid secretion in patients with type 2 diabetes and healthy controls

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Abstract

AIM: Gastric inhibitory polypeptide is secreted from intestinal K-cells in response to nutrient ingestion and acts as an incretin hormone in human physiology. While animal experiments suggested a role for GIP as an inhibitor of gastric secretion, the GIP effects on gastric acid output in humans are still controversial.

METHODS: Pentagastrin was administered at an infusion rate of 1 μg . kg^{-1} . h^{-1} over 300 min in 8 patients with type 2 diabetes (2 female, 6 male, 54 ± 10 years, BMI 30.5 ± 2.2 kg/m^2; no history of autonomic neuropathy) and 8 healthy subjects (2/6, 46 ± 6 years., 28.9 ± 5.3 kg/m^2). A hyperglycaemic clamp (140 mg/dl) was performed over 240 min. Placebo, GIP at a physiological dose (1 pmol . kg^{-1} . min^{-1}), and GIP at a pharmacological dose (4 pmol . kg^{-1} . min^{-1}) were administered over 60 min each. Boluses of placebo, 20 pmol GIP/kg, and 80 pmol GIP/kg were injected intravenously at the beginning of each infusion period, respectively. Gastric volume, acid and chloride output were analysed in 15-min intervals. Capillary and venous blood samples were drawn for the determination of glucose and total GIP. Statistics were carried out by repeated-measures ANOVA and one-way ANOVA.

RESULTS: Plasma glucose concentrations during the hyperglycaemic clamp experiments were not different between patients with type 2 diabetes and controls. Steady-state GIP plasma levels were 61 ± 8 and 79 ± 12 pmol/l during the low-dose and 327 ± 35 and 327 ± 17 pmol/l during the high-dose infusion of GIP, in healthy control subjects and in patients with type 2 diabetes, respectively ($P = 0.23$ and p 0.99). Pentagastrin markedly increased gastric acid and chloride secretion ($P < 0.001$). There were no significant differences in the rates of gastric acid or chloride output between the experimental periods with placebo or any dose of GIP. The temporal patterns of gastric acid and chloride secretion were similar in patients with type 2 diabetes and healthy controls ($P = 0.86$ and $P = 0.61$, respectively).

CONCLUSION: Pentagastrin-stimulated gastric acid secretion is similar in patients with type 2 diabetes and healthy controls. GIP administration does not influence gastric acid secretion at physiological or pharmacological plasma levels. Therefore, GIP appears to act as an incretin rather than as an enterogastrone in human physiology.

Key words: Gastric inhibitory polypeptide; Gastric acid secretion; Type 2 diabetes; Hyperglycemic clamp; Pentagastrin-stimulated acid secretion

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INTRODUCTION

Gastric inhibitory polypeptide (GIP) is secreted from intestinal K-cells in response to nutrient ingestion and acts to augment insulin secretion from pancreatic beta-cells[1-4]. Together with glucagon-like peptide 1 (GLP-1) it confers approximately 60 % of total postprandial insulin secretion, thus acting as an incretin hormone[5, 6]. Since the insulinotropic effect of GIP appears to be dependent on the prevailing plasma glucose concentrations[7], the term "glucose-

dependent insulinotropic polypeptide" was coined later to denominate the peptide in accordance with its major physiological role[8]. In addition, GIP administration augments glucagon secretion under certain circumstances[7, 9]. While there is little doubt regarding GIP effects on endocrine pancreatic secretion, the case is different for its role in the regulation of gastrointestinal functions. In fact, original studies in canine gastric preparations pointed to an inhibition of gastric acid secretion by GIP[10, 11] and subsequent *in vivo* experiments demonstrated an increase in gastric acid output following antibody blockade of endogenous GIP in dogs, suggesting a physiological importance of GIP in the stomach[12]. These inhibitory effects of GIP on gastric acid secretion were ascribed to the local antral release of somatostain[13].

In humans, a significant inhibition of gastric acid output was only reported during the administration of highly supraphysiological doses of GIP[14, 15], whereas no effects were observed at physiological GIP plasma levels[15, 16].

While GIP is the major mediator of the incretin effect in healthy subjects[1, 5], the incretin activity of GIP is almost absent in patients with type 2 diabetes[17-19]. It is yet unclear, whether this is due to a general impairment in beta-cell function in type 2 diabetes or whether the reduced insulinotropic effect of GIP reflects a specific defect, for example of the GIP receptor, in patients with type 2 diabetes[20, 21]. In the latter case, one would expect a loss of GIP action not only with respect to insulin secretion, but also regarding other physiological actions like the inhibition of gastric acid secretion. Therefore, in the present study, the effects of GIP on pentagastrin-stimulated gastric acid secretion at both physiological and supraphysiological plasma levels were investigated in patients with type 2 diabetes and healthy controls. The effects of GIP on insulin secretion have been reported in a separate communication[22].

MATERIALS AND METHODS

Study protocol

The study protocol was approved by the ethics committee of the medical faculty of the Ruhr-University, Bochum on 01-24-2002. Written informed consent was obtained from all participants.

Study design

At a screening visit a clinical examination was performed and laboratory parameters were screened. If the subjects met the inclusion criteria (see below), they were recruited for the following procedure: Pentagastrin-stimulated gastric acid secretion was assessed over 300 min. A hyperglycemic clamp aiming at a steady capillary plasma glucose concentration of 140 mg/dL (7.8 mmol/L) was performed over 240 min. Placebo (1 % human serum albumin in 0.9 % NaCl), GIP at a low infusion rate (1.0 pmol/(kg · min)), and GIP at a high infusion rate (4.0 pmol/(kg · min)) were administered consecutively, each over 60 min. Boluses of placebo, GIP at a low dose (20 pmol/kg) and GIP at a high dose (80 pmol/kg) were administered intravenously at the beginning of each infusion period (at 0, 60, and 120 min, respectively).

In order to exclude a time-dependent order effect on gastric secretion, five of the control subjects were studied on an additional occasion with the administration of placebo only (1 % human serum albumin in 0.9 %NaCl from 0 to 180 min) instead of GIP. Boluses of placebo were administered at 0, 60, and 120 min.

Table 1 Characteristics of the participants (mean±SE)

Parameter (unit)	Healthy controls	Patients with type 2 diabetes	Significance (*P*-value)
Sex (female/male)	2/6	2/6	1.0
Age (years)	46±6	54±10	0.07
Body mass index (kg/m^2)	28.9±5.3	30.5±2.2	0.43
Waist-to-hip ratio (cm/cm)	0.97±0.08	1.00±0.05	0.40
Blood pressure			
Systolic (mmHg)	118±13	138±21	0.044
Diastolic (mmHg)	79±10	92±10	0.026
HbA_{1c} (%)[1]	6.0±0.5	8.3±2.2	0.013
Diabetes duration (years)	-	9 ± 4	-
Total-cholesterol (mg/dL)	223±52	210±35	0.57
HDL-cholesterol (mg/dL)	49±22	34±14	0.15
LDL-cholesterol (mg/dL)	145±37	164±31	0.32
Triglycerides (mg/dL)	161±110	167±92	0.90
Creatinine (mg/dL)	0.86±0.13	1.05±0.29	0.12

Statistics: ANOVA or χ^2 test. [1]Normal range: < 6.5

Subjects/patients

Eight patients with type 2 diabetes and eight healthy control subjects without a family history of diabetes were studied. Patient/subject characteristics are presented in Table 1.

Subjects with anemia (hemoglobin < 11 g/dl), elevation in liver enzymes more than double the respective upper normal limits, or with elevated creatinine concentrations (> 1.3 mg/dl) were excluded. Among the patients with type 2 diabetes, 2 were treated with metformin, whereas the other 6 were on a dietary regimen. All antidiabetic treatment was withdrawn at least 48 hours prior to the experiments. None of the participants had a history of gastrointestinal disease or was taking any kind of medication with a known influence on gastric secretion. In addition, none of the patients had a history or any clinical signs of autonomic or sensory neuropathy. No abnormalities in the responsiveness of both the patellar and Achilles tendon reflexes, as assessed using a reflex hammer, were found. The results of a vibration perception threshold performed at both medial malleoli were within the normal limits

Peptides

Synthetic GIP was purchased from PolyPeptide Laboratories GmbH, Wolfenbüttel,
Germany, and processed for infusion as described[18]. Pentagastrin was purchased from Cambridge Laboratories, UK, Lot-No. 1TT.

Experimental procedures

The tests were performed in the morning after an overnight fast. Two forearm veins were punctured with a teflon cannula, and kept patent using 0.9 % NaCl (for blood sampling and for glucose and peptide administrations, respectively). Both ear lobes were made hyperemic using Finalgon® (Nonivamid 4 mg/g, Nicoboxil 25 mg/g). A

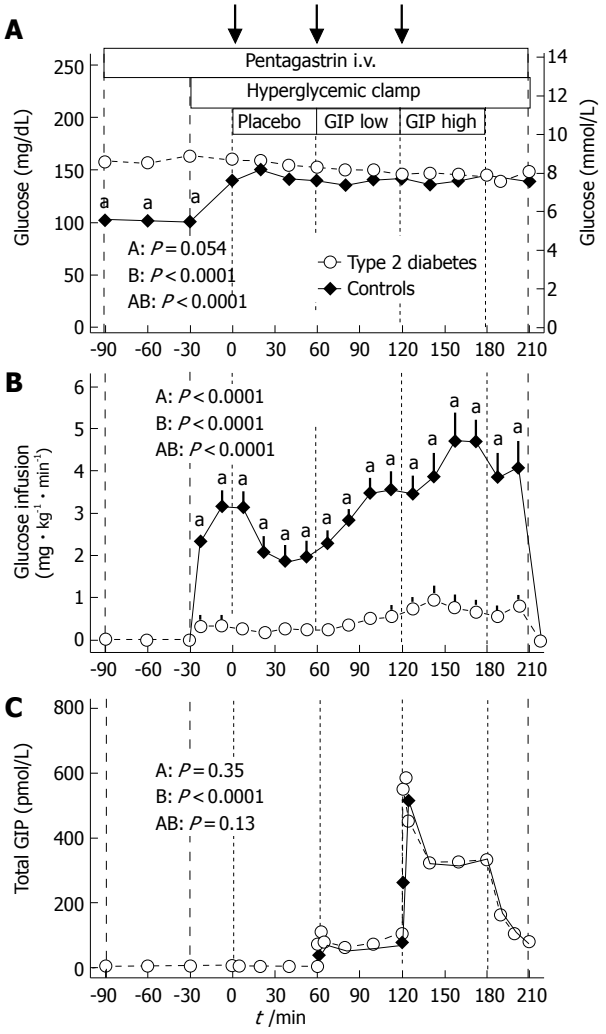

Figure 1 Plasma concentrations of glucose (**A**), glucose infusion rates (**B**) and plasma concentrations of total GIP (C) during hyperglycemic clamp experiments with the intravenous infusion of pentagastrin (1 μg . kg^{-1} . h^{-1}; -90 to 210 min), placebo (0 - 60 min), GIP at a low dose (1 pmol . kg^{-1} . min^{-1}; 60-120 min), and GIP at a high dose (4 pmol . kg^{-1} . min^{-1}; 120 -180 min) in eight patients with type 2 diabetes (open circles) and eight healthy controls (filled diamonds). Arrows indicate bolus administrations of placebo, 20 pmol GIP/kg and 80 pmol GIP/kg, respectively. Data are presented as means ± SEM; *P*-values were calculated using repeated measures ANOVA and one-way ANOVA and denote **A:** differences between the groups, **B:** differences over time and AB: differences due to the interaction of group and time. $^{a}P < 0.05$ patients with type 2 diabetes at individual time points.

nasogastric tube (Total length: 144 cm, diameter 6 mm, Sherwood Medical, Gosport, UK) was positioned for removing gastric acid by intermittent suction at 15 min intervals (HICO Gastrovac, Hirtz & Co., Germany). Basal gastric acid output was assessed over 15 min following a 30 min equilibration period. From -90 to 210 min, pentagastrin was administered intravenously at an infusion rate of 1 μg · kg body $weight^{-1}$ · h^{-1}. All participants remained in a semi-recumbent position, turned to their left side throughout the experiments.

A hyperglycemic clamp test aiming at a steady capillary plasma glucose concentration of 7.8 mmol/l (140 mg/dl) was started by injecting 40 % glucose as a bolus at -30 min and maintained by infusing glucose (20 % in water, weight/vol.) until 210 min, as appropriate, based on glucose determinations performed every 5 min and until 210 min. At 0 min, an intravenous infusion of placebo (1 % human serum albumin in 0.9 % NaCl) was started and maintained until 60 min. At 60 min, an intravenous infusion of GIP was started with an infusion rate of 1.0 pmol · kg^{-1} · min^{-1}. At 120 min, the GIP infusion rate was increased to 4.0 pmol · kg^{-1} · min^{-1} and maintained until 180 min. At 0 min an intravenous bolus of placebo was administered. GIP was injected as a bolus at 60 min (20 pmol/kg) and at 120 min (80 pmol/kg). Venous blood samples were drawn frequently, as seen in Figure 1.

Blood specimen

Venous blood was drawn into chilled tubes containing EDTA and aprotinin (Trasylol®; 20 000 KIU/ml, 200 μl per 10 ml blood; Bayer AG, Leverkusen, Germany) and kept on ice. After centrifugation at 4 ℃, plasma for hormone analyses was kept frozen at -28 ℃. Capillary blood samples collected from the ear lobe (approximately 100 μl) were stored in NaF (Microvette CB 300; Sarstedt, Nümbrecht, Germany) for the immediate measurement of glucose.

Laboratory determinations

Glucose was measured with a Glucose Analyser 2 (Beckman Instruments, Munich, Germany). GIP immunoreactivity was determined as described[18] using a C-terminal assay involving antiserum R65, which reacts fully with intact GIP (1-42) and the truncated metabolite (3-42), but not with so-called 8-Ku GIP, of which the chemical nature and molecular relation to GIP is uncertain. The assay has a detection limit of less than 2 pmol/l and an intra-assay variation of approximately 6 %. Human GIP (Peninsula Laboratories, Europe, Ltd.) was used as standard, and radiolabeled GIP was obtained from Amersham Pharmacia Biotech Ltd. (Aylesbury, UK).

Gastric volume output was measured in 15-min fractions to the nearest 0.5 ml. Acidity was determined by titration to pH 7.0 using phenol red as an indicator dye. Chloride concentration was measured by a Corning EEL 920 chloride meter (CIBA Corning Diagnostics, Fernwald, Germany).

Statistical analysis

Results are reported as mean ± SEM. Statistical calculations were carried out using repeated-measures analysis of variance (RM-ANOVA), using Statistica Version 5.0 (Statsoft Europe, Hamburg, Germany). This analysis provides p-values for differences between groups (A), differences over time (B), and for the interaction of groups with time (AB). If a significant interaction of treatment and time was documented ($P < 0.05$), values at single time points were compared by one-way ANOVA. A *P*-value < 0.05 was taken to indicate significant differences.

RESULTS

Fasting plasma glucose concentrations were significantly higher in patients with type 2 diabetes compared to control subjects ($P < 0.001$; Figure 1). During the hyperglycemic clamp period, similar glucose levels were obtained in both groups. As expected, glucose infusion rates required to

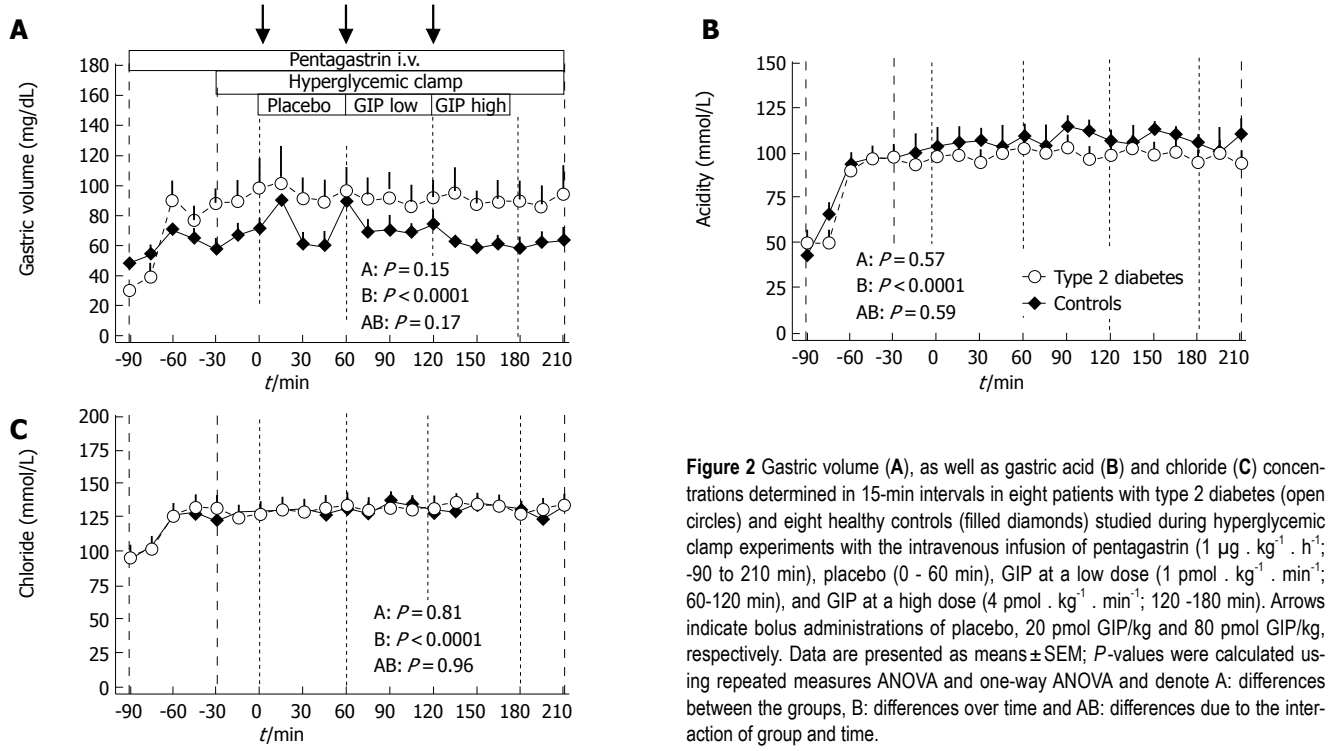

Figure 2 Gastric volume (**A**), as well as gastric acid (**B**) and chloride (**C**) concentrations determined in 15-min intervals in eight patients with type 2 diabetes (open circles) and eight healthy controls (filled diamonds) studied during hyperglycemic clamp experiments with the intravenous infusion of pentagastrin (1 μg . kg^{-1} . h^{-1}; -90 to 210 min), placebo (0 - 60 min), GIP at a low dose (1 pmol . kg^{-1} . min^{-1}; 60-120 min), and GIP at a high dose (4 pmol . kg^{-1} . min^{-1}; 120 -180 min). Arrows indicate bolus administrations of placebo, 20 pmol GIP/kg and 80 pmol GIP/kg, respectively. Data are presented as means ± SEM; *P*-values were calculated using repeated measures ANOVA and one-way ANOVA and denote A: differences between the groups, B: differences over time and AB: differences due to the interaction of group and time.

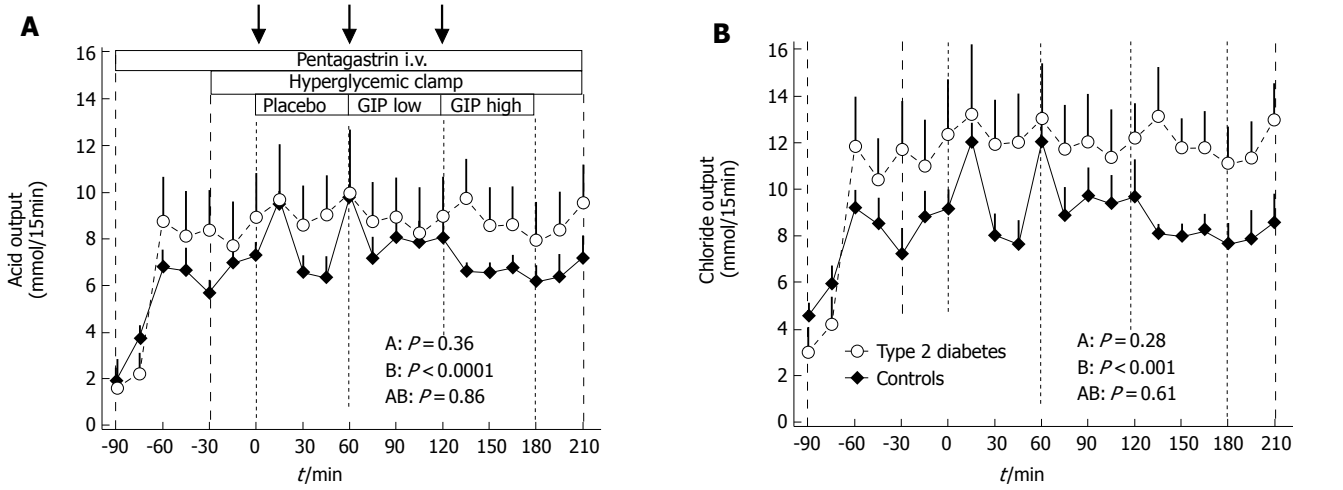

Figure 3 Gastric acid (**A**), and chloride (**B**) secretion rates per 15 min in eight patients with type 2 diabetes (open circles) and eight healthy controls (filled diamonds) studied during hyperglycemic clamp experiments with the intravenous infusion of pentagastrin (1 μg . kg^{-1} . h^{-1}; -90 to 210 min), placebo (0 - 60 min), GIP at a low dose (1 pmol . kg^{-1} . min^{-1}; 60-120 min), and GIP at a high dose (4 pmol . kg^{-1} . min^{-1}; 120 -180 min). Arrows indicate bolus administrations of placebo, 20 pmol GIP/kg and 80 pmol GIP/kg, respectively. Data are presented as means ± SEM; p-values were calculated using repeated measures ANOVA and one-way ANOVA and denote **A:** differences between the groups, **B:** differences over time and AB: differences due to the interaction of group and time.

maintain the hyperglycaemic clamp conditions were much higher in the controls than in the patients with type 2 diabetes ($P < 0.0001$).

GIP plasma levels increased to steady-state levels of 61 ± 8 and 79 ± 12 pmol/l during the low-dose and 327 ± 35 and 327 ± 17 pmol/l during the high-dose infusion of GIP, in healthy control subjects and in patients with type 2 diabetes, respectively ($P = 0.23$ and $P = 0.99$).

Basal gastric acid output was 1.9 ± 0.4 mmol/15 min and 1.6 ± 0.4 mmol/15 min (in healthy controls and in patients with type 2 diabetes, respectively; $P = 0.56$). The secretion of both gastric acid and chloride was markedly increased during the administration of pentagastrin ($P < 0.001$; Figures 2 and 3). In contrast, intravenous glucose administration had no effects on gastric acid or chloride output (Figure 2 and 3; Table 2). There were no differences in the rates of gastric acid or chloride output between the experimental periods with the administration of placebo, the low GIP dose, and the high GIP dose (Figure 3; Table 2). The lack of GIP effect on gastric secretion was confirmed in the five control experiments with the administration of placebo only and with GIP ($P = 0.87$ for acid output and $P = 0.59$ for chloride output, respectively; details not shown). The temporal pattern of

Table 2 Gastric acid and chloride secretion in patients with type 2 diabetes and healthy controls (mean±SE)

Experimental period: Time (min):	Basal (-90)	Pentagastrin (-45 to -30)	Hyperglycemia (-15 to 0)	Placebo (45 to 60)	GIP low 105 to 120)	GIP high (165 to 180)	Significance (*P* -value)
Healthy controls							
H^+ -secretion [mmol/15 min]	1.9±0.4	6.2±0.9	7.2±0.7	8.1±1.1	7.9±1.0	6.5±0.5	< 0.001
Cl^- -secretion [mmol/15 min]	1.9±0.4	7.9±1.0	9.0±0.8	9.9±1.3	9.6±1.2	8.0±0.7	< 0.001
Patients with type 2 diabetes							
H^+ -secretion [mmol/15 min]	1.6±0.4	8.3±1.7	8.3±1.7	9.5±1.8	8.6±1.7	8.2±1.6	< 0.01
Cl^- -secretion [mmol/15 min]	1.6±0.4	11.1±1.4	11.7±2.1	12.5±2.1	11.8±1.7	11.4±1.5	< 0.001

Statistics: ANOVA

gastric acid and chloride secretion was similar in patients with type 2 diabetes and healthy controls ($P = 0.86$ and $P = 0.61$, respectively; Figure 3).

DISCUSSION

The present experiments were undertaken to investigate the influence of GIP on pentagastrin-stimulated gastric acid secretion in patients with type 2 diabetes and in healthy controls. No effects of GIP on gastric acid output were observed at both physiological and pharmacological plasma concentrations.

A role for GIP in the regulation of gastric acid secretion was suggested from initial experiments in dogs[10-13]. Moreover the reduced rates gastric of acid secretion observed after jejuno-ileal shunt operation, when GIP plasma levels are increased, lent support to the concept of GIP as a negative regulator of gastric secretion[23]. In humans however, inhibitory effects of GIP on gastric acid secretion were only described, when supraphysiological GIP doses were used[14, 15], whereas GIP plasma levels that resembled those typically reached following meal ingestion failed to inhibit gastric acid secretion[15, 16]. In the present experiments, no effect of GIP on gastric acid output was observed even at supraphysiological plasma concentrations. The differences between the present and some of the previous studies[14, 15] may be explained by the use of different GIP preparations. In fact, both studies demonstrating inhibitory effects of GIP on gastric acid secretion in humans employed porcine GIP[14, 15], which differs from human GIP in two amino acid positions[24]. Moreover, some earlier peptide preparations contained impurities with cholecystokinin (CCK)-33 and -39, thereby limiting the interpretation of those studies[25]. Therefore, all aspects considered, the role of GIP in the regulation of gastric acid secretion in humans appears negligible.

The present data give rise to reconsider the role of GIP in human physiology. In fact, the peptide was initially considered to act as an enterogastrone[10, 11]. This term was proposed to describe (hormonal) factors from the duodenum that are secreted in response to nutrient ingestion and inhibit upper GI-functions at their typical plasma concentrations[26]. Such effects have been demonstrated for GLP-1 as well as for peptide-YY (PYY)[27-30]. In contrast, GIP does not appear to have any effects on gastric emptying[31] or gastric acid secretion in humans, and therefore does not fulfil the criteria for an enterogastrone. Rather, GIP acts as an incretin hormone, as demonstrated by numerous previous experiments[1, 5, 32]. Of note, a potent augmentation of insulin secretion by GIP was observed in the present experiments as well[22], thereby affirming the activity of the GIP preparations used.

The lack of GIP effect on gastric acid output represents another interesting difference in the biological actions of GIP and the other incretin hormone GLP-1, which is known as a potent inhibitor of gastric secretion[27, 33]. In fact, even though both hormones are secreted almost simultaneously[5, 34] and act in concert to augment glucose-stimulated insulin secretion[5, 32], they do exhibit a number of characteristic differences: Thus, GLP-1 suppresses pancreatic glucagon release[35, 36], whereas GIP has no effect or, at normoglycemic fasting conditions, even stimulates glucagon release[7, 9]. In addition, GLP-1 dose-dependently decelerates gastric emptying, thereby reducing the rise in glycemia following meal ingestion[30, 33, 37, 38]. In contrast, GIP administration has no effect on the velocity of gastric emptying[31]. Taken together, GLP-1 apparently possesses both incretin and enterogastrone activity, whereas GIP mainly acts as incretin hormone.

Another purpose of the present experiments was to compare gastric acid secretion in patients of type 2 diabetes and healthy controls. Since hyperglycemia itself has been shown to inhibit gastric acid output[39], hyperglycaemic clamp conditions were applied to match plasma glucose concentrations in both groups. Under these conditions, no differences occurred in the rates of gastric acid or chloride output. It is important to point out that in the present study only patients without a history or clinical signs of neuropathy were included. In fact, a number of previous studies have demonstrated reduced rates of gastric acid secretion in diabetic patients with overt autonomic neuropathy[40] [41-43]. In contrast, no abnormalities in gastric secretion were reported in patients with diabetes without signs of neuropathy[41, 43]. Therefore, it appears that, unless neuropathy develops, gastric acid secretion is similar in patients with type 2 diabetes and non-diabetic controls, when plasma glucose levels are matched.

In conclusion, gastric inhibitory polypeptide has no effect on gastric acid secretion in patients with type 2 diabetes and healthy controls. Therefore, GIP seems to act as an incretin rather than as an enterogastrone in human physiology.

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BASIC RESEARCH

Reconstruction of liver organoid using a bioreactor

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Abstract

AIM: To develop the effective technology for reconstruction of a liver organ *in vitro* using a bio-artificial liver.

METHODS: We previously reported that a radial-flow bioreactor (RFB) could provide a three-dimensional high-density culture system. We presently reconstructed the liver organoid using a functional human hepatocellular carcinoma cell line (FLC-5) as hepatocytes together with mouse immortalized sinusoidal endothelial cell (SEC) line M1 and mouse immortalized hepatic stellate cell (HSC) line A7 as non parenchymal cells in the RFB. Two x 10^7 FLC-5 cells were incubated in the RFB. After 5 d, 2 x 10^7 A7 cells were added in a similar manner followed by another addition of 10^7 M1 cells 5 d later. After three days of perfusion, some cellulose beads with the adherent cells were harvested. The last incubation period included perfusion with 200 nmol/L swinholide A for 2 h and then the remaining cellulose beads along with adherent cells were harvested from the RFB. The cell morphology was observed by transmission electron microscopy (TEM) and scanning electron microscopy (SEM). To assess hepatocyte function, we compared mRNA expression for urea cycle enzymes as well as albumin synthesis by FLC-5 in monolayer cultures compared to those of single-type cultures and cocultures in the RFB.

RESULTS: By transmission electron microscopy, FLC-5, M1, and A7 were arranged in relation to the perfusion side in a liver-like organization. Structures resembling bile canaliculi were seen between FCL-5 cells. Scanning electron microscopy demonstrated fenestrae on SEC surfaces. The number of vesiculo-vacuolar organelles (VVO) and fenestrae increased when we introduced the actin-binding agent swinholide-A in the RFB for 2h. With respect to liver function, urea was found in the medium, and expression of mRNAs encoding arginosuccinate synthetase and arginase increased when the three cell types were cocultured in the RFB. However, albumin synthesis decreased.

CONCLUSION: Co-culture in the RFB system can dramatically change the structure and function of all cell types, including the functional characteristics of hepatocytes. Our system proves effective for reconstruction of a liver organoid using a bio-artificial liver.

Key words: Liver organoid; Organ reconstruction; Bio-artificial liver; Coculture; Liver sinusoidal endothelial cell; Hepatocytes; Fenestrae; Vesiculo vacuolar organelles; Radial flow bioreactor

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INTRODUCTION

Liver regeneration technology has made many advances in recent years. Efforts now are being made toward development of embryonic stem cells (ES cells), differentiation of hemopoietic stem cells, and development of isolation and culture methods for somatic stem cells originating from different organs. Hemopoietic stem cells, hepatoblasts originating from fetal liver, hepatocytes, and pancreatic duct epithelial cells have been included in the list of candi-

date cells for liver regeneration[1]. Development of immortalized cells by introduction of the simian virus 40 (SV40) large T antigen gene or human telomerase reverse transcriptase (hTERT) is also under investigation[2]. To date, however, no technique for regenerating and reconstructing parenchymal organs using these cells has been established. Conventional cell culture methods have achieved this goal clinically for skin, cornea, and bone tissue[3,4].

Reconstruction of organs such as the liver requires maintenance of viable cells at a high density and coculture under conditions favorable to several different cell types that constitute a liver. To make a culture system is important in reconstructing a liver organoid. Conventional stationary culture techniques are not well suited to the culture of cells in a layered form, i.e., in a structural and functional organoid a simple air/CO_2 incubator does not deliver adequate oxygen supply to layered cells. Furthermore, high-density culture cannot be maintained with the limited nutrients available in conventional cultures. For these reasons, construction of a bioreactor that allows 3-dimensional growth in a high-density perfusion culture has been advocated for reconstructing a liver organoid. In our study a radial-flow bioreactor (RFB) developed in Japan was used as a candidate model for high-density perfusion culture. Filled with a porous carrier, this bioreactor permits culture at a cell density 10 times higher than that allowed by a hollow-fiber culture system[5, 6]. Another important point is to select a cell source. Clinically, cells using bio-artificial liver are required to be highly functional and supplied quickly in large quantities. Therefore we established a functional human hepatocellular carcinoma cell line (FLC-5), which can express drug-metabolized enzymes (e.g., human-type carboxyl esterase or cytochrome) and liver-specific proteins such as albumin. *In vitro* this cell line retains its three-dimensional form, developing distinct microvilli on the surface. These cells can be cultured in serum-free ASF104 medium (Ajinomoto, Tokyo). A liver organoid cannot be reconstructed with hepatocytes only. At minimum, coculture of hepatocytes with nonparenchymal cells, such as sinusoidal endothelial cell (SEC) and hepatic stellate cell (HSC) is required. So we established immortalized SEC line M1[7] and an immortalized HSC line A7[8] by isolating nonparenchymal cells from an H-2Kb-tsA58-transgenic mouse liver transfected with the SV40 large T antigen gene[9].

Reconstruction of the liver sinusoid is important for activity of the liver organoid as a functional unit. Also, the open pores on the surfaces of SEC in fenestrae have an important functional role in the liver sinusoid. Fenestrae are the most remarkable characteristics of SEC, as first described by Wisse in 1970[10] using transmission electron microscopy (TEM). Diameters of these pores vary between species, ranging from 100 to 200 nm[11]. These fenestrae facilitate the transport of materials and solutes from the luminal to the abluminal side of the liver parenchymal cells and *vice versa*[12]. The process and mechanism of formation of these pores remain largely unclarified[13, 14]. The presence of actin filaments at the margin of these pores has been demonstrated by electron microscopic studies[15, 16]. Swinholide A, a most potent microfilament-disrupting drug available, has been demonstrated to increase the number of SEC fenestrae[13]. However, when immortalized SEC was treated in a monolayer culture or as a monoculture in the RFB, an increase in number of fenestrae could not be observed when the Swinholide A was introduced. The potential for drug-induced increase also has been reported to disappear in long-term cultures[7].

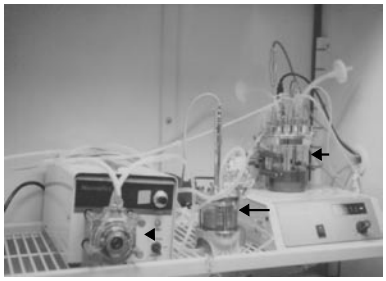

Figure 1 The system of radial-flow bioreactor. A 15-mL radial-flow bioreactor (large arrow), a mass flow controller (arrow head), and a reservoir (small arrow) are connected each other. Culture medium is perfused in the RFB. Medium conditions (PH, oxygen, CO_2 and temperature) are controlled by computer.

In developing a high functioning organoid using a bioartificial liver, the function, form and reactivity of pharmacological agent should be near *in vivo*. In the present study, we reconstructed a functional liver organoid using immortalized cell lines in the RFB.

MATERIALS AND METHODS

Cell culture and medium

We used the three cell lines mentioned above, FLC-5, M1, and A7. As reported, culture of M1 cells was possible in serum-free conditions while supplementation of ASF104 medium with 2% fetal bovine serum (FBS) was required for culture of A7 cells. Therefore, in coculture experiments, ASF104 medium was enriched with 2% FBS.

Coculture in radial-flow bioreactor

As reported elsewhere, the RFB system is composed of a 15-mL radial-flow chamber (RA-15; ABLE, Tokyo), a mass flow controller (RAD925, ABLE), a reservoir (Figure 1), a computer, and a tissue incubator as described previously[17] (Figure 1). The culture medium was oxygenated within the reservoir, and the pH was adjusted automatically to 7.4. Oxygen pressure in the culture medium was measured both within the reservoir and at the outlet of the bioreactor. Relative oxygen consumption was monitored on the basis of the oxygen pressure gradient. During the study the temperature within the reservoir was kept constantly at 37 ℃. Two $\times 10^7$ FLC-5 cells were inoculated into the reservoir. The bioreactor was perfused in a closed circuit for 2 h to aid cells in adhering to the porous carrier cellulose beads (Asahi Kasei, Tokyo). Subsequently the bioreactor was switched to the open-circuit mode, and incubation was continued with addition of fresh culture medium to the reservoir. After 5 d, 2×10^7 A7 cells were added in a similar manner followed by another addition of 10^7 M1 cells 5 d later. Retinol (10^{-6}mol/L) was added during the first 2 d. After three days of perfusion cellulose beads with the adherent cells were harvested, and cells deposited at the bottom of the bioreactor also were recovered. Beads with attached cells were fixed in 1.2% or 2.0% glutaraldehyde as described below.

Swinholide A experiments

We cultured the three cell lines as described above. The

last incubation included perfusion with 200 nmol/L swinholide A (Sigma catalog number S9810; S) for 2 h. The cellulose beads along with adherent cells were harvested from the bioreactor. Beads with attached cells were fixed in glutaraldehyde and prepared for morphologic observation as follows.

Electron microscopy

For scanning electron microscopy (SEM), cultured cells were fixed with 1.2% glutaraldehyde in 0.1 mol/L phosphate buffer (PB) at pH 7.4 and postfixed with 1% OsO_4 in 0.1mol/L PB. The fixed cells were rinsed twice with PBS, subsequently dehydrated in ascending concentrations of ethanol, critical point-dried using carbon dioxide, and coated by vacuum- evaporated carbon and ion-spattered gold. Specimens were observed under JSM-35 scanning electron microscope (JEOL, Tokyo) at an accelerated voltage of 10 kV.

For transmission electron microscopy (TEM), cultured cells were fixed with 2.0% glutaraldehyde in 0.1mol/L PB for 1 h and postfixed with 1% OsO_4 in 0.1mol/L PB for 1 h at 4 ℃. Specimens were dehydrated in ethanol and subsequently embedded in a mixture of Epon-Araldite. Thin sections (60 nm) were cut with a diamond knife mounted on an LKB ultratome, and stained with aqueous uranyl acetate. Specimens were examined under a JEOL 1200EX electron microscope.

Amino acid analysis of supernatants

For analysis of amino acid fractions by high-performance liquid chromatography (HPLC), supernatants were collected from FLC-5 alone and from cocultures of the three cell types in the bioreactor. Supernatants were mixed with 5% sulfosalicylic acid and allowed to stand at 4 ℃ for 15 min. After centrifugation to precipitate protein, supernatants were injected into amino acid analysis columns (L-8500, Hitachi, Tokyo).

Quantitative TaqMan RT-PCR

We measured mRNA expression for the urea cycle enzymes, carbamoyl phosphate synthetase (CPS1), ornithine carbamoyltransferase (OCT), argininosuccinatesynthetase (ASS), argininosuccinatelyase (ASL), and arginase (ARG), as well as mRNA expression for albumin, hepatocyte nuclear factor (HNF)-1 and HNF-4, by quantitative TaqMan reverse transcription polymerase chain reaction (RT-PCR). RT-PCR was performed on the ABI PRISM 7700 sequence detection system using random hexamers from TaqMan reverse transcription reagents and the RT reaction mix (Applied Biosystems, Rockville, M) to reverse-transcribe RNA. TaqMan universal PCR Master Mix and Assays-on-Demand gene expression probes (Applied Biosystems) were used for PCR. A standard curve for serial dilution of 18S rRNAs was generated similarly. A relative standard curve method (Applied Biosystems) was used to calculate the amplification difference in urea cycle-related enzymes between cocultured and control cells, and elongation factor 1 (EF1), for each primer set and between albumin, HNF-1, HNF-4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Specificity was evaluated using GAPDH mRNA as an internal control (4310884E; Perkin-Elmer Applied Biosystems). Each amplification was performed in triplicate, and averages were obtained.

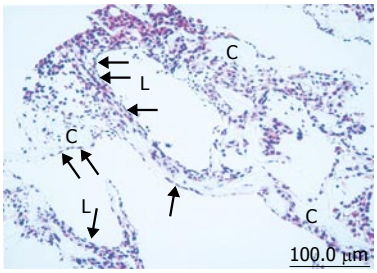

Figure 2 Light microscopic image of coculture in the RFB. High-density and layered cells attached on the cellulose beads (C). Sinusoid-like lumen structure (L) could be observed. SEC was observed with flat shape on surface of the lumen and perfusion side (arrow).

Based on DNA sequences in GenBank, primers and the TaqMan probe for albumin, HNF-1, and HNF-4 were designed using the primer design software Primer Express TM (Perkin-Elmer Applied Biosystems, Foster City, CA). AmpliTaq DNA polymerase extended the primer and displaced the TaqMan probe through its 5'-3' exonuclease activity. Probes were labeled with a reporter fluorescent dye either 6-carboxy-fluorescein (FAM) or 2, 7 dimethoxy-4,5-dichloro-6-carboxy-fluorescein (JOE) at the 5' end and a quencher fluorescent dye [6-carboxytetramethyl-rhodamine (TAMRA)] at the 3' end.

Primers/probes were as follows: ornithine transcarbamoylase (OTC) forward primer 5'-CCAGGCAATAAAAGAGTCAGGATT-3', reverse primer/ 5'-TTATCAAAG TCCCCTGGTTAGAGATACT-3', probe/ 5'-(FAM)- TTCAAATGCTCCTACACCCTGCCCTG-(TAMRA)-3'; arginosuccinase (ASL) forward primer/ 5'-TGGCCAAGGAGGTCGTCA-3', reverse primer 5'-TTCCTCGTCGTCCGGAAG-3', probe 5'-(FAM)-TGTCTTCCAGACCCGGAGACCGAA-(TAMRA)-3'; albumin forward primer/ 5'-CGATTTTCTTTTTAGGGCAGTAGC-3', reverse primer/ 5'-TGGAAACTTCTGCAAACTCAGC-3', probe/ 5'-(FAM)-CGCCTGAGCCAGAGATTTCCCA-(TAMRA)-3'; HNF-1 forward primer/ 5'-AGCGGGAGGTGGTCGATAC-3', reverse primer/ 5'-CATGGGAGTGCCCTTGTTG-3', probe/ 5'-(FAM)-TCAACCAGTCCCACCTGTCCCAACA-(TAMRA)-3'; HNF-4 forward primer/ 5'-GGTGTCCATACGCATCCTTGA-3', reverse primer/ 5'-TGGCTTTGAGGTAGGCATACTCA-3', probe/ 5'-(FAM)-CCTTCCAGGAGCTGCAGATCGATGAC-(TAMRA)-3'; GAPDH forward primer/ 5'-CTCCCCACACACATGCACTTA-3', reverse primer/ 5'-CCTAGTCCCAGGGCTTTGATT-3', probe/ 5'-(VIC)-AAAAGAGCTAGGAAGGACAGGCAACTTGGC-(TAMRA)-3'.

RESULTS

Structure of cells cultured in bioreactor

In the bioreactor, cells cultured in high density assumed layered form on the cellulose beads. Lumen-like structure was observed. Endothelial cells were exits with flat shape at the surface of the lumen and the perfusion side (Figure 2). Multiple layers of FLC-5 cells adhered to the cellulose beads, while A7 and M1 cells were predominantly localized to the side where perfusion occurred. Layered cells were seen in a hole of porous cellulose beads. Sinusoid-like lumen was observed at perfusion side in the cellulose beads

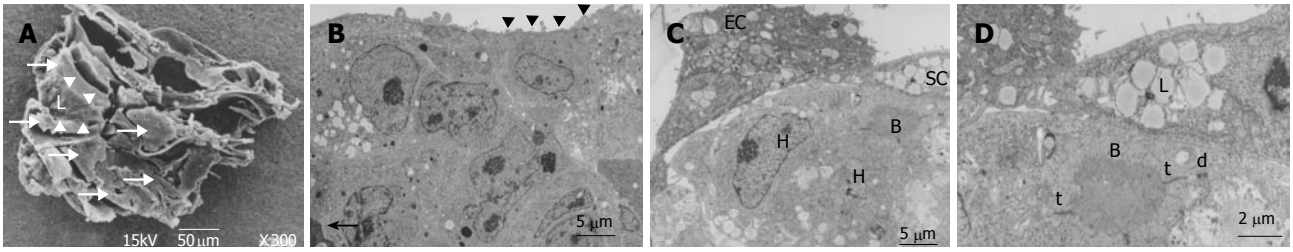

Figure 3 Transmission electron microscopic images of cocultures in the RFB. **A**: The cells are arrayed on the cellulose beads. Several cell clusters could be seen in a gap of cellulose beads (arrow). Vascular lumen structure surrounding cell clusters could be seen in the beads (small arrow). Culture media flow through inside of lumen structure; **B**: The cells are arrayed in layers on cellulose beads. Part of a cellulose bead (arrow) is visible at the bottom of the layer. A process of a sinusoidal endothelial cell (arrowhead) is seen at the perfusion side. Scale bar: 5 μm; **C**: Sinusoidal endothelial cells (EC) can be seen at the perfusion side. Hepatic stellate cells (SC) containing fatty vitamin A droplets are seen overlying the FLC-5 cells (H). FLC-5 cells (H) below EC and SC show bile-canaliculus-like structures (B). Scale bar: 5 μm. **D**: Bile canaliculus-like structures (B) containing electron dense bile components. Tight junctions (t) and desmosomes (d) are visible, as are fatty vitamin A droplets (L). Scale bar: 2 μm.

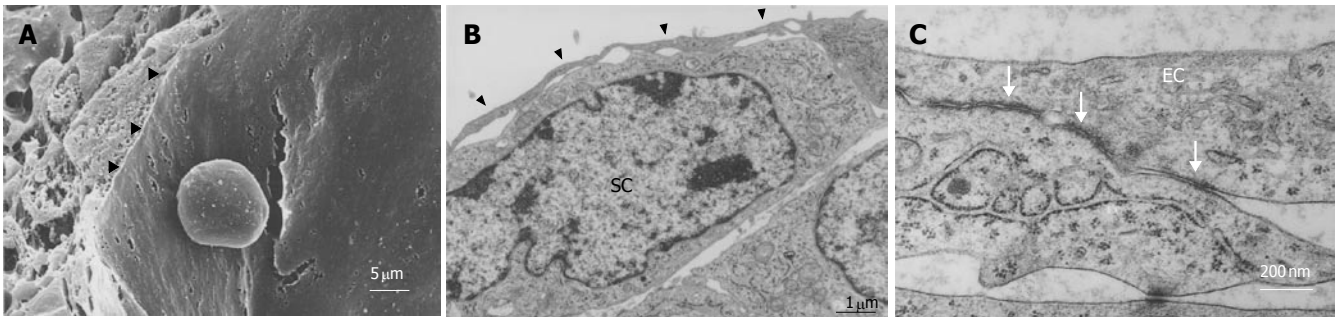

Figure 4 Ultrastructure of sinusoidal endothelial cells. **A**: Scanning electron microscopic image of sinusoidal endothelial cells localized at the perfusion side. They form a thin layer (arrowhead), showing the typical appearance of a sinusoid-like vascular structure. Scale bar: 5 μm; **B**: Transmission electron microscopic image showing sinusoidal endothelial cell growth at the perfusion side forming a thin layer (arrowhead) overlying the A7 cells (SC). Scale bar: 1 μm; **C**: Transmission electron microscopic view showing tight junctions (arrow) between sinusoidal endothelial cells (EC). Scale bar: 200 nm.

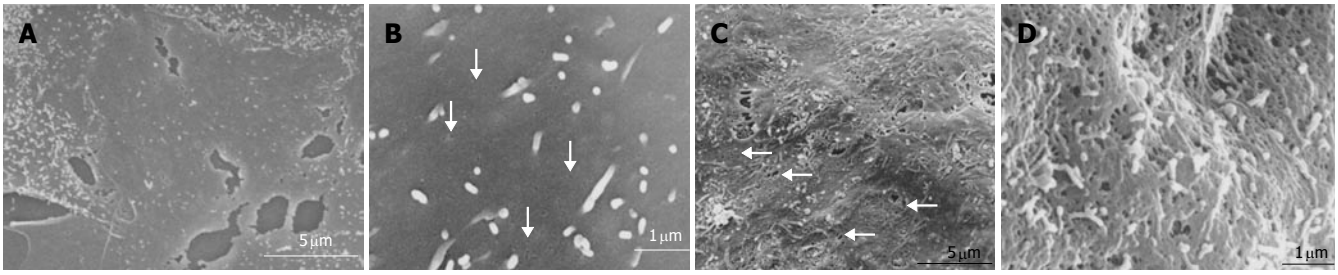

Figure 5 Scanning electron microscopic image of the surface of sinusoidal endothelial cells. **A**: Low-magnification scanning electron microscopic images of the surface of sinusoidal endothelial cells cultured on plastic dishes. The sinusoidal endothelial cells formed a thin layer on the plastic dish substrate. Scale bar: 5 μm; **B**: High-magnification scanning electron microscopy images of the surface of sinusoidal endothelial cells cultured on plastic dishes. Fenestrae could not be detected on the surface of endothelial cells. Only small pits are seen (arrow). Scale bar: 1 μm; **C**: Low-magnification scanning electron microscopic view of the surface of sinusoidal endothelial cells cocultured in the RFB. Fenestrated pores could be observed (arrow). Scale bar: 5 μm; **D**: High-magnification scanning electron microscopic view of the surface of sinusoidal endothelial cells cocultured in the RFB. Pores have a diameter of 100 - 200 nm. Scale bar: 1 μm.

(Figure 3A). TEM showed that cocultured cells assumed layered form from cellulose beads to the perfusion side (Figure 3B). M1 and A7 cells containing vitamin A-laden fat droplets were seen mainly at the perfusion side, while dense layers of FLC-5 cells were observed beneath (Figure 3C). At sites where the three cell lines were in contact with each other bile canaliculus-like structures were present between neighboring FLC-5 cells. Lumens of these structures contained electron-dense bile components, tight junctions and desmosomes also could be observed (Figure 3D). This side showed growth of endothelial cells with the formation of sinusoid-like vascular structures (Figures 4A and 4B). Tight junctions were seen between endothelial cells (Figure 4C). Fenestrae which are characteristic of SEC *in vivo*, were absent in monocultures of M1 cells on plastic dishes (Figures 5A and 5B). Because a long time subculture would change the character of M1 cells, pores were represent on the surface of M1 cells co-cultured in the RFB system (Figure 5C). The pores had a diameter of 100 to 200 nm, being similar in morphology and size to those of fenestrae shown by SEC *in vivo* (Figure 5D).

Morphology of M1 cells incubated with swinholide A

Cells incubated for 2 h with 200 nmol/L of the actin-disrupting agent swinholide A showed the increased number of pores (Figure 6), while some pores were dilated (about

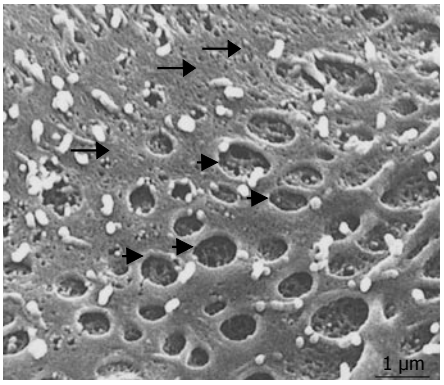

Figure 6 Scanning electron micrographs of the surface of swinholide A - treated SEC cells in the RFB culture system. Large open pores have a fenestra-like appearance (short arrow). Small pores were detectable in the nonfenestrated area (long arrow). Scale bar: 1 μm.

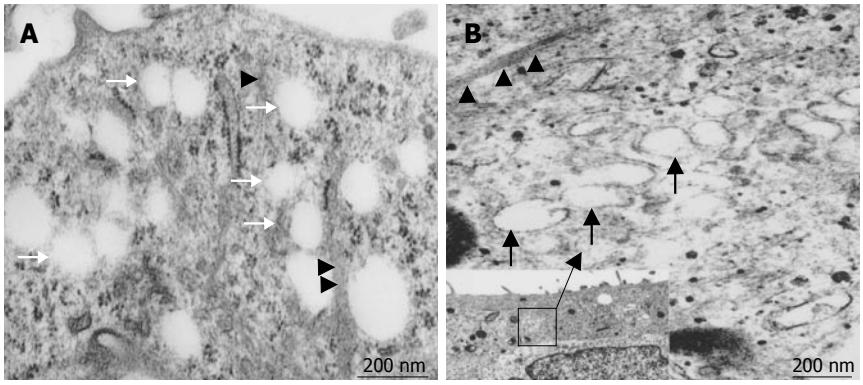

Figure 7 Transmission electron micrographs of sectioned SEC cells after swinholide A - treatment; **A**: Numerous open pores or fenestrae in the cytoplasm (arrow). Fine cytoskeletal elements showing a close spatial relationship with these pores (arrowhead). Scale bar: 200 nm; **B**: VVO could be observed in SEC cells (arrows) in response to stress or actin fibers (arrowheads). Scale bar: 200 nm. Inset shows the overall composition of the cells. Scale bar: 1 μm.

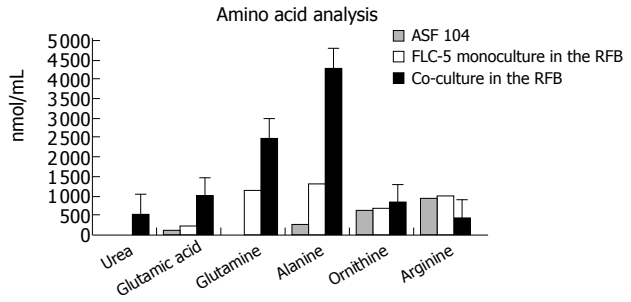

Figure 8 Amino acid and urea analysis in supernatants. Urea was detectable only in coculture, at 523 nmol/ml. ASF 104 designates culture medium. Mean value ± SD.

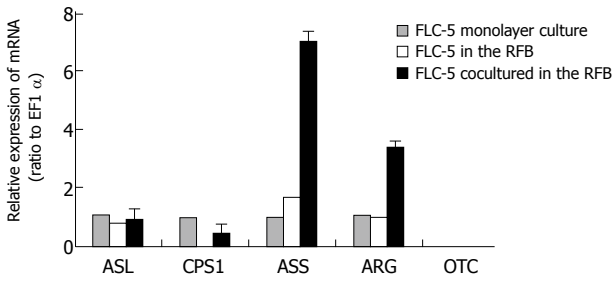

Figure 9 Comparison of expressions of CPS1, OTC, ASS, ASL, and ARG mRNA in FLC-5 incubated under different conditions as assessed by TaqMan 1-step RT-PCR. The mRNA expression of each enzyme in different conditions is relative to that in monolayer cultures. Mean value ± SD.

1 μm). Small pores (tens of nanometers in size) that probably resembled coated pits were abundant in the nonfenestrated areas.

TEM investigation showed that treatment with swinholide A resulted in fenestrated pores with a diameter between 100 and 200 nm. The pores fused with each other formed labyrinthine structures (Figure 7A). In addition, vacuoles with a diameter of about 200 nm, similar to previously described vesiculo vacuolar organelles (VVO), were noted. These structures typically were seen in areas where relatively regular overlap was seen in FLC-5, A7, and M1. The number of VVO increased when cells were treated with 200 nmol/L swinholide A, which was associated with partial fusion (Figure 7B).

Amino acid fractions from supernatants

At the end of culture, the supernatant was subjected to amino acid analysis. Urea production was not seen in monocultures of FLC-5 cells in the RFB, while FLC-5 cells cocultured with M1 and A7 cells produced 523 nmol urea /mL in the culture medium, suggesting that the urea cycle was activated in the coculture RFB system (Figure 8). Several amino acids were increased in the medium.

We compared mRNA expression of CPS1, OTC, ASS, ASL, and ARG in FLC-5 monolayer cultures with those of monocultures in the RFB system. In addition, mRNA expression in cocultures in the RFB also was assessed. We could not detect OTC in any type of culture. Expression of other urea cycle enzymes showed no notable difference between monolayer culture of FLC-5 and monoculture of FLC-5 in the RFB. However, ASS and ARG expressions in co-culture in the RFB were about 7 and 3 times greater than those in FLC-5 monolayer culture (Figure 9).

Albumin synthesis and expression of nuclear factors

We compared mRNA expression of albumin and HNF-1 and HNF-4 as transcription regulation factors between experimental conditions. Expression of mRNA encoding the three proteins was less in FLC-5 co-cultures in the RFB system than in FLC-5 RFB monocultures or in FLC-5 cells in monolayer culture (Figures 10A-10C). In a previous study, albumin production was enhanced in the RFB using the immortalized cell line[17]. However it was different cell line in this study.

DISCUSSION

Introduction of a functional human hepatocellular carcinoma cell line (FLC) in our system can allow the cells to be cultured at high density in a layered array and maintain viability for long periods[17,18].

Immortalized cells can be used for artificial liver. The reason is that it can supply cells in large quantities and quickly. Immortalized cells lose several characteristics in

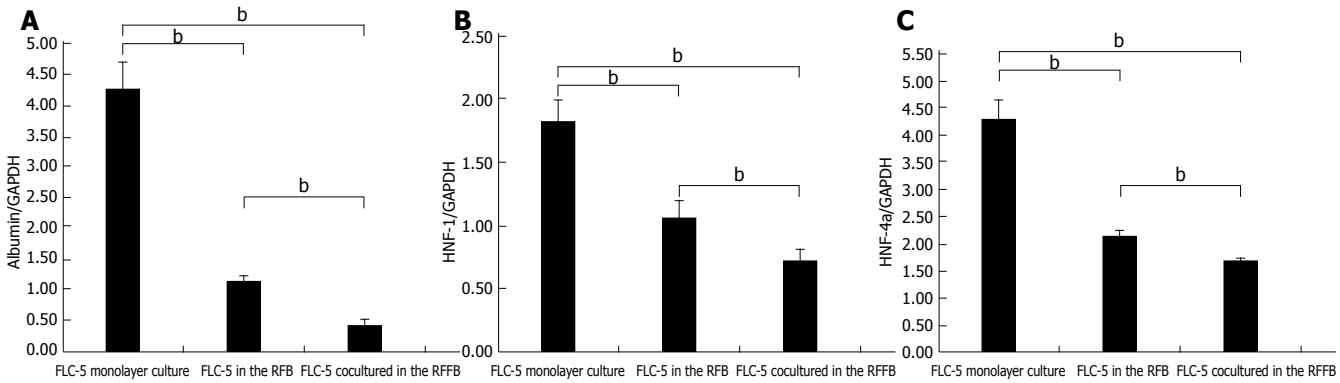

Figure 10 Expression of mRNA for albumin (**A**), HNF-4 (**B**) and HNF-1 (**C**) as transcription regulation factors in each condition. Messenger RNA expression for these three proteins decreased in cocultured FLC-5 in the RFB compared with FLC-5 monocultures in the RFB and FLC-5 cultured in a monolayer. Mean value ± SD. The ratio of mRNA for each protein versus GAPDH is shown. Differences with respect to each condition were statistically significant ($^bP < 0.01$) according to Student's *t*-test.

morphology and function. However, three cell lines were studied in our RFB culture system, including their fine structure according to electron microscopy. Layers of FLC-5, A7, and M1 were arranged respectively from the carrier attachment side to the perfusion side. In some areas, liver-like architectures, sinusoid-like lumen structure, bile-canaliculi and functional complex, were observed, comparable to *in vivo* tissue relationship. The M1 cell line well covered the perfusion side, mimicking vascular structures, indicating that this cell type forms an arrangement similar to that *in vivo*. Furthermore, M1 cells in monolayer culture did not express fenestrae, probably reflecting a long culture or subculture time[19]. In a previous study, we found that M1 cells also lack fenestrae in monocultures in the RFB[7]. In contrasts, fenestrated pores were seen in M1 cells cocultured according to the present RFB experimental design. Coculture and cell to cell contact have an influence on these morphological changes. Because fine structures *in vivo* could be observed better than monoculture in the RFB.

The electron microscopic observations in the present study clearly showed that if an appropriate environment for cell growth was provided in a perfusion culture system, the individual cell types could arrange themselves according to their *in vivo* characteristics, even in a high-density layered culture.

This study also examined the numerical dynamics of fenestrae. For this we exposed the cocultures to the actin-disrupting drug swinholide A[13]. When the cells were treated with swinholide A, the number of pores with a diameter of about 100 to 200 nm increased 2 h after swinholide A treatment. Furthermore, by TEM, cytoplasmic vesicles about 200 nm in diameter could be seen and were much larger than the caveolae in the cytoplasm, and their number increased in the presence of swinholide A. These vacuolar-like vesicles probably represent the vesiculo vacuolar organelle (VVO) as described by Feng *et al*[20]. The VVO is an organelle contributing to transport of macromolecules between luminal and abluminal sides of endothelial cells, thus increasing transcellular permeability. Vascular permeability factor and vascular endothelial growth factor (VPF/VEGF) can induce formation of VVO[21]. FLC-5 used in this study, could express VPF/VEGF (data not shown). The presence of vascular factors may partially explain why VVO is noted in cocultures and why fenestrae could be observed in our experiments[22]. VVO is thought to be formed by fusion of caveolae, when multiple VVOs fuse together, a structure extending from the luminal to the abluminal sides of endothelial cells is formed. In the present study, fused VVOs also were seen in swinholide A - treated specimens by transmission electron microscopy, suggesting that this fusion represents a process culminating in formation of the labyrinthine structures in SEC[23]. The mechanism of pore formation in immortalized SEC and under cocultured perfusion conditions remains unknown from the present study. However, pore formation may result from multiple effects or factors working in concert upon endothelial cells, such as cytoskeletal dynamics represented by actin and/or the influence of a yet unknown factor secreted by other cell types present in the cocultures such as VEGF. The observation that hepatic endothelial cells maintain one of their typical morphological features (i.e. an abundant number of membrane-bound coated-pits, uncoated vesicles/vacuoles and fenestrae) is an indication that the bioreactor mimics a nearby physiological cultivation environment for the various liver cell types. However, the mechanism by which the bioreactor and its culture environment bring about and maintain these membrane-bound vesicles and fenestrae in endothelial cells remain to be elucidated and consequently open up new directions for future experiments.

To assess hepatocyte function, we compared mRNA expression for urea cycle enzymes and albumin synthesis by FLC-5 in monolayer culture compared to these single-type cultures and cocultures in the RFB. Previously, we have demonstrated hepatocyte functions such as albumin synthesis and cytochrome expression are enhanced in the RFB[24, 25]. Urea production is among the most primitive functions of liver cells. We could not detect urea in medium from monolayer cultures or monocultured FLC-5 in the RFB. In contrast, FLC-5 cells cocultured in the RFB exhibit ability to produce urea, and mRNA expression for ASS and ARG is enhanced. The medium used in this experiment, ASF 104 contained arginine, so urea production was observed in cocultures in the RFB although OTC was not expressed. One report showed that urea produc-

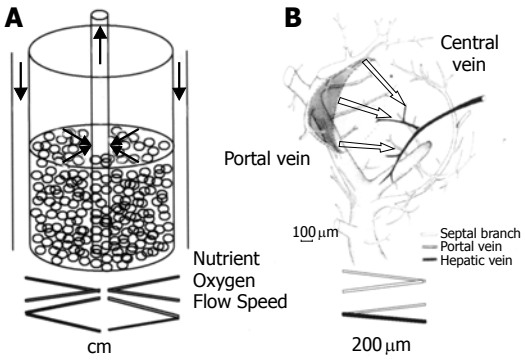

Figure 11 RFB and intact organ. **A**: In the RFB system, culture medium flows from outside the column toward the center of the reactor. Medium flows faster at the center than at the periphery. Biases in distribution of oxygen and nutrition at inflow and outflow are minimized. **B**: In the hepatic lobe, blood flows from the portal vein to central vein. The RFB system is similar to the organization of the hepatic primary lobe[31]. Figure 11B is reproduced from Figure 9 in reference 31.

tion in OTC-deficient mice could be detected under the same condition[26]. Glutamic acid, glutamine and alanine were also increased in supernatant co-cultured in the RFB, indicating that amino acid metabolism becomes active.

It was reported that three-dimensional spherical culture induces albumin synthesis, a particularly important hepatocytic function[27, 28]. However, in the present study, mRNA expression of albumin was decreased under coculture conditions in the RFB. Nuclear transcriptional factors HNF-4 and HNF-1, which regulate albumin synthesis, were decreased under coculture conditions in the RFB. Albumin in supernatant was also decreased during culture (data not shown). The results suggest that the culture environment (cell-to-cell communication, cell polarity, shear stress, and other factors) can control manifestations of intracellular nuclear transcription factors and therefore dramatically influence albumin production by liver cells. Immortalized cells can be used for artificial liver. The reason is that it can supply cells in large quantities and quickly. But immortalized cells may change the characteristics of its original cells. In this study, albumin synthesis was decreased. It was not useful for artificial liver. In future study, we have to try other cell sources (ES cell, oval cell, and other immortalized cell lines).

Finally, several points should be noted concerning our culture system. First, controlling the mixture ratio of the three cell types used is very difficult since each type possesses its own potential for active growth. Thus, growth rates vary between cell types and are difficult to control. For examples, A7 cells grew less rapidly and tended to be less than the other two cell types in the coculture system. Second, the hepatic lobule spans about 140 μm *in vivo*, extending from the portal to the central area, toward which portal blood flows in a radial manner. According to Matsumoto *et al*[29], the liver is an organ composed of numerous groups of microscopic three-dimensional units (minimal radial-flow bioreactors) extending from the inflow side (composed of combinations of parabola-shaped inflow fronts) to the central vein[30]. According to this model, the liver microcirculation as observed *in vivo* could not be reproduced faithfully with a radial-flow bioreactor, since the distance between inflow and outflow sides in the bioreactor is about 1.5 cm (Figure 11). Third, bile canaliculus-like structures are formed between hepatocytes. Since we did not use the bile duct cells in this study, whether different cell types can reconstruct bile ducts remains to be elucidated[31]. Finally, although several questions remain, the results of the present study suggest that liver reconstruction is possible *in vitro*. Such organ reconstruction technology is expected to contribute greatly to the development of sophisticated artificial livers and other organs for transplantation. Our culture system may be a very important tool to maintain liver organ.

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BASIC RESEARCH

Interleukin-2 gene-encoded stromal cells inhibit the growth of metastatic cholangiocarcinomas

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Abstract

AIM: To demonstrate bone marrow stromal cells (BMSCs) can be used as an attractive target for genetic modification in the treatment of malignant diseases.

METHODS: Using a hamster model of biliary cancer, we investigated the therapeutic effects of interleukin-2 (IL-2) gene-modified BMSCs. Syrian golden hamsters were injected via the femoral vein with 5×10^5 cells of the KIGB-5 biliary cancer cell line (n=20). One week later, the hamsters were injected intraperitoneally with BMSCs containing Ad/hIL-2 and Ad/ΔE1, unmodified BMSCs, or RPMI only (control) and observed for 12 wk (n=5 /each group).

RESULTS: All hamsters treated with BMSCs containing Ad/hIL-2 survived with no evidence of the disease during this period. In contrast, hamsters in the other three groups showed disseminated metastases involving the lungs as early as 4 wk.

CONCLUSION: Ad/IL-2 therapy is effective in the treatment of biliary cancer.

Key words: Bone marrow stromal cell; Adenovirus/hIL-2; Biliary cancer

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INTRODUCTION

Cholangiocarcinoma arising from bile duct epithelium is the second most common primary liver cancer in the world[1]. In the United States, 2000-3000 new cases of cholangiocarcinoma are diagnosed per year[2]. At present, only surgical excision of all detectable tumors can improve 5-year survival[3-5]. However, most patients are not candidates for surgery and undergo only endoscopic or percutaneous biliary drainage procedures, such as plastic or metal stents. Systemic chemotherapy and radiation therapy are not able to enhance the survival of patients with cholangio carcinoma[6-11].

Introduction of specific genes into the body by infusion of vehicle cells that have been modified *ex vivo* offers a potential therapy for a number of diseases. Vehicle cells most commonly used for gene transfer include hematopoietic stem cells, peripheral blood lymphocytes and bone marrow stromal cells (BMSCs). Among these, BMSCs have several advantages including ease of culture and gene transduction, as well as specificity to hematopoietic organs such as bone marrow, spleen, and liver. These qualities make BMSCs ideal for gene transfer in hematologic diseases[12].

BMSCs have been shown to support hematopoiesis and have the potential to differentiate into cells of multiple lineages, including osteogenic, chondrogenic and adipogenic cells[13-15]. Human BMSCs can be easily obtained and grown in conventional *ex vivo* culture. In addition, BMSCs can be infused safely and have been shown to home to the bone marrow, spleen, lung, liver and kidney. Using a murine lymphoma model, we found that immunocell therapy with ad/hIL-2 encoded stromal cells shows promising therapeutic results[16]. Here we further investigated immunocell therapy for biliary cancer induced by the KIGB-5 cell line in Syrian golden hamsters, and found that Ad/hIL-2 genetically-modified BMSCs could provide adoptive immunocell therapy for biliary cancer.

MATERIALS AND METHODS

Animals

Female Syrian golden hamsters aged 6-8wk, purchased from Harlan (Indianapolis, Indiana, USA) were housed in the specific pathogen-free unit of the Animal Resource Center at the Asan Institute for Life Sciences and Technology.

Cholangiocarcinoma cell line

The KIGB-5 cell line, a gallbladder carcinoma cell line derived from Syrian golden hamsters, was established and kindly provided by Dr. Tajima (Nagasaki University, Nagasaki, Japan). This cell line was maintained in complete RPMI-1640 (GIBCO BRL, Grand Island, NY, USA) supplemented with 100 mL/L fetal bovine serum (FBS) (GIBCO BRL, Grand Island, NY, USA), 100 U/mL penicillin (Sigma, St. Louis, MO, USA), 0.1 mg/mL streptomycin (Sigma), and 10^{-5} mol/L β-mercaptoethanol (Sigma)[17]. The 293 cell line, derived from human embryonic kidney, was maintained in high glucose DMEM (GIBCO BRL) and 100 mL/L FBS (GIBCO BRL).

BMSC culture

Bone marrow cells were obtained by flushing the femurs and tibias of 6-8 wk old Syrian golden hamsters with RPMI-1640 medium (GIBCO BRL) using 2mL syringes with 23-guage needles. After a single-cell suspension was prepared, the cells were plated at a concentration of 5.0×10^5 /mL in 75 cm^2 plastic tissue culture flasks, and cultured in McCoy's 5A medium (Sigma) supplemented with 125 mL/L FBS, 125 mL/L horse serum (HS), 200mmol/L L-glutamine, 0.05 mg/mL hydrocortisone, 1 ng/mL recombinant human basic fibroblast growth factor (rh bFGF, Invitrogen Corporation, Groningen, Netherlands), and 0.5ng/mL rh IL-1α (Invitrogen) at 37 ℃ in 50 mL/L CO_2 and 950 mL/L air. The cultures were fed weekly by replacing 500 mL/L of the supernatant with fresh culture medium. When the cells became confluent, they were trypsinized and harvested for passage. Gene transduction was performed on cells after 5 passages.

Preparation of recombinant adenovirus

Three replication-deficient adenoviral vectors were used, namely Ad/Lac-Z containing the β-galactosidase gene, Ad/ΔE1 and Ad/hIL-2 harboring the human IL-2 gene. Each of these vectors was constructed from human adenovirus serotype 5 by homologous recombination. The expression of the inserted genes was driven by a CMV promoter. The recombinant adenoviruses were propagated into human 293 embryonic kidney cells, and the adenoviral titers were determined by a plaque-forming assay with these cells. Recombinant adenoviruses were diluted to a titer of 5.0×10^8 PFU/mL in viral supernatant and stored at -70 ℃.

Stromal cell transduction

Stromal cells (SCs) were seeded at a density of 1.0×10^5 /mL in 75 cm^2 plastic tissue culture flasks and allowed to grow. The medium was removed, the cells were washed twice with PBS, and the modified adenovirus was added at various multiplicities of infection (MOI). The cells were cultured at 37℃ for 24h in a humidified atmosphere of 50 mL/L CO_2.

The transduction efficiency of the recombinant adenoviruses was determined by an X-gal assay. BMSCs were transduced with Ad/Lac Z for 24h at 0, 10, 20, 50, 100 MOI. After one round of transfection the transduction efficiency was determined by the percentage of blue-stained cells, after subtraction of the percentage of non-specifically stained, uninfected BMSCs[7]. Stained positive cells were 5-10% at 10 MOI, 10-15% at 20 MOI, 40-50% at 50 MOI, 60-65% at 100 MOI (Figure 1).

From the results of the preliminary experiments we decided to use 50 MOI for further experiments.

X-gal assay

BMSCs were seeded in 12-well plates and transfected 24 h later with Ad/Lac Z. The cells were cultured for 24 h at 37 ℃ in a humidified atmosphere containing 50 mL/L CO_2. The media were removed and the cells were washed with PBS. Fixing solution was added to the transfected cells at room temperature for 5 min and removed. The cells were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside(X-gal) overnight at 37℃ in a humidified atmosphere containing 50 mL/L CO_2.

Stromal cell transplantation

To test for homing of gene-transduced stromal cells, 6-8 wk old Syrian gold hamsters were infused intraperitoneally (IP) or injected via the femoral vein with transduced stromal cells mixed in 0.1 mL PBS at a dose of 2.5×10^6 cells per animal. After one week, the hamsters were sacrificed and the bone marrow, spleen, liver, kidneys, and lungs were obtained. Single cells prepared from these organs using collagenase (GIBCO BRL) were cultured for four days and stained with X-gal to test for homing of the transfected cells.

RT-PCR

Total RNA was extracted using Trizol (Life Technologies, Invitrogen) following the manufacturer's instructions. The extracted RNA was resuspended in diethylpyrocarbonate-treated water at a concentration of 0.5 μg/μL. Two ug of each total RNA preparation was reverse-transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Boeringer, Manheim, Germany) in 20 μL reaction mixture containing 50 mmol/L DTT, 1 μg oligo (dT) and 0.125 mmol/L dNTPs, as described by the manufacturer. The cDNAs were stored at -20℃ or directly used for subsequent amplification. Amplification of human IL-2 mRNA was performed using primers: 5' -TTGCATTGCACTAAGTCTTGC-3' (forward) and 5' -CAATGGTTGCTGTCTCATCAG-3' (reverse), whereas amplification of GAPDH mRNA was performed using primers: 5'-ACCACAGTCCATGCCATCAC-3' (forward) and 5'-TCCACCACCCTGTTGCTGTA-3' (reverse). PCR was performed in reaction mixtures containing 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 200 μmol/L of each dNTP, 1 μmol/L of each primer, 0.5 units of Taq polymerase, and 1×concentration of PCR buffer, in a total volume of 50 μL. The amplification pro-

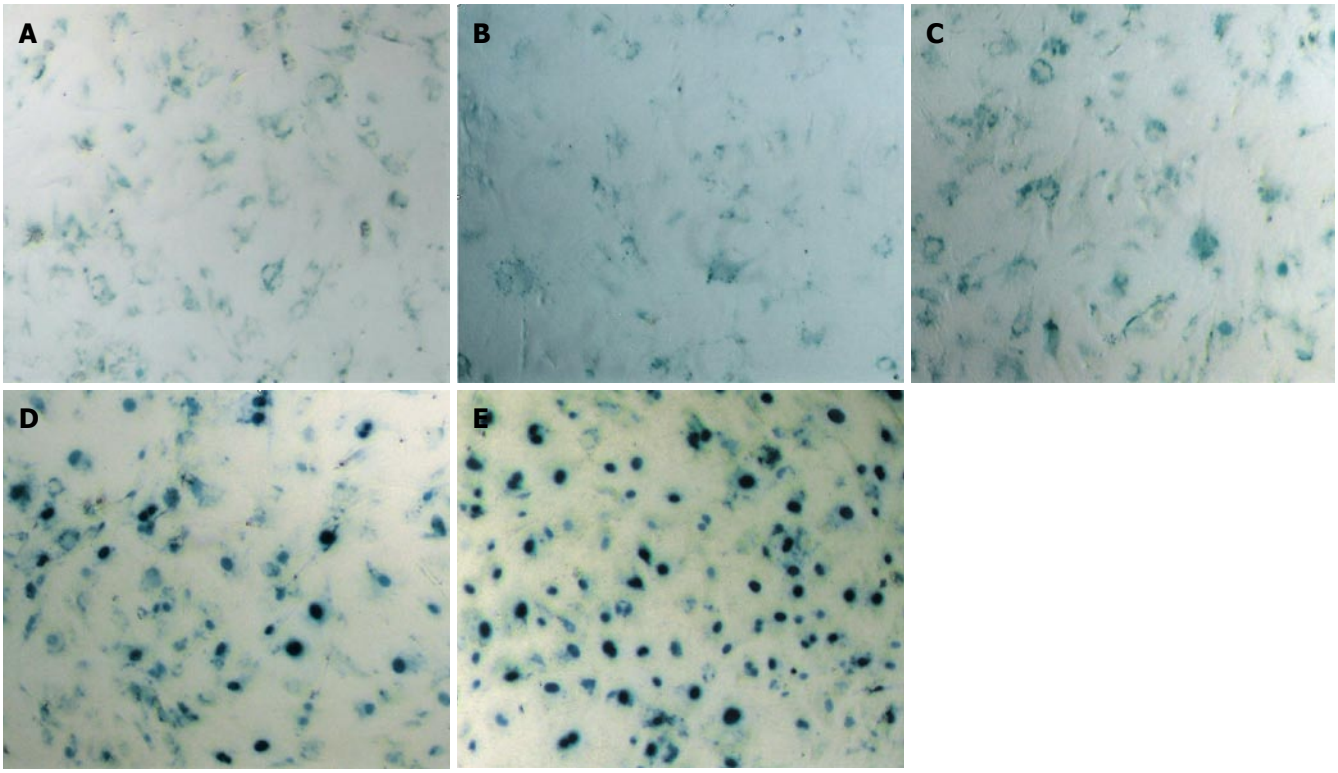

Figure 1 X-gal assay of hamster BMSCs transfected with Ad/ Lac Z at 0 (**A**), 10 (**B**), 20 (**C**), 50 (**D**), and 100 (**E**) MOI.

tocol consisted of 32 cycles of denaturation at 94℃ for 30 s, annealing at 55℃ (IL-2) or at 65℃ (GAPDH) for 30 s, and extension at 72℃ for 40 s (IL-2) or 80 s (GAPDH), using a Perkin-Elmer 9600 thermal cycler. The PCR products were separated on 15 g/L agarose gels stained with ethidium bromide.

hIL-2 ELISA

BMSCs were seeded at a concentration of 2.5×10^5 cells/mL and transduced with Ad/hIL-2 for 1, 2, 3, or 5 d .The supernatants were collected each day, filtered and tested for the presence of IL-2 by ELISA (Endogen, Woburn, MA, USA).

Preparation of cholangiocarcinoma-bearing hamster model

KIGB-5 cells were cultured for 6-7 passages, washed three times and resuspended in PBS. Each hamster was infused through the femoral vein with 5.0×10^5 KIGB-5 cells. After seven days, the tumor-bearing hamsters were divided into four groups and injected intraperitoneally with PBS ($n = 5$), unmodified BMSCs ($n = 5$), BMSCs+Ad/hIL-2 ($n = 5$) or BMSCs+Ad/ΔE1 ($n = 5$). Hamsters were sacrificed 4, 8, and 12 wk after tumor inoculation, and the number of metastatic lesions in each was assessed.

RESULTS

Evaluation of BMSC homing

Following transduction of BMSCs with Ad/LacZ at 50 MOI for 24 h, 2.5×10^6 cells were transplanted into each hamster. After one week, the hamsters were sacrificed and various organs including bone marrow, spleen, kidneys, liver, and lungs, were obtained. Single cell preparations were made from each of these organs and examined by X-gal assay. We found that the BMSCs made in our laboratory homed primarily into the bone marrow, spleen and liver, with fewer in the lungs and kidneys (Figure 2).

Expression of human IL-2

The Ad/hIL-2 construct was designed to express human IL-2 protein in BMSCs. To confirm hIL-2 expression in BMSCs transduced with Ad/hIL-2, we tested these cells by RT-PCR and ELISA. Both showed IL-2 expression in transduced BMSCs (Figure 3).

Gross and microscopic findings of metastatic lesions

Following injection of KIGB-5 cells into hamsters, we observed gross metastatic lung lesions after 8 wk (Figure 4).

Hamsters subsequently injected with RPMI, unmodified BMSCs, or BMSCs containing Ad/ΔE1 had multiple metastatic lung lesions 8 and 12 wk after tumor cell injection, but no metastatic lesions were observed in the liver or other organs. In contrast, no evidence of metastatic tumors was observed in hamsters treated with BMSCs containing Ad/hIL-2. These findings were further confirmed by microscopic examination (Figure 5).

DISCUSSION

Cholangiocarcinoma is an extremely aggressive tumor most frequently detected at an advanced stage. Fewer than 50% of patients with cholangiocarcinoma have a chance of curative surgery. Patients with advanced disease have a

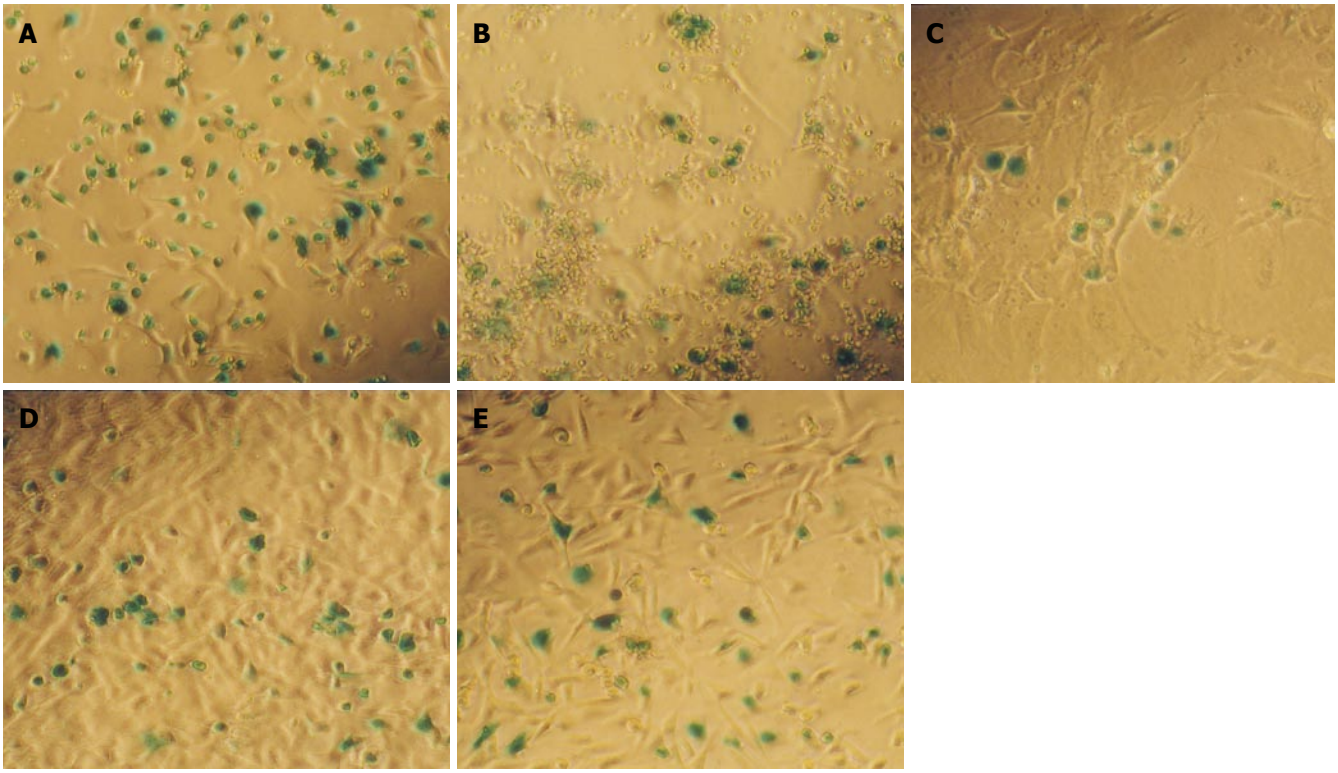

Figure 2 X-gal assay of post -transfection in BM (**A**), spleen (**B**), kidney (**C**), lung (**D**), and liver (**E**).

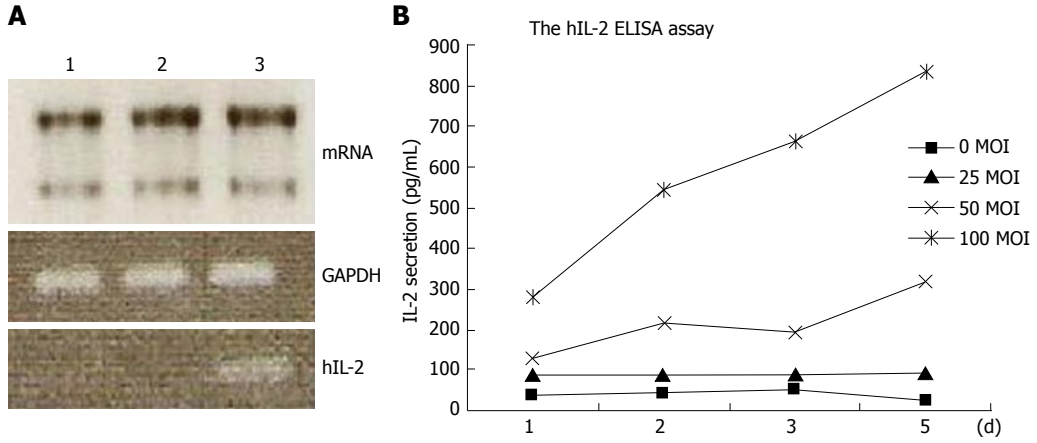

Figure 3 Expression and quantitative analysis of human IL-2. **A:** RT-PCR of in vitro cultured BMSC. Lane 1: $1x10^6$ stromal cells; lane 2: $1x10^6$ stromal cells containing 20 MOI Ad/ΔE1; lane 3: $1x10^6$ stromal cells containing 20 MOI Ad/hIL-2; **B:** ELISA assay of IL-2 secretion from in vitro cultured BMSCs (2.5 X 10^5/mL) of Syrian golden hamster after transduction with Ad/hIL-2 at MOI of 0(◆), 25(■), 50(▲), and 100 (●). Culture supernatants were harvested on different days and tested for hIL-2 secretion levels by ELISA.

median survival time of less than 6 mo and respond poorly to conventional chemotherapy[18]. Patients who undergo curative surgery have a 5 year-survival rate ranging from 16% to 32% because of early lymph node metastases[19, 20].

There have been no clinical trials using adoptive immunotherapy (IL-2 cytokine or dendritic cell and tumor lysates) for biliary cancer. We previously showed that BMSCs can be targeted to bone marrow in mice as well as to other organs, including spleen, liver, kidneys and lungs. Moreover, BMSCs containing Ad/hIL-2, with a low calculated dose of IL-2, are therapeutically effective for malignant lymphoma, because animals treated with this recombinant demonstrate 100% survival with no evidence of the disease, whereas control mice show extensive metastases[16]. These findings encouraged us to investigate whether Ad/hIL-2 would be effective in hamsters bearing biliary cancer induced by the KIGB-5 cell line. IL-2 is a cytokine with multiple biologic effects, including enhancement of the cytotoxic activity of cytotoxic T lymphocytes (CTLC). Previous clinical trials have shown that IL-2 has anti-tumor modulating effects in melanoma, renal cell carcinoma and hematologic malignancies[22]. In each of these models, the IL-2 dose is extremely high and has moderate to severe clinical side effects whereas the response rate ranges from 20-25%[21, 22].

Adenoviruses can bind efficiently to epithelial cells and efficiently transfer genes into both replicating and non-replicating cells, although the transfection efficiency varies depending on cell type. Therapy with BMSCs containing Ad/hIL-2 has potential advantages for gene delivery *in vivo* due to their ability to home to specific organs and to secrete IL-2 into the local microenvironment. This may en-

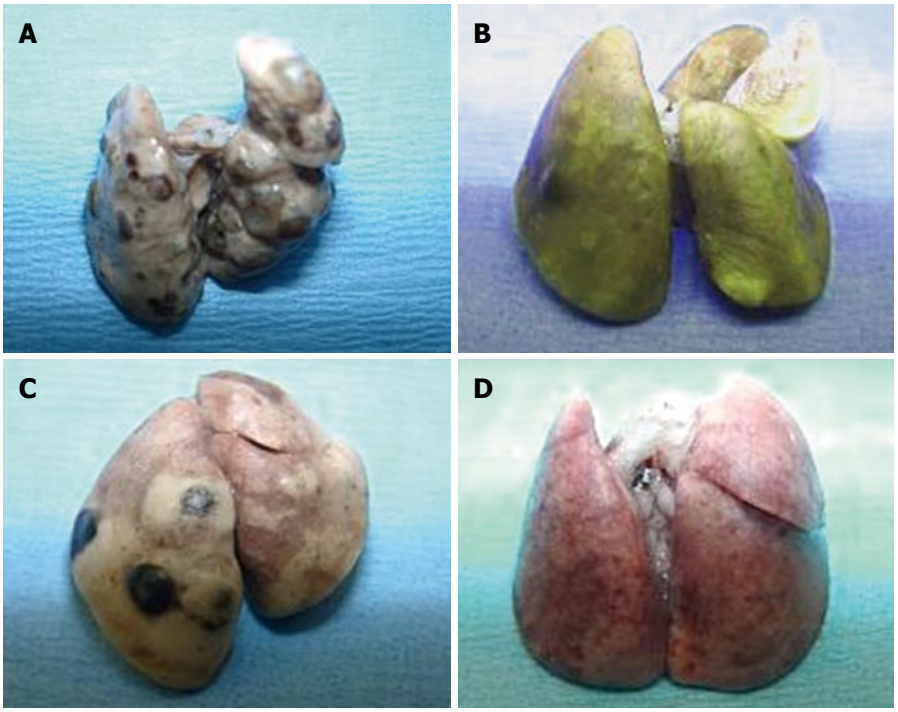

Figure 4 Gross findings of metastatic lung lesions in Syrian hamsters 8 wk after injection of KIGB-5 tumor cells in RPMI control (**A**), BMSCs (2.5×10^6 cells/d) (**B**), BMSCs containing 50 MOI Ad/ΔE1 (2.5×10^6 cells/day) (**C**), and BMSCs containing 50 MOI Ad/hIL-2 (2.5×10^6 cells/d) (**D**). Hamsters injected with RPMI, BMSCs, or BMSCs containing Ad/ΔE1 showed multiple metastatic lung lesions 8 wk after tumor injection, whereas hamsters injected with BMSCs containing Ad/IL-2 showed no evidence of disease.

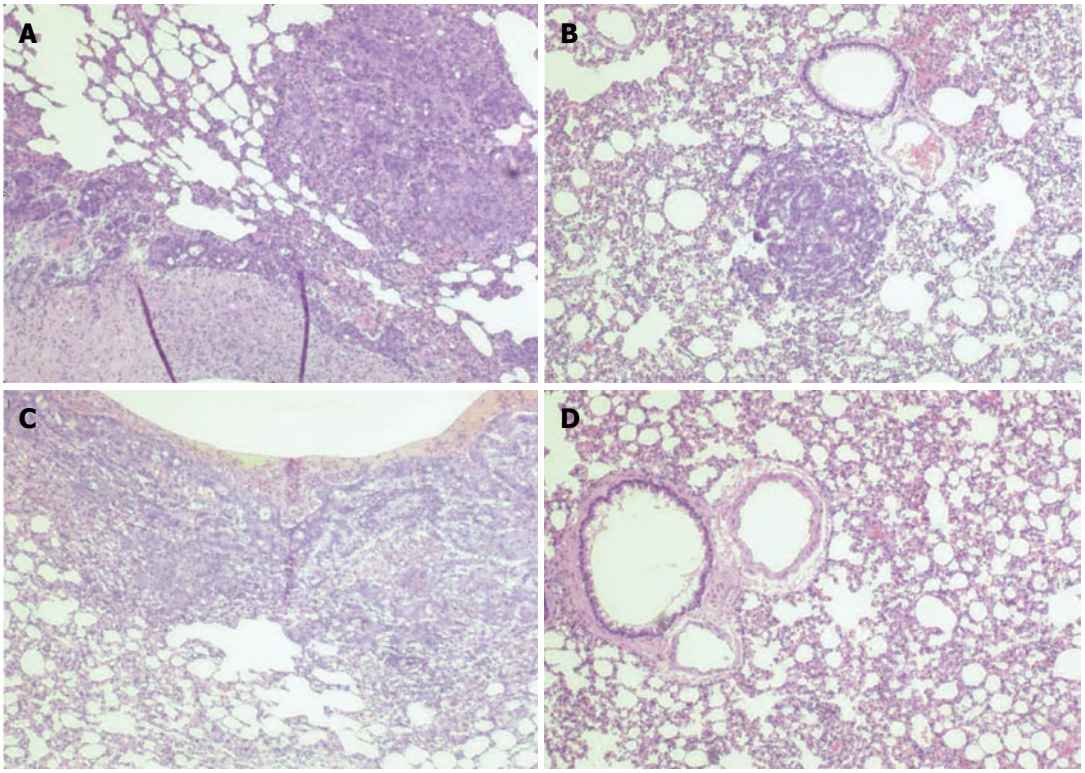

Figure 5 Microscopic findings in Syrian hamsters 8 wk after injection of KIGB-5 tumor cells in RPMI control (**A**), BMSCs (2.5×10^6 cells/d) (**B**), BMSCs containing 50 MOI Ad/ΔE1 (2.5×10^6 cells/d) (**C**), and BMSCs containing 50 MOI Ad/hIL-2 (2.5×10^6 cells/d) (**D**). Hamsters injected with RPMI, BMSCs, or BMSCs containing Ad/ΔE1 showed multiple metastatic lesions in both lungs, whereas hamsters injected with BMSCs containing Ad/IL-2 showed no evidence of disease.

hance cytotoxicity against tumor cells while reducing side effects.

Using BMSCs containing Ad/Laz ((β-galactosidase gene), we observed strong positive X-gal staining in the bone marrow, spleen, liver, lungs and kidneys. The transduction efficiency of these BMSCs was 70% at 50 MOI and 90% at 100 MOI. At 50 MOI, BMSCs containing Ad/hIL-2 showed effective anti-tumor effects. These animals showed no evidence of the disease, whereas the control animals developed widespread metastases during the 12 wk observation period. In addition, hamsters treated with BMSCs containing Ad/hIL-2 showed no evidence of side effects until the time of sacrifice. These findings suggest that BMSCs containing Ad/hIL-2 can implant into various organs and that locally-transplanted IL-2 stimulates and activates T4 and T8 cells leading to an attack on tumor cells via paracrine effects, thus killing tumor cells at metastatic sites.

One limitation of this study is that BMSCs were infused prior to the formation of measurable tumor mass

in hamsters, thus not allowing us to determine whether the effect of IL-2 was dependent on tumor burden. However, given that BMSCs containing Ad/hIL-2 could suppress cholangiocarcinoma development in hamsters, this therapy may be effective as an adjuvant treatment after curative resection in minimizing residual disease after debulking surgery. These findings suggest that treatment with BMSCs containing Ad/hIL-2 may be one of the potential modalities for cholangiocarcinoma, especially for eradicating residual metastatic biliary cancer.

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BASIC RESEARCH

S-adenosyl-methionine decreases ethanol-induced apoptosis in primary hepatocyte cultures by a c-Jun N-terminal kinase activity-independent mechanism

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Abstract

AIM: To determine the role of c-Jun N-terminal kinase (JNK) activity in ethanol-induced apoptosis and the modulation of this signaling cascade by S-Adenosyl-methionine (AdoMet).

METHODS: Primary hepatocyte cultures were pretreated with 100 μmol/L SP600125, a selective JNK inhibitor, 1 mL/L DMSO or 4 mmol/L AdoMet and then exposed to 100 mmo/L ethanol. Hepatocyte apoptosis was determined by the TUNEL and DNA ladder assays. JNK activity and its inhibition by SP600125 and AdoMet were determined by Western blot analysis of c-jun phosphorylation and Bid fragmentation. SP600125 and AdoMet effects on the apoptotic signaling pathway were determined by Western blot analysis of cytochrome c release and pro-caspase 3 fragmentation. The AdoMet effect on glutathione levels was measured by Ellman's method and reactive oxygen species (ROS) generation by cell cytometry.

RESULTS: The exposure of hepatocytes to ethanol induced JNK activation, c-jun phosphorylation, Bid fragmentation, cytochrome c release and pro-caspase 3 cleavage; these effects were diminished by SP600125, and caused a significant decrease in ethanol-induced apoptosis ($P < 0.05$). AdoMet exerted an antioxidant effect maintaining glutathione levels and decreasing ROS generation, without a significant effect on JNK activity, and prevented cytochrome c release and pro-caspase 3 cleavage.

CONCLUSION: The JNK signaling cascade is a key component of the proapoptotic signaling pathway induced by ethanol. JNK activation may be independent from ROS generation, since AdoMet which exerted antioxidant properties did not have a significant effect on JNK activity. JNK pathway modulator agents and AdoMet may be components of promising therapies for alcoholic liver disease (ALD) treatment.

Key words: Alcoholic liver disease; c-Jun N-terminal kinase; Apoptosis; SP600125; S-Adenosyl methionine; Bid; Reactive oxygen species

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INTRODUCTION

Ethanol abuse increases the risk of developing liver damage, such as fatty liver, hepatitis, cirrhosis and the development of viral hepatitis and hepatocarcinomas[1-3]. Alcoholic liver disease (ALD) is a common health problem in Western countries, therefore the diagnostic evaluation and clinical management of ALD are major issues of concern. Clinical management depends on the extent of ALD and, although alcohol abstinence and supportive care are the classical treatments for ALD, the development of new therapies to improve ALD outcome are being examined. Among these new therapeutic agents are small c-jun N–terminal kinase (JNK) inhibitors, such as SP600125 and S-Adenosyl methionine (AdoMet), the principal biological methyl donor and also a glutathione (GSH) precursor in the liver[4-6]. However, an incomplete knowledge of the molecular mechanisms regulated by these agents in ALD development has complicated the validation of these new therapeutic approaches.

The JNK signaling pathway, a member of the mitogen-activated protein kinases family, regulates cell differentiation, proliferation, inflammation, cell survival and cell death in response to stress signals[7,8]. Acute ethanol exposure induces JNK activation, promoting hepatocyte apoptosis[9-11], which has been strongly correlated with liver disease and postulated as the "nexus of liver injury and fibrosis"[12]. Small molecule JNK inhibitors, such as SP600125[13], have been proved to exert a protective effect in reperfusion liver injury and are suggested as optional treatment for liver disease[14-16]. Thus, the JNK signaling transduction pathway has emerged as an interesting element in liver disease development, opening the possibility that its modulation may represent an important approach for ALD treatment. Besides specific small molecule inhibitors, JNK activity may be modulated by other therapeutic agents, such as antioxidants[17-19]. Indeed, in diverse aspects of cellular function, JNK is implicated as a redox stress cell sensor[20]. Alcohol ingestion promotes oxidative stress[21-24] and causes changes in intrahepatic GSH[25,26]. This events may be related to JNK activation induced by ethanol. AdoMet, which has been shown to inhibit hepatocyte apoptosis[27], is increasingly used for liver disease treatment, although its protective mechanisms still remain unclear. A possible hepatoprotective mechanism has been attributed to AdoMet effects on the regulation of cytosol and mitochondrial GSH levels[28-30]. The up-regulation of GSH levels may have a down-regulatory effect on JNK signaling, since GSH acts as a key cell signaling modulator[31,32].

Although JNK has been identified as a pro-apoptotic signaling pathway involved in ALD development, the mechanism by which JNK induces programmed cell death remains far less clear[9,14,16], limiting the identification and validation of JNK-activity modulator agents as a potential ALD treatment. In this study, we determined the effect of the small JNK inhibitor SP600125 and AdoMet on the apoptotic pathway activated by ethanol and a possible modulating effect of AdoMet on JNK activity as part of its hepatoprotective mechanism. We found that JNK activation played a critical role in ethanol-induced apoptosis by means of its effect on Bid fragmentation, a key pro-apoptotic target of JNK. Even though AdoMet increased GSH concentration and protected against ethanol-induced apoptosis, we did not find an effect of AdoMet on JNK activity. These results strongly suggest that JNK activation induced by ethanol is independent of ROS generation, and that the AdoMet protective effects lie mainly at mitochondrial level.

MATERIALS AND METHODS

Materials

Anti-JNK-P and DeadEnd™ Colorimetric TUNEL System were obtained from Promega (Madison, WI). Antibodies against JNK and c-jun-P were purchased from Cell Signaling. c-jun, Bid, caspase 3 and cytochome c antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin monoclonal antibody was kindly provided by Dr. Manuel Hernández (CINVESTAV, IPN México). JNK inhibitor SP600125 and caspase 8 inhibitor Z-IETD-fluoromethyl ketone were purchased from Calbiochem (San Diego, CA). Collagenase, ethanol, dimethyl sulfoxide (DMSO), AdoMet in p-toluene sulfonate salt form, 2', 7' -dichloro-dihydrofluorescin diacetate (DCFH-DA) and all other reagents were purchased from Sigma Chemical Company (St. Louis,MO). Electrophoresis reagents and Protein assay kit were from Bio-Rad (Richmond, Calif.)

Animals

Male Fisher 344 rats with an average weight of 200 g were obtained from the Lab Animal Facility UPEAL, CINVESTAV. Animal care was performed according to the guidelines established by the Institutional Animal Care Committee

Hepatocyte isolation and treatments

Rat hepatocytes were isolated from male Fisher rats (weighing 180-200 g) by collagenase perfusion as previously described[33]. Viable hepatocytes were separated by Percoll gradient centrifugation at 1500 r/min for 2 min at 4 °C. Subsequently, cell viability was examined by trypan blue exclusion, and was over 90%. Hepatocytes were plated in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 100 mL/L bovine serum (Gibco-BRL). After 2 h incubation, the medium was changed to William's E medium containing 100 mL/L bovine serum, 1.5 U/mL insulin, 20 nmol/L dexamethasone, 100 μg/mL strep tomycin and 100 U/mL penicillin. Cells were cultured overnight at 37 °C in a humidified atmosphere containing 50 mL/L CO_2. The day after plating, cells were washed with Hank's balanced salt solution (HBSS), and incubated in William's E medium (supplemented with 1 mL/L of fetal calf serum, 100 μg/mL streptomycin and 100 U/mL penicillin) with either 100 μmol/L SP600125, 25 μmol/L Z-IETD-FMK or 1 mL/L DMSO as a vehicle control for 2 h. Another set of cultures was pre-incubated with 4 mmol/L AdoMet for 1 h. Then 100 mmol/L ethanol was added and the dishes were sealed with parafilm to prevent evaporation. The cells were incubated for different periods of time at 37 °C before collection.

Cell viability assessment

Cell viability was determined using the MTT (methyl thiazole tetrazolium) assay as previously described[34]. Briefly, at indicated times after pretreatments and ethanol exposure, media were removed and MTT solution (0.4 mg/mL in media, filter sterilized) was added and the cells were incubated for 2 h at 37 °C. Plates were washed twice with PBS. The resultant formazan product was dissolved by addition of 500 μL DMSO to the plate. Optical density was measured at $A_{595\ nm}$ and cell survival was expressed as percentage of absorbance.

DNA gel electrophoresis

After different pretreatments and 24 h of ethanol exposure, hepatocytes (detached and attached) were harvested and washed in ice-cold PBS by centrifugation at 4°C at 1500 r/min for 5 min in an Eppendorf microcentrifuge. Approximately 8×10^5 cells were resuspended in lysis buffer (200 mmol/L EDTA; 100 mmol/L Tris, pH 8.0;

8 g/L sodium lauryl sarcosine; 20 μg/mL DNase-free RNasa) and incubated at 37 ℃ for 2 h, followed by addition of proteinase K (200 μg/mL) and another overnight incubation at 50 °C. DNA was electrophoresed on 18 g/L agarose gel at 35 mv, stained with ethidium bromide and visualized under UV light[35].

TUNEL assay

The TUNEL assay was performed using a DeadEnd colorimetric apoptosis detection system kit. Rat hepatocytes were cultured on Falcon Chamber slides pre-coated with poly-L-lysine. After different pretreatments and 24 h ethanol exposure, slides were fixed by immersion in 40 g/L paraformaldehyde in PBS for 25 min at room temperature. Then, the TUNEL assay was performed as previously described[36] following the manufacturer's instructions. One hundred cells were counted in three randomly selected microscopic fields and cell apoptotic rate was expressed as a percentage of the total cells counted.

Isolation of mitochondria

Mitochondria were isolated from 4.8×10^6 cells. Cells were washed twice with HBSS, scrapped and centrifuged for 10 min at 1 500 r/min at 4 ℃ in an Eppendorf microcentrifuge. Cell pellets were resuspended in permeabilizing buffer (210 mmol/L D-manitol, 10 mmol/L HEPES, 0.2 mmol/L EGTA, 50 mmol/L succinate, and addition just before use of 70 mmol/L sacarose, 1.5 g/L bovine serum albumin and 80 μg/mL digitonine, pH 7.2) and incubated at 4 ℃ for 20 min. Permeabilized cells were centrifuged at 170 r/min for 10 min at 4 ℃. The supernatant was centrifuged at 13 000 *g* for 10 min at 4 ℃, the second supernatant obtained contained the cytosolic fraction. The pellet resultant from the first centrifugation after the permeabilization step was incubated with 1 mL/L Triton X-100 in PBS for 20 min at 4 ℃ and centrifuged at 13 000 *g* for 10 min at 4 ℃, thus the supernatant contained the mitochondrial fraction. Supernatants were precipitated with 50 g/L sulfosalicylic acid and resuspended in PBS[37]. Protein concentration in the supernatant was determined by the Lowry method.

Immunoblot analysis

Cellular protein was extracted at 4 ℃ in kinase lysis buffer (20 mmol/L Hepes pH 8.0, 136 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 mmol/L KCl, 2 mmol/L $MgCl_2$, 50 mmol/L sodium fluoride, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 2 g/L SDS) or Chaps buffer for samples to determine Bid and pro-caspase cleavage (50 mmol/L Tris-HCl, 2 mmol/L EDTA, 1 g/L Chaps, 1 mmol/L phenylmethylsulfonyl fluoride and Complete (BioRad) 25×). Cell lysates were centrifuged at 12 000 *g* for 10 min at 4 ℃. The supernatant protein concentration was determined by Lowry method. Whole cell extracts and cytosolic or mitochondrial fractions were resolved on SDS-PAGE and transferred onto nitrocellullose membrane. Blots were incubated overnight at 4 ℃ with antibodies against JNK, phospho JNK, c-jun, phospho c-jun, Bid, caspase 3 (Bid antibody detected full length Bid and caspase 3 antibody recognized the precursor of pro-caspase 3 form) and cytochome c. Then, blots were incubated with horseradish peroxidase-conjugated antibody and developed by chemiluminescence.

Intracellular GSH content determination

Hepatocytes were incubated under control conditions in William's E medium only, or were pretreated with 4 mmol/L AdoMet for 1 h and then exposed to ethanol, or only exposed to ethanol. After the indicated times, cells were harvested and washed with HBSS buffer (pH 7.4). Cells (3×10^6) were sonicated for 1 min in ice-cold 50 g/L sulfosalicylic acid, incubated for 20 min on ice and centrifuged at 12 000 *g* at 4 ℃ for 30 min. The resultant thiol extract was assayed by the method of Ellman with previously reported modifications[38,39]. The pellet obtained from the centrifugation was washed twice with 50 g/L sulfosalicylic acid and resuspended in 0.5 mol/L NaOH. Protein concentration was determined by the method of Lowry. The GSH content (including other eventual thiols) was expressed as nmol per mg of protein.

Measurement of intracellular reactive oxygen species (ROS)

The level of intracellular ROS was measured by the change in fluorescence resulting from oxidation of DCFDA. After treatment for indicated times, hepatocytes were washed with HBSS and incubated with 5 μmol/L DCFH-DA for 30 min at 37 ℃. Hepatocytes were harvested and resuspended in HBSS, and then 5 g/L propidium iodide was added to detect dead cells. Intracellular ROS levels were measured on a Benckton Dickinson FACS Calibur. Ten thousand events were recorded for the analysis[40].

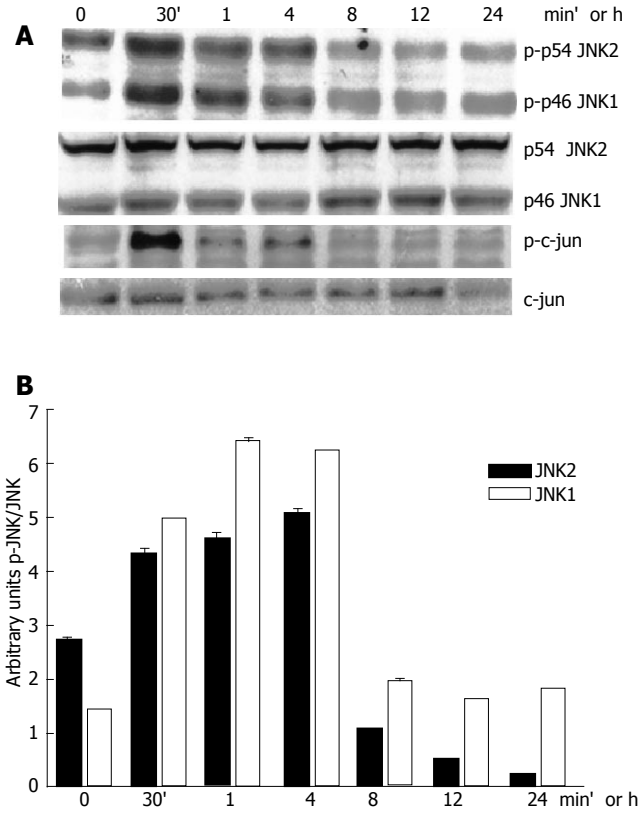
Statistical analysis

Data were analyzed using one-way ANOVA to determine differences between all independent groups. Differences between 2 groups were tested using two-tailed unpaired Student's *t*-test. $P<0.05$ was considered statistically significant.

RESULTS

Inhibition of ethanol-induced JNK activation by SP600125

We first confirmed that 100 mmol/L ethanol exposure promoted JNK activation in our system. Ethanol induced JNK phosphorylation. We detected that the JNK1 basal activity was lower than JNK2, while JNK1 activity increment was higher than that for JNK2. Maximal JNK activation induced by ethanol was detected after 30 min and remained above control levels until 8 h after, as detected by c-jun phosphorylation, one of the most commonly measured parameters of JNK activity (Figure 1A-B). Next, we determined the effect of 100 μmol/L SP600125 on JNK activity. JNK activity significantly decreased in SP600125-pretreated hepatocytes compared to hepatocytes exposed to ethanol only ($P<0.05$), as determined by a decrease in c-jun phosphorylation (Figure 2A-B). The Western blot and densitometric analyses shown are representative of three individual experiments.



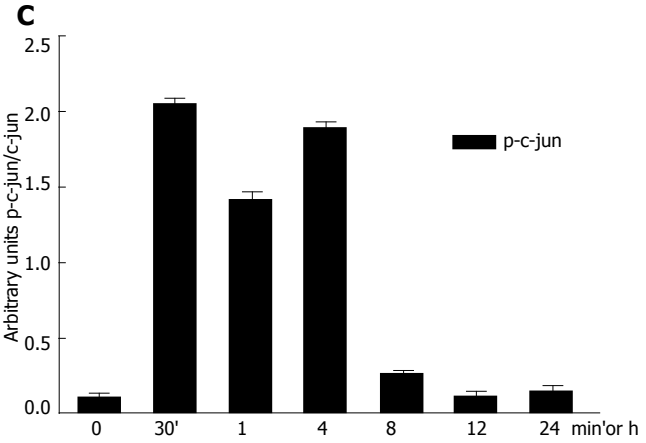


Figure 1 Inhibition of 100 mmol/L ethanol-induced JNK activation by SP600125. **A:** Western blot; **B:** JNK; **C:** P-c-jun densitometric analysis.

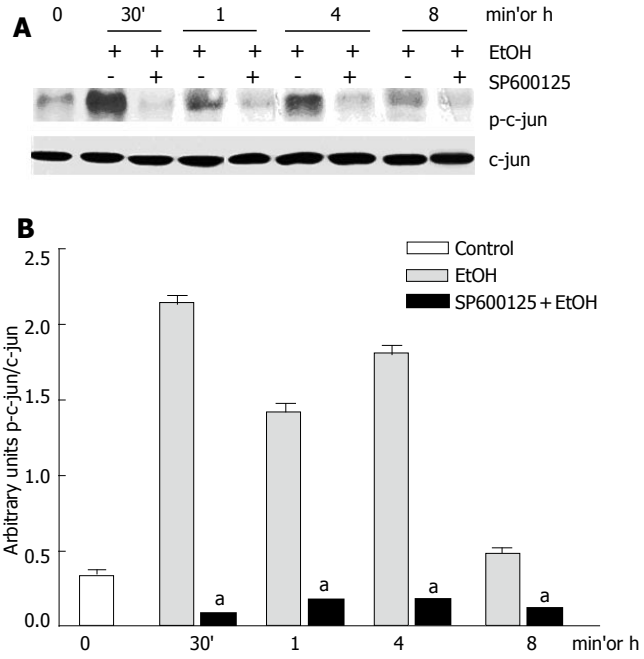


Figure 2 c-jun phosphorylation inhibition by 100 μmol/L SP600125. **A:** Western blot; **B:** Densitometric analysis. Each bar represents mean±SEM of $n = 3$. [a]indicates a significant difference ($P < 0.05$) between c-jun phosphorylation in hepatocytes exposed to ethanol only and SP600125-pretreated hepatocytes.

Inhibition of ethanol-induced apoptosis by SP600125 and AdoMet

Once we identified an effect of both ethanol and SP600125 on JNK activity, we determined the effect of SP600125 and AdoMet on the apoptosis process induced by ethanol. Ethanol-induced apoptosis was significantly reduced ($P < 0.05$) in hepatocyte cultures pretreated with 100 μmol/L SP600125 or 4 mmol/L AdoMet, but not in hepatocytes pretreated with 1 g/L DMSO, the vehicle for SP600125, as shown by the DNA laddering assay (Figure 3A) or TUNEL assay (Figure 3B). A 24-h exposure to 100 mmol/L ethanol produced approximately 47% cell death in non-pretreated hepatocytes as compared to 23% and 25% cell death observed in SP600125- and AdoMet-pretreated cultures, respectively. Cells pretreated with DMSO but not stimulated with ethanol showed 5% cell death (Figure 1C).

Protection of SP600125 and AdoMet on ethanol hepatocyte injury

In order to determine whether or not the survival of hepatocytes exposed to 100 mmol/L ethanol was modulated by SP600125 and AdoMet, we determined survival rates after different periods of 100 mmol/L ethanol exposure. Cell metabolic activity, determined by the MTT assay, significantly decreased after 12 h in cells only exposed to ethanol ($P < 0.05$) compared to the cells pre-incubated with AdoMet, SP600125 or incubated under control conditions. Cell metabolic activity, measured as the percentage of tetrazolium salt reduction, decreased from 75% observed in pretreated hepatocytes to 40% observed in hepatocytes exposed to ethanol only (Figure 4).

AdoMet effect on GSH intracellular levels from ethanol-incubated hepatocytes

The JNK signaling pathway activation may be modulated by antioxidants. AdoMet, a GSH precursor in liver, prevented ethanol-induced apoptosis as aforementioned. We tested if, through an antioxidant effect, AdoMet may be related to an inhibitory action of JNK activity as a possible hepatoprotective mechanism. We first determined the effect of 4 mmol/L AdoMet pretreatment on GSH intracellular levels in ethanol-incubated hepatocytes. Pre-incubation with 4 mmol/L AdoMet significantly prevented GSH decrease induced by the exposure of cultured hepatocytes to 100 mmol/L ethanol ($P < 0.05$), and moreover, maintained GSH levels above those found in control hepatocyte cultures. A significant increase in GSH concentra-

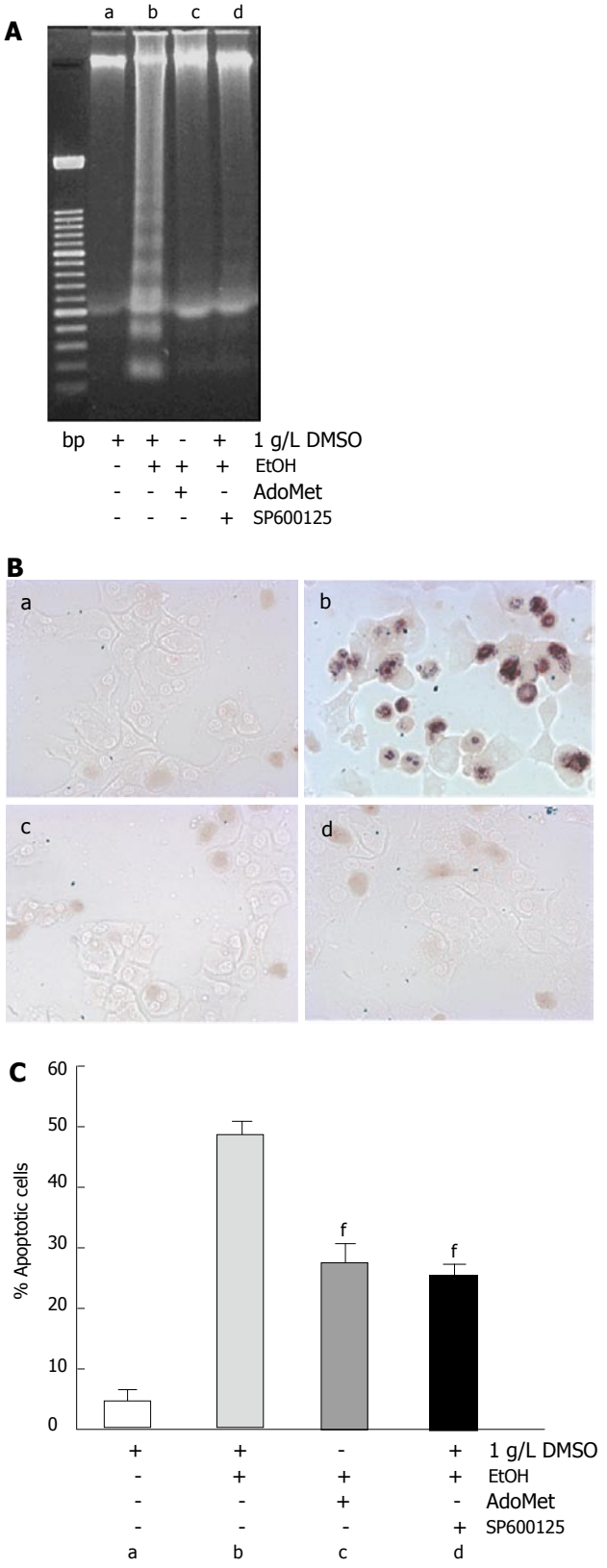


Figure 3 Effect of SP600125 and AdoMet in apoptosis induced by ethanol. **A:** DNA ladder assay; **B:** 40x TUNEL assay; **C:** data from TUNEL assay; a: control; b: ethanol; c: AdoMet+ ethanol; d: SP600125 + ethanol. The data represent mean ±SE of $n = 3$. [f]indicates a significant difference ($P < 0.05$) between SP600125 or AdoMet-pretreated cells and cells only pretreated with DMSO and exposed to ethanol.

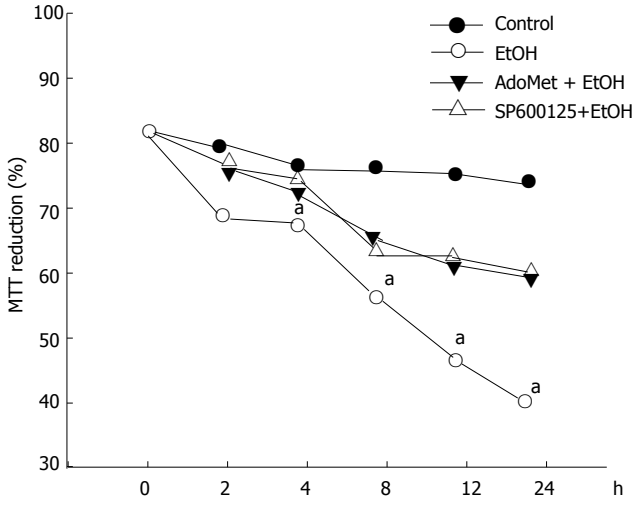


Figure 4 Protective effect of SP600125 and AdoMet on ethanol hepatocyte injury. Data represent percentage of MTT reduction in mean±SEM of $n = 3$. [a]represents a significant difference ($P < 0.05$) in MTT reduction between pretreated and non-pretreated cells.

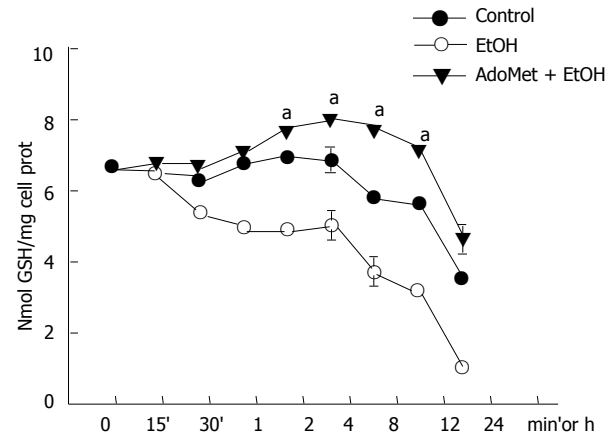


Figure 5 Prevention of GSH decrease in hepatocyte cultures exposed to ethanol by AdoMet. Data represent percentage change of ROS levels in mean±SEM of $n = 3$. [a]represents a significant difference ($P < 0.05$) in ROS generation between pretreated cells and cells exposed to ethanol only.

tion was observed between 8 and 12 h in hepatocytes pretreated with AdoMet compared to hepatocytes incubated in control medium (Figure 5) ($P < 0.05$). Furthermore, ROS generation in hepatocytes pretreated with 4 mmol/L AdoMet and then exposed to ethanol was maintained as in control cells in comparison to hepatocyte cultures exposed to ethanol only, in which a significant ROS generation was observed (Figure 6) ($P < 0.05$).

Effect of AdoMet on JNK activity

Once we determined that AdoMet had antioxidant effects, we tested if AdoMet exerted an inhibitory action on JNK activity. We first measured AdoMet effect on c-jun phosphorylation. AdoMet pretreatment slightly decreased JNK activity induced by ethanol treatment by 25.4% with respect to the cultures exposed to ethanol only, contrasting with the effect of the selective JNK inhibitor which produced a c-jun phosphorylation inhibition of approximately 68.8% with respect to ethanol- treated samples. So, the decrease on c-jun phosphorylation induced by AdoMet was

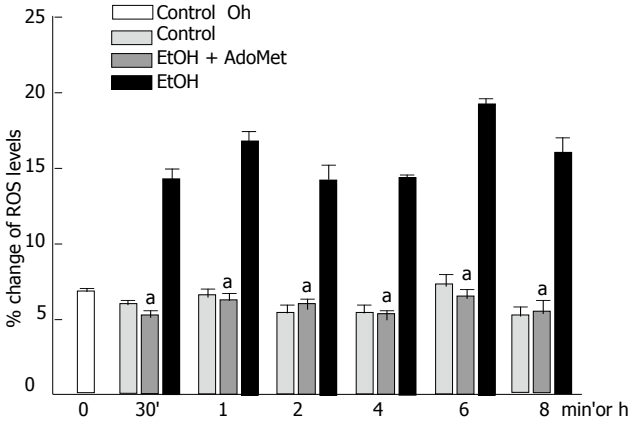


Figure 6 Decrease of ethanol-induced ROS generation in hepatocyte cultures by AdoMet. ROS generation in hepatocytes pretreated with Ado Met and ethanol was significantly reduced compared to hepatocyte cultures exposed to ethanol only ($^aP<0.05$).

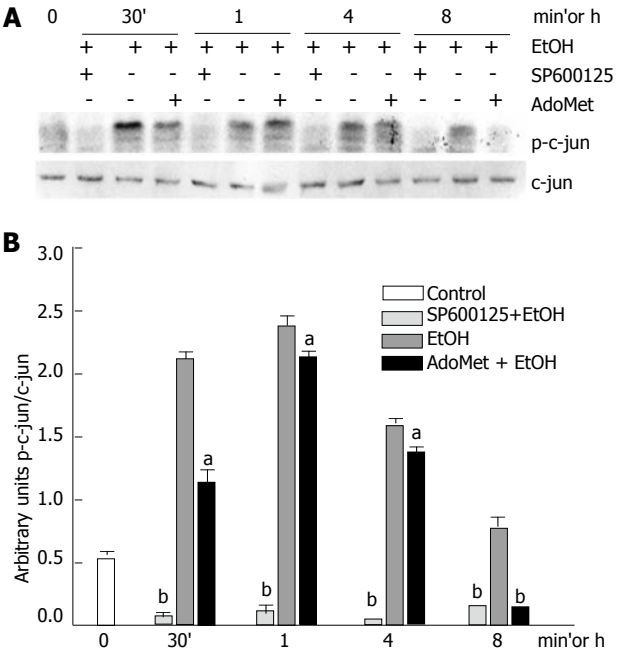


Figure 7 Effect of AdoMet on JNK activity. **A:** Western blot; **B:** densitometric analysis. Data represent mean±SEM of $n = 3$. ($^aP < 0.05$) or ($^bP < 0.01$) indicates a significant difference in c-jun phosphorylation between SP600125- or AdoMet-pretreated cells and non-pretreated cells exposed to ethanol.

not comparable to the decrease produced by SP600125, a selective JNK inhibitor (Figure 7).

Effects of SP600125 and AdoMet on Bid fragmentation, another JNK target

We next determined the effects of SP600125 and AdoMet on Bid cleavage. Bid is a proapoptotic member of the Bcl-2 family, which may represent another important JNK target. We observed that 100 mmol/L ethanol hepatocyte exposure promoted Bid cleavage between 4 h and 24 h. SP600125 hepatocyte pretreatment significantly prevented Bid fragmentation compared to cells stimulated with ethanol only between 4 h to 24 h ($P < 0.05$). Bid has been reported as a caspase 8 target. Indeed, we found that pre-

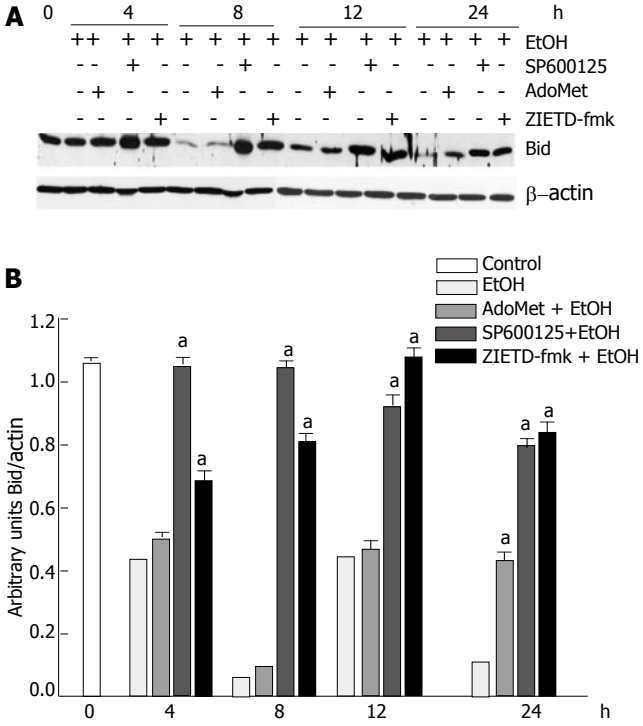


Figure 8 Preventive effect of SP600125 and AdoMet on Bid cleavage, a component of the JNK signaling pathway. **A:** Western blot; **B:** densitometric analysis. The data represent mean± SE for $n = 3$. ^{a} indicates a significant difference in Bid fragmentation between SP600125- or Z-IETD-fmk-pretreated cells and cells exposed to ethanol only ($P < 0.05$).

incubation with 25 μmol/L Z-IETD-fmk, a selective caspase 8 inhibitor, significantly decreased Bid fragmentation induced by ethanol (Figure 8) ($P < 0.05$). These results suggested that Bid cleavage induced by ethanol not only depends on JNK, but also on caspase 8 activity. Nevertheless, even if AdoMet exerted antioxidant properties, it did not prevent Bid fragmentation induced by ethanol. Western blot and densitometric analyses shown are representative of three independent experiments.

Decrease in ethanol-induced cytochrome c release and caspase 3 activation by SP600125 and AdoMet

Apoptosis induced by 100 mmol/L ethanol involves Bid fragmentation, which is responsible for cytochrome c release with the subsequent activation of caspase 3. We determined the effect of SP600125 and AdoMet on these apoptotic components. Pretreatment of hepatocyte with SP600125 prevented Bid cleavage induced by ethanol and decreased cytochrome c release from mitochondria between 8 h and 24 h after ethanol exposure. Even though AdoMet did not have an effect on Bid fragmentation, it decreased cytochrome c release from mitochondria (Figure 9). Cytochrome c release contributes to pro-caspase 3 fragmentation, so we observed that cytochrome c release prevention by SP600125 and AdoMet also decreased pro-caspase 3 fragmentation (Figure 10). These results indicated that the hepatoprotective effects of SP600125 and AdoMet converge at mitochondrial level, but AdoMet antiapoptotic action mainly lies in an inhibition of the mitochondrial apoptotic pathway.

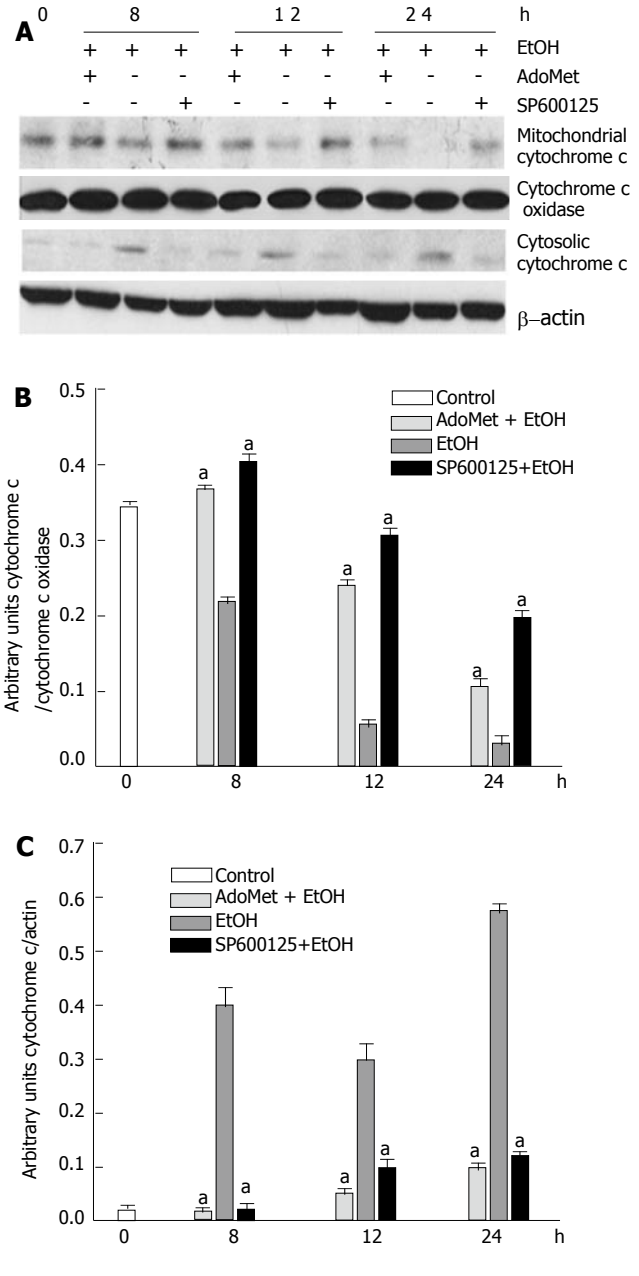


Figure 9 Prevention of cytochrome c release by AdoMet and SP600125. **A:** Western blot; **B:** mitochondrial cytochrome c; **C**: cytosolic cytochrome c densitometric analysis. [a]represents a significant decrease ($P < 0.05$) in cytochrome c release in AdoMet- and SP600125-pretreated hepatocytes in comparison with non-pretreated cells.

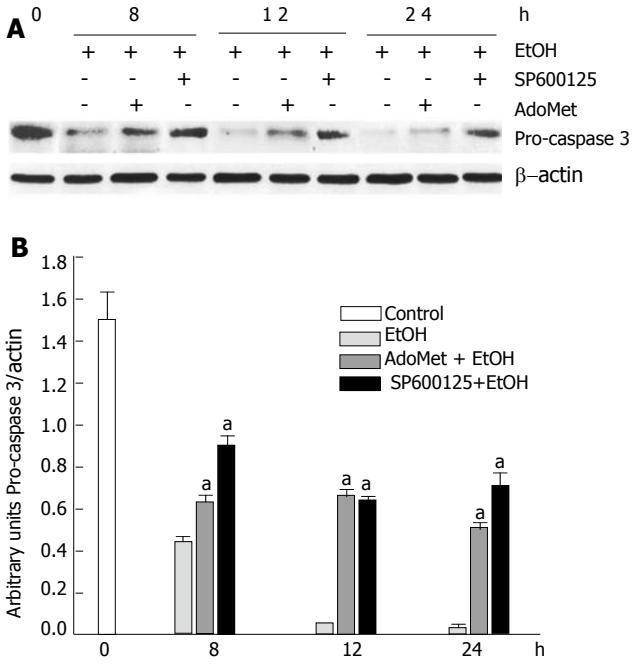


Figure 10 Decrease of ethanol-induced pro-caspase 3 fragmentation by SP600125 and AdoMet. **A:** Western blot; **B:** densitometric analysis. [a]indicates a significant difference ($P < 0.05$) between SP600125- and AdoMet-pretreated hepatocytes compared to non-pretreated hepatocytes.

DISCUSSION

This study points out that modification of the JNK signaling transduction pathway by selective JNK inhibitors and mitochondrial function preservation by AdoMet are among several key factors that must be considered to develop successful therapies for ALD treatment. We determined the role of JNK activity in the apoptotic process induced by ethanol in primary hepatocyte cultures and the possible modulator effect of JNK activity by AdoMet, an agent increasingly used in ALD treatment.

JNK activity is related to proapoptotic responses, but it is also linked to antiapoptotic signals and cell proliferation[41,42], thus the functions and components of this signaling cascade still remain controversial. We exposed hepatocytes to 100 mmol/L ethanol, considering previous reports which indicate that ethanol levels in the peripheral blood of some alcoholic patients have been exceeded 100 mmol/L[43]. In the present study, the exposure of primary hepatocyte cultures to 100 mmol/L ethanol promoted JNK activation. We detected that JNK1 basal activity was lower than JNK2 basal activity, while the JNK1 activity increment was higher than JNK2. These findings are in agreement with previous reports[9,44] and strongly suggest that JNK1 activity is related to the proapoptotic function of this signaling pathway activation by ethanol. We determined ethanol JNK activation by measuring c-jun phosphorylation, the most common parameter of JNK activity. In our system, JNK activity was significantly inhibited by a 2-h pretreatment with 100 μmol/L SP600125, a concentration previously reported for the selective inhibition of JNK[15]. Indeed, c-jun phosphorylation was significantly decreased by 100 μmol/L SP600125 in comparison with cells pretreated with 1 g/L DMSO only, as a vehicle control. We measured a decrease in c-jun and not in JNK phosphorylation because previous reports have indicated that SP600125 specifically inhibits c-jun phosphorylation and does not down-regulate p-JNK levels[14,15]. In our study, selective JNK inhibition by SP600125 allowed us to confirm that JNK functioned as a proapoptotic signaling pathway in hepatocyte cultures exposed to 100 mmol/L ethanol for 24 h, since in SP600125-pretreated hepatocyte cultures, DNA ladder and positive TUNEL cells significantly decreased. Our results, in agreement with previous reports[9,15], indicated that JNK might be a key pathway involved in apoptosis induction by ethanol. However, other signaling events must be involved in the apoptotic pathway induced by ethanol, since apoptotic cells in SP600125-pretreated hepatocytes were not completely abolished,

suggesting that JNK activity contributes to but is not the only factor required for the apoptotic process induced by ethanol. MAPK p38 is also a key candidate in this process, since there are several reports indicating that TNF-alpha, which plays a critical role in ALD development, induces p38 activation[45]. Recently, Bid, a pro-apoptotic member of the bcl-2 family, has been reported as a JNK target in different systems[46,47]. We observed that the incubation of primary hepatocyte cultures with 100 mmol/L ethanol induced Bid cleavage, which was decreased by SP600125. These results strongly suggested Bid as a key target of the pro-apoptotic JNK activity induced by ethanol. Even though maximal JNK activity, measured by c-jun phosphorylation, was observed between 30 min and 4 h after ethanol treatment, Bid fragmentation was not observed until 8 h after ethanol exposure, when c-jun phosphorylation was slightly above control. This slow kinetic of JNK-stimulated Bid cleavage may indicate that several steps separate JNK activation from Bid fragmentation[48]. Since JNK is not a protease, it is likely that one of these steps corresponds to a protease induction by JNK[49,50]. Thus, the exact target of JNK involved in Bid fragmentation is yet to be established[51]. Bid cleavage was also decreased when cultures were exposed to Z-IETD-fmk, a selective caspase 8 inhibitor, indeed it has been reported that Bid is a caspase 8 target[52]. Fas-mediated apoptosis has been related to FasL induction by c-jun activation, and downstream to caspase 8 and Bid fragmentation. Our result suggests that Bid fragmentation in the apoptosis process induced by ethanol lies at least in two distinct pathways, and not only depends on JNK activity. In accordance with this result, a previous report indicates that Bid fragmentation by JNK does not depend on transcription, and in addition, SP600125 does not affect Fas-mediated apoptosis[16]. JNK and other kinases that regulate JNK activity are known to be responsive to redox changes[20], and oxidative stress is critically involved in ALD development[22-24]. We observed that treatment of hepatocyte culture with 100 mmol/L ethanol induced a peak of ROS generation between 6 h and 8 h after ethanol exposure, which coincided with a significant decrease in GSH levels. These redox alterations may be resulted from two of the main components of alcohol metabolism, alcohol dehydrogenase (ADH) and cytochrome P450 2E1, since it has been reported under similar culture conditions that 4-methyl pyrazole, an ADH inhibitor, led to the reduction of H_2O_2 generation[21, 53]. Besides, different non-enzymatic free radical pathways and the mitochondrial dysfunction after the reduction of GSH content are involved in ROS generation induced by ethanol. Therefore, JNK activation in response to ethanol may be related to the oxidative metabolism of ethanol in the liver and to the resultant alterations in intracellular redox state, primarily reflected by GSH changes. AdoMet, an increasingly used agent for liver disease treatment, has wide physiological and pharmacological actions, including its effect on GSH metabolism. However, its hepatoprotective mechanisms have not been completely elucidated[27,54]. We observed that AdoMet, as SP600125, significantly reduced ethanol-induced apoptosis. AdoMet has been identified as a GSH precursor, a major cell antioxidant, and JNK activity has been reported to be down-regulated by antioxidant agents. Thus, our study suggested that AdoMet may down-regulate JNK activity as part of its hepatoprotective mechanism through an antioxidant effect. We found that 4 mmol/L AdoMet was able to prevent GSH decrease induced by ethanol in primary hepatocyte cultures and even maintained intracellular GSH levels above those found in non-treated cells. Furthermore, AdoMet pretreatment also diminished ethanol- induced ROS generation. These results indicated that AdoMet might be related to the repletion of GSH levels. There are contradictory results about the effect of AdoMet on GSH levels; while a previous study found no effect of AdoMet on GSH levels[27], present results are in agreement with others which reported an increment of GSH levels resulted from AdoMet pretreatment[29,55]. Even though AdoMet exerted a redox state regulating effect, surprisingly, it only decreased c-jun phosphorylation by 25.5% in comparison to the inhibition induced by SP600125, which corresponded to 62.8%. We also tested the effect of AdoMet on Bid fragmentation and did not observe an inhibitory effect of AdoMet until 24 h after ethanol exposure. At this time, JNK activity was below control levels, so it is possible that this decrease was not related to JNK activity. These results indicated that even though AdoMet exerted an antioxidant action, it did not have a significant effect on JNK activity, at least on c-jun or Bid, the measured JNK targets. Thus, we suggest that JNK activation may be independent of oxidative stress, in accordance with previously reported results in which cell pretreatment with N-acetyl-cysteine failed to prevent JNK activation[10]. Other reports suggest that JNK activation may be independent of ethanol metabolism, since ethanol activates JNK in HepG2 cells which do not express detectable levels of Cyp2E1 and express low levels of alcohol dehydrogenase activity[56]. However, further studies are required to determine the regulatory mechanisms of JNK activity independent of ROS generation. Even though AdoMet did not have a significant effect on JNK activity as it was measured by its action on c-jun and Bid, it preserved the mitochondrial metabolic activity as it was measured by the MTT assay. In hepatocytes exposed to ethanol only, the mitochondrial function was significantly reduced compared to AdoMet-pretreated hepatocytes. Downstream AdoMet prevented cytochrome c release and pro-caspase 3 fragmentation. In agreement with previous reports which indicated that AdoMet maintained mitochondrial membrane stability and corrected the reduction of GSH content both in cytosol and mitochondria[29,30,56], these results suggest that the AdoMet protective effect mainly lies at mitochondrial level.

In summary, our results point out that ALD is a complex and multifactorial process, and that the mechanisms involved in ALD development must be thoroughly explored for the design of successful therapeutic strategies. We found that AdoMet, although protected against ethanol apoptosis induction and exerted an antioxidant effect, did not show any inhibitory action on Bid fragmentation, a key proapoptotic target of JNK. This finding is in agreement with the suggestion that the mechanisms which lead to JNK activation are independent of ROS generation. We conclude that the JNK signaling pathway modulation by selective inhibitors

and mitochondrial integrity maintenance by AdoMet are promising components of therapeutic approaches for ALD.

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BASIC RESEARCH

Oral administration of *S*-nitroso-*N*-acetylcysteine prevents the onset of non alcoholic fatty liver disease in rats

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Abstract

AIM: To evaluate the potential of *S*-nitroso-*N*-acetylcysteine (SNAC) in inhibition of lipid peroxidation and the effect of oral SNAC administration in the prevention of nonalcoholic fatty liver disease (NAFLD) in an animal model.

METHODS: NAFLD was induced in Wistar male rats by choline-deficient diet for 4 wk. SNAC-treated animals (n=6) (1.4 mg/kg/day of SNAC, orally) were compared to 2 control groups: one (n=6) received PBS solution and the other (n=6) received NAC solution (7 mg/kg/d). Histological variables were semiquantitated with respect to macro and microvacuolar fat changes, its zonal distribution, foci of necrosis, portal and perivenular fibrosis, and inflammatory infiltrate with zonal distribution. LOOHs from samples of liver homogenates were quantified by HPLC. Nitrate levels in plasma of portal vein were assessed by chemiluminescence. Aqueous low-density lipoprotein (LDL) suspensions (200 μg protein/mL) were incubated with $CuCl_2$ (300 μmol/L) in the absence and presence of SNAC (300 μmol/L) for 15 h at 37 ℃. Extent of LDL oxidation was assessed by fluorimetry. Linoleic acid (LA) (18.8 μmol/L) oxidation was induced by soybean lipoxygenase (SLO) (0.056 μmol/L) at 37 ℃ in the presence and absence of N-acetylcysteine (NAC) and SNAC (56 and 560 μmol/L) and monitored at 234 nm.

RESULTS: Animals in the control group developed moderate macro and microvesicular fatty changes in periportal area. SNAC-treated animals displayed only discrete histological alterations with absence of fatty changes and did not develop liver steatosis. The absence of NAFLD in the SNAC-treated group was positively correlated with a decrease in the concentration of LOOH in liver homogenate, compared to the control group (0.7±0.2 nmol/mg vs 3.2±0.4 nmol/mg protein, respectively, P<0.05), while serum levels of aminotransferases were unaltered. The ability of SNAC in preventing lipid peroxidation was confirmed in *in vitro* experiments using LA and LDL as model substrates.

CONCLUSION: Oral administration of SNAC prevents the onset of NAFLD in Wistar rats fed with choline-deficient diet. This effect is correlated with the ability of SNAC to block the propagation of lipid peroxidation *in vitro* and *in vitro*.

Key words: Nitric oxide; *S*-nitroso-*N*-acetylcysteine; Oxidative stress; Nonalcoholic fatty liver disease

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INTRODUCTION

Nonalcoholic steatohepatitis (NASH) is considered a particular type of a large spectrum of nonalcoholic fatty liver disease (NAFLD), which includes fat alone and fat with nonspecific inflammation[1,2]. Although several predisposing factors such as obesity and diabetes, are related to NAFLD, the pathogenesis of NAFLD and its progression to fibrosis and chronic liver disease are still unclear[3-5]. One of the main hypotheses is that the mechanism of hepatocyte injury in NASH is associated with oxidative stress

and lipid peroxidation resulting from the imbalance between pro-oxidant and antioxidant chemical species[6]. Such an imbalance is associated with increased β-oxidation of fatty acids by mitochondria, peroxisomes, and cytochrome P450 2E1 (CYP2E1) pathways. These oxidative processes produce free electrons, H_2O_2, and reactive oxygen species (ROS) while depleting the potent antioxidants, glutathione and vitamin E[1]. The increased levels of free fatty acids present in the fatty liver provide a perpetuating and propagating mechanism for oxidative stress via lipid peroxidation, with secondary damage to cellular membranes and key organelles such as mitochondria[6]. Lipid peroxidation usually leads to the formation of peroxyl radicals, which are central species in the peroxidation chain reaction. Enzymatic lipid peroxidizing systems include lipoxygenases (LOXs), which are a family of nonheme iron-containing dioxygenases and able to induce enzymatic peroxidation of polyunsaturated fatty acids using atmospheric oxygen (O_2) as a second substrate. In contrast to lipid monooxigenases like cytochrome P-450, whose main catalytic activity is the hydroxylation of substrates, LOXs are able to introduce peroxides in lipid substrates, forming reactive fatty acid hydroperoxides (LOOH). In general, LOXs contain an essential iron atom, which is present as Fe^{2+} in the inactive enzyme form. Enzymatic activation occurs through hydroperoxide-driven oxidation of Fe^{2+} to Fe^{3+}. Among LOXs, 15-LOX is of particular interest, which can also oxidize esterified fatty acids in biological membranes and lipoproteins and has been implicated in the pathogenesis of atherosclerosis[7-9]. Site-specific oxidation of lipidic substrates can also be performed in model systems when metal ions (Cu(I)/Cu(II)) or Fe(II)/Fe(III)) are used to generate radicals in the absence of chelant species[10].

Nitric oxide (NO) can act as a potent inhibitor of the lipid peroxidation chain reaction by scavenging propagatory lipid peroxyl radicals and by inhibiting many potential initiators of lipid peroxidation, such as peroxidase enzymes[11]. However, in the presence of superoxide ($O_2^{\bullet -}$), NO forms peroxynitrite ($OONO^-$), a powerful oxidant, which is able to initiate lipid peroxidation[12]. An excess of NO is expected to exert a protective effect against lipid peroxidation, while an excess of $O_2^{\bullet -}$, or equimolar concentrations of NO and $O_2^{\bullet -}$ are expected to induce lipid peroxidation[13]. Thus, the balance between NO and $O_2^{\bullet -}$ may have important implications in NAFLD, where oxidative stress seems to have a pivotal role in the onset and/or progression of the disease[12,13]. NO is believed to coexist in cells with S-nitrosothiols (RSNOs) which are considered endogenous NO carriers and donors in mammals[14]. NO covalently bound to the sulfur atom in RSNOs resists oxidant inactivation by oxyhemoglobin and has the same physiological properties of free NO, including its protective action on oxidative stress[15]. RSNOs have been considered potential therapeutic agents in a variety of pathologies in which NO may be involved[16] and S-nitroso-N-acetylcysteine (SNAC) is a relatively stable RSNO and a potent vasodilator[17]. SNAC is among the RSNOs, which can be synthesized through the S-nitrosation of the corresponding free thiol (in this case, N-acetylcysteine, NAC). Free thiols (R-SH) play also an important role *in vivo* as antioxidants. Hydrogen abstraction from thiol group is particularly fast compared to hydrogen abstraction from carbon atoms or alkoxyl radicals[18-21]. At physiological pH values, thiyl radicals ($R\text{-}S^{\bullet}$) formed can react with excess thiol anions ($R\text{-}S^-$) to give disulphide radical anions ($R\text{-}SS\text{-}R^{\bullet -}$), or can dimerize giving rise to inter or intramolecular RS-SR cross-links in a termination process. Compared to free thiols, RSNOs can be more powerful terminators of radical chain-propagation reactions by reacting directly with $ROO^{\bullet}$ radicals, yielding nitro derivatives (ROONO) as end products as well as dimmers RS-SR.

The aim of this study was to evaluate the role of SNAC as an NO donor, in the prevention of NAFLD in an animal model where NAFLD was induced by a choline deficient diet. Our results show, for the first time, that SNAC is able to block the onset of NAFLD in this animal model. This result was correlated with *in vitro* experiments which have confirmed the ability of SNAC to prevent the oxidation of low-density lipoprotein (LDL) and linoleic acid (LA) as model substrates, by Cu(II) ions and soybean lipoxygenase (SLO), respectively.

MATERIALS AND METHODS

Materials

N-acetyl-L-cysteine (NAC), linoleic acid, sodium nitrite, hydrochloric acid, human lyophilized LDL, soybean lipoxygenase, sodium dodecil sulfate (SDS), phosphate buffer saline (PBS, pH 7.4) and copper (II) chloride (Sigma, St. Louis, MO) were used in this study. All experiments were carried out using analytical grade water from a Millipore Milli-Q gradient filtration system.

SNAC synthesis

SNAC was synthesized through the S-nitrosation of N-acetyl-L-cysteine (Sigma Chemical, St. Louis, MO) in an acidified sodium nitrite solution[17]. Stock SNAC solutions were further diluted in PBS. Solutions were diluted to 2.4 x 10^{-4} mol/L in PBS (pH 7.4) before administration.

Nitrate quantification

Nitrate (NO_3^-, a stable metabolite of NO) levels in plasma of portal vein of the animals were assessed by chemiluminescence using a Sievers nitric oxide analyzer (NOA-280, Boulder, CO) according to a method described elsewhere[22]. Higher nitrate concentrations were found in the plasma of animals which received SNAC orally (10.8 μmol/L) then intraperitoneally (4.2 μmol/L). This result was used as a criterion to choose oral administration as a protocol to achieve greater SNAC absorption.

Effect of NAC and SNAC on in vitro LDL oxidation

Oxidation of LDL was induced through the addition of $CuCl_2$ (300 μmol/L) to oxygenated aqueous LDL suspensions (200 μg/mL) in the absence and presence of SNAC (300 μmol/L). Aqueous LDL suspensions were prepared by diluting solid LDL to 200 μg protein/mL with EDTA-free PBS and incubated with $CuCl_2$ (300 μmol/L) for 15 h at 37 ℃. The extent of LDL oxidation was assessed by measuring the fluorescence intensity of LDL suspensions. Oxidation of LDL resulted in derivatization of lysine residues of apolipoprotein B by lipid peroxide decomposition

products, leading to fluorescent free and protein-bound Schiff base conjugates as previously described[23,24]. In all cases, fluorescence spectra of such conjugates were firstly recorded in the range 430-600 nm, in order to characterize the shape and position of the emission peak. All the spectrofluorimetric measurements were performed using a Perkin-Elmer LS-55 luminescence spectrometer with a temperature-controlled cuvette holder thermostatized at 37℃. Spectra of the solutions were obtained in 1 cm quartz cuvette. The excitation and emission wavelengths were 360 and 433 nm, respectively. Native LDL (200 μg/mL) served as the control.

Effect of NAC and SNAC on in vitro LA oxidation

Oxidation of LA was induced through the addition of SLO to aqueous LA dispersions. LA was dispersed in SDS solution (0.01 mol/L). The final LA concentration was 18.8 μmol/L. LA was aliquoted into a quartz cuvette, flushed with O_2 for 1 min and SLO (0.056 μmol/L) was added with a syringe to start the oxidation. The oxidation reactions were monitored in the absence or presence of NAC and SNAC (56 and 560 μmol/L) at 37℃ through the increase in absorbance at 234 nm, due to conjugated diene formation. A Hewlett Packard spectrophotometer, model 8453 (Palo Alto, CA, USA) with a temperature-controlled cuvette holder, was used to monitor the spectral changes in the range 200-600 nm in the dark and at 37℃. Spectra of the solutions were obtained in 1 cm quartz cuvette referenced against air, under stirring (1 000 r/min). Each point in the kinetic curves of absorbance *vs* time was the average of two experiments with the error bars expressed by their standard deviations (SD).

Animals

Male Wistar rats, weighing 300 to 350 g, were housed in cages with a controlled light/dark cycle, receiving free water. Fatty liver was induced in the animals by choline deficient diet for four weeks. The animals were randomly divided into three groups: control group ($n = 6$) fed with choline deficient diet plus oral administration of vehicle (0.5 mL of PBS), SNAC group ($n = 6$) fed with choline-deficient diet plus oral administration of SNAC solution (0.5 mL of SNAC solution, reaching 1.4 mg/kg/day), and NAC group ($n = 6$) fed with choline-deficient diet plus oral administration of NAC solution (0.5 mL of NAC solution, reaching 7 mg/kg per day). After four weeks of treatment, plasma samples were collected, animals were sacrificed, and their livers were collected for histological examination and lipid peroxidation analysis. All procedures for animal experimentation were in accordance to the Helsinki Declaration of 1975 and the Guidelines of Animal Experimentation from the School of Medicine of the University of São Paulo.

Biochemical analysis

Serum alanine aminotransferase (AST), aspartate aminotransferase (ALT), cholesterol and triglycerides were analyzed by standard methods[25].

Histological analysis

Fragments of liver tissue previously fixed by immersion in formaldehyde saline (10%) solution were processed and submitted to hematoxylin-eosin (HE) and Masson trichrome staining for histological analysis. Scharlach red (O-tolylazo-o-tolylazo-β-naphthol) fat staining[26] was used for more accurate evaluation of fatty change. Histological variables were blindly semiquantitated from 0 to 4+ with respect to macro and microvacuolar fatty change, its zonal distribution, foci of necrosis, portal and perivenular fibrosis as well as inflammatory infiltrate with zonal distribution.

Lipid peroxidation

Samples of liver homogenates were extracted with a mixture of acetonitrile : hexane (4 : 10, v/v). The contents were vortexed for 2 min and centrifuged at 2 500 r/min for 10 min for phase separation. The hexane phase containing cholesteryl ester derived hydroperoxides (LOOH), was collected and evaporated under nitrogen. The residue was dissolved in methanol : butanol (2 : 1, v/v), filtered through a 22 μm Millex filter (Millipore, São Paulo, Brazil) and analyzed by HPLC (Perkin-Elmer series 200, Beaconsfield, Buckinghamshire, England) using an LC18DB column (Supelco, Bellefonte, PA, USA). LOOHs were eluted in methanol : butanol 2 : 1 (v/v) at a flow rate of 1.0 mL/min through a pump (Perkin-Elmer series 200) and an LC-240 fluorescence detector (Perkin-Elmer) with the excitation source switched off. A solution of 100 mmol/L borate buffer pH 10/methanol 3 : 7 (v/v) containing microperoxidase (25 mg/L) was used as the reaction solution for the postcolunm reaction[27]. Peaks were identified using external standards prepared from their respective oxidation products as previously described[27] and quantified using the package Turbochrom Navigator software (Perkin-Elmer). Results were expressed as nmol of lipid hydroperoxides/mg of protein.

Statistical analysis

All data were expressed as mean ± SE or as mean ± SD. Statistical significance was evaluated using the one-way ANOVA test for comparisons among three groups (Control *vs* NAC *vs* SNAC-LOOH quantification) and t-test for the comparison between two means (Control *vs* SNAC - biochemical analysis). $P < 0.05$ was considered statistically significant.

RESULTS

Figure 1 shows the micrographs of liver tissue extracted from animals treated with choline-deficient diet, which received vehicle or SNAC solutions for four weeks. A moderate macro and microvacuolar steatosis in periportal zone could be seen in the control group (Figure 1A) while in the SNAC-treated group the animals did not develop liver steatosis (Figure 1B). Scharlach staining showed a fatty change (positive staining) in the control group (Figure 1C), whereas in the SNAC-treated group no fat change was detected (negative staining) (Figure 1D). In both animal groups, necroinflammatory activity was minimal and no fibrosis was detected. In the NAC-treated group there was a macro and microvacuolar steatosis in periportal zone (data not shown).

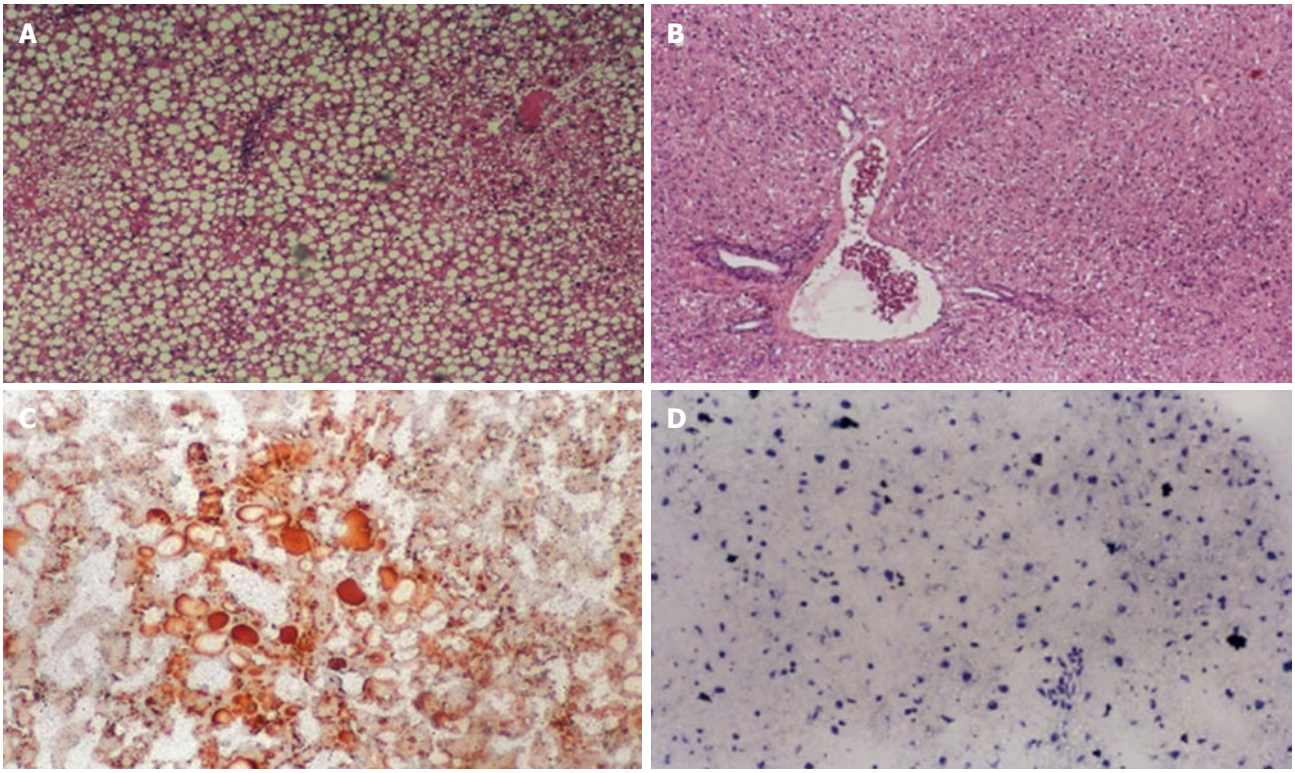

Figure 1 Histological features of liver tissue of rats fed with choline-deficient diet. **A**: Control group showing a moderate macro and microvacuolar steatosis in periportal zone; **B**: SNAC-treated animals showing normal liver in periportal zone (hematoxylin-eosin stain-HE); **C**: Control group showing positive Scharlach staining; **D**: SNAC-treated animals showing negative Scharlach staining.

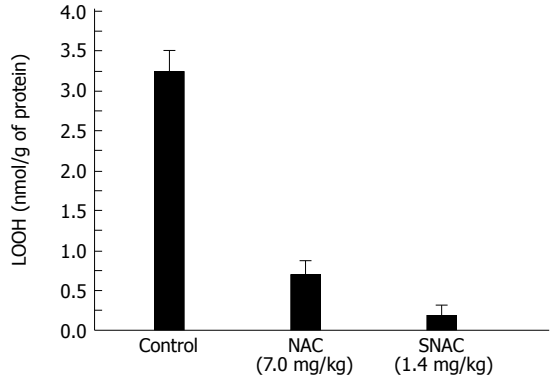

Figure 2 Concentration of hydroperoxides (LOOH) in liver homogenates of the control group, NAC and SNAC-treated animals.

Table 1 Levels of alanine aminotransferase (AST), aspartate aminotransferase (ALT), cholesterol and triglycerides in serum of rats fed with choline-deficient diet (mean±SD)

Group	Number of animals	AST (U/L)	ALT (U/L)	Cholesterol (U/L)	Triglyceride (U/L)
Control[1]	6	108±3	40±1	36±1	88±3
SNAC[2]	6	95±4	37±8	35±1	70±1

Normal values in U/L for AST: 10-34; ALT: 10-44; mg/dL: cholesterol and triglyceride: 45-89. [1]Control - animals fed with choline-deficient diet. [2]SNAC –animals fed with choline-deficient diet and treated daily with oral SNAC administration.

Figure 2 shows that SNAC prevented the rise of LOOH concentration in the liver of the SNAC-treated group, compared to the control group (0.3 ± 0.1 *vs* 3.2 ± 0.4 nmol/mg protein, respectively). The protective effect of NAC was also expressed by a reduction of hydroperoxide formation that could be seen in the *ca.* 4.6-fold reduction in LOOH formation (0.7 ± 0.2 nmol/mg protein *vs* 3.2 ± 0.4 nmol/mg protein, respectively).

On the other hand, the levels of AST and triglycerides were increased to a similar extent in the control and SNAC-treated groups. SNAC treatment of the choline-deficient fed rats did not lead to changes in ALT and cholesterol levels (Table 1)

Figure 3 shows the emission spectra of human LDL suspension (200 μg/mL) in PBS. The two emission peaks at *ca.* 410 and 440 nm (Figure 3A) could be assigned to the partial oxidation of the freshly prepared LDL suspension. It could be seen that these two peaks increased after incubation of LDL with $CuCl_2$ (300 μmol/L) (Figure 3B) reflecting the oxidation of LDL catalyzed by Cu (II) ions. However, incubation of LDL with $CuCl_2$ under the same condition, but in the presence of SNAC (300 μmol/L) completely blocked the growth of the 410 and 440 nm peaks (Figure 3C). In fact, the peak at 440 nm was extinguished in this case.

Figure 4 shows the effect of SNAC on the kinetics of LA oxidation by SLO. This effect could be evaluated through the analysis of two kinetic parameters: initial rate and extent of the peroxidation reaction until the achievement of the chemical equilibrium. Kinetic curves were ob-

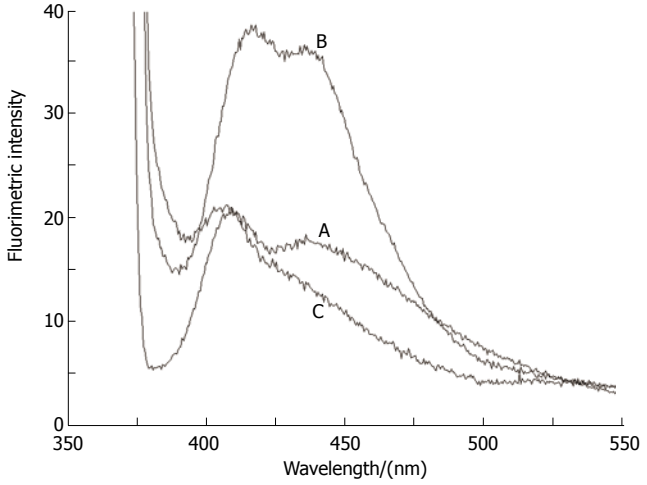

Figure 3 Emission spectra of human LDL (200 μg/mL) suspended in aerated PBS. **A**: Freshly prepared suspension; **B**: after incubation with $CuCl_2$ (300 μmol/L) for 15 h; **C**: after co-incubation with $CuCl_2$ (300 μmol/L) and SNAC (300 μmol/L). The excitation and emission wavelengths were 360 and 433 nm, respectively.

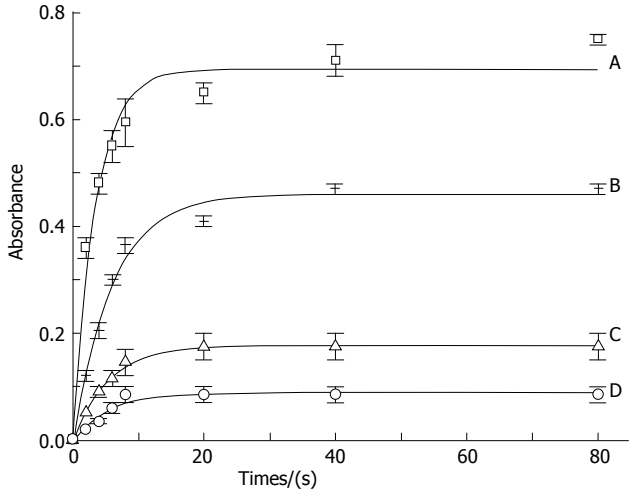

Figure 4 Kinetic curves of linoleic acid (18.76 μmol/L) peroxidation catalyzed by SLO (**A**) (0.056 μmol/L), SLO co-incubated with NAC (**B**) (560 μmol/L), SLO co-incubated with SNAC (**C**) (56 μmol/L) and SLO co-incubated with SNAC (**D**) (560 μmol/L). Absorbance changes were monitored at 234 nm at 37 ℃.

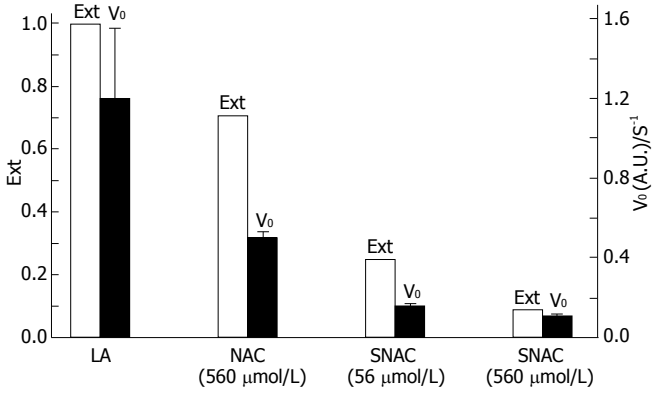

Figure 5 Barr graph showing the extent (Ext) and initial rates (V_0) of the peroxidation reaction of linoleic acid (LA) by SLO. Data were extracted from the curves of Figure 4.

tained from the corresponding spectral changes in the UV, monitored through the band with maximum at 234 nm. This band was characteristic of conjugated dienes and could thus be taken as a marker of LA peroxidation. While the initial rates of reactions correspond to the inclination of the initial sections of the curves (*ca.* 10 s), and the extents of reactions corresponded to the absorbance values at the plateaus. It could be seen that both parameters were maximum when LA (18.76 μmol/L) was incubated with SLO (0.056 μmol/L) (Figure 4A). Co-incubation with NAC (560 μmol/L) reduced the extent and rate of oxidation (Figure 4B), but this reduction was much more pronounced in the co-incubation with SNAC at a concentration ten times lower than NAC (56 μmol/L) (Figure 4C). The reduction was further increased in the presence of SNAC (560 μmol/L) (Figure 4D). These effects could also be evaluated in the bar graph of Figure 5, where the initial rates of reaction and the extents of reaction were extracted from the kinetic curves of Figure 4. It could be seen in Figure 5 that both the rates and the extents of reaction in the presence of SNAC were reduced to about half of those obtained in the presence of NAC at a concentration ten times higher.

DISCUSSION

Choline-deficient diet is a classical general model of NAFLD, where Cyp2E1 is up regulated and the animals develop steatosis, steatohepatitis and hepatic fibrosis[28]. The results obtained in this animal model show a strong inhibitory effect of SNAC on fatty change, which is the initial step of NAFLD. The protective effect of SNAC observed here could be analyzed according to the suggested role of oxidative stress in the pathology of NAFLD[29-31]. Although the exact role of antioxidants in the prevention of NAFLD is not well established yet, a number of studies have shown that markers of oxidative stress are increased, while levels of endogenous antioxidants (e.g. vitamin E and glutathione, GSH) are decreased in NAFLD[29, 30]. The microsomal enzymes CYPs 2E1 and 4A are believed to be involved in the fatty acid oxidation in the liver of humans with NASH, contributing to the pathogenesis of this disease[31]. In the present case, formation of lipid hydroperoxides (LOOH), which are one of the main products of the lipid peroxidation process, was observed to be expressively reduced in the liver tissue of the SNAC-treated animals, indicating that SNAC acts as a potent inhibitor of lipid/lipoprotein oxidation. This result is in accordance with the reactivity of NO from SNAC and the ability of NO to block the propagation of radical chain reactions by forming nitrated lipid derivatives as end products[32-36].

SNAC-induced inhibition of LDL oxidation by Cu(II) as a model system, was confirmed in the *in vitro* experiments (Figure 3). The emission peaks at 410 and 440 nm in the fluorescence spectra of LDL suspensions were assigned to adduct formation (Schiff bases) between oxidation products of the lipid content of LDL particles (mainly malondialdehyde, MDA) and amino groups of the apolipoprotein (mainly Apo-B-100), which are well known markers of LDL oxidation[37,38]. The inhibition of their formation in the co-incubation of LDL with Cu (II) and SNAC, showed that SNAC could block LDL oxidation under this condition. The protective effect of SNAC was also confirmed *in vitro* using LA as a second model compound

in which peroxidation was catalyzed by SLO (Figures 4 and 5). The co-incubation of LA with SNAC (56 μmol/L) and its correspondent reduced thiol, NAC (560 μmol/L) highlighted the much more potent effect of SNAC in the inhibition of LA peroxidation, once SNAC at a concentration ten times lower than NAC exerted a much more important antioxidant effect. The fact that an increase in SNAC concentration to 560 μmol/L did not lead to a proportional reduction in the kinetic parameters associated with LA peroxidation, is probably due to the relatively fast initial steps of LA peroxidation.

As SNAC does not react directly with aldehydes or ketones, the protective effect observed here must be associated with the termination of lipid radical chain propagation reactions, through the inactivation of alkoxyl ($LO^{\bullet}$) and peroxyl ($LOO^{\bullet}$) intermediates, which have already been demonstrated to be converted into inactive ROONO products by NO[32-36] *in vivo*. A general equation for these reactions can be written as:

$$2RS\text{-}NO + LO^{\bullet} / LOO^{\bullet} \rightarrow LONO / LOONO + RS\text{-}SR \quad (1)$$

where RSNO can be any primary S-nitrosothiol and RS-SR is the corresponding oxidized thiol yielded as a dimmer. The same RS-SR dimmers are formed if the RSNOs release NO primarily according to[39]:

$$2\ RSNO \rightarrow RS\text{-}SR + 2NO \quad (2)$$

Free NO released in equation 2 is also capable of reacting with $LO^{\bullet}/LOO^{\bullet}$ species[35], leading to the same termination products of equation 1.

Although NAC (the precursor of SNAC) has also an important antioxidant action due to the ease of hydrogen abstraction from its thiol group (data not shown), the protective action of SNAC cannot be assigned to its conversion into NAC. Such a reaction does not take place in an oxidative environment. Under such conditions, the anti-oxidant effect of SNAC can be assigned mainly to the lability and reactivity of NO, according to equations 1 and 2. This statement is supported not only by the greater antioxidant action of SNAC compared to NAC in the *in vitro* experiments with LDL and LA, but also by the *in vivo* results showing that the daily oral administration of NAC at a concentration five times higher than SNAC, did not prevent the development of liver steatosis in the present animal model and led to a lower reduction in the LOOH level in the liver tissue. The protective action of NAC in this animal model is not entirely dissimilar to that obtained with other more classical anti-oxidants. However, ascorbic acid which reduces liver steatosis in rats on choline-deficient diet, is not able to inhibit the onset of this pathology, and α-tocopherol (vitamin E) does not even reduce fat accumulation in the hepatic tissue in the same animal model[40].

The important protective action of an NO donor in this model suggests that NAFLD can be associated with an impairment of endogenous NO production in the liver. Since the production of endothelium-derived NO has already been demonstrated to be impaired in other diseases related to oxidative stress, like atherosclerosis[41, 42], the effects of NO in NAFLSD can involve other mechanisms in addition to those associated solely with oxidative stress. NO is also known to be a signal transduction mediator and accumulating data suggest that S-nitrosation and nitrosilation reactions performed by NO may be a ubiquitous posttranslational modification involved in signal transduction regulation[43]. The absence of correlation between the reduction of LOOH concentration and the occurrence of macro and microvacuolar steatosis in the NAC-treated group, is an evidence that protective mechanisms other than the inhibition of lipid peroxidation, are operative when SNAC is administered to choline deficient animals. Such mechanisms are probably associated with the biochemical/signaling actions of NO and can be specifically linked to the biochemistry of RSNOs. In contrast to other NO donors which are already in widespread clinical use, like organic nitrates, nitrites and sodium nitroprusside, few clinical studies have been reported for RSNOs. Therefore, the use of RSNOs as exogenous NO sources in the treatment of NAFLD can bring new perspectives for understanding the pathogenesis of this disease.

In conclusion, oral administration of SNAC as an exogenous NO source, can block the onset of NAFLD and the reduction of LOOH production in liver tissue as a result of this treatment can be associated with the ability of SNAC to block the lipid peroxidation. These findings have clinical implications, regarding novel therapeutic strategies for the treatment of NAFLD.

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BASIC RESEARCH

Effect of WeiJia on carbon tetrachloride induced chronic liver injury

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Abstract

AIM: To study the effect of WeiJia on chronic liver injury using carbon tetrachloride (CCl_4) induced liver injury animal model.

METHODS: Wistar rats weighing 180-220g were randomly divided into three groups: normal control group (Group A), CCl_4 induced liver injury control group (Group B) and CCl_4 induction with WeiJia treatment group (Group C). Each group consisted of 14 rats. Liver damage and fibrosis was induced by subcutaneous injection with 40% CCl_4 in olive oil at 3 mL/kg body weight twice a week for eight weeks for Groups B and C rats whereas olive oil was used for Group A rats. Starting from the third week, Group C rats also received daily intraperitoneal injection of WeiJia at a dose of 1.25 μg/kg body weight. Animals were sacrificed at the fifth week (4 male, 3 female), and eighth week (4 male, 3 female) respectively. Degree of fibrosis were measured and serological markers for liver fibrosis and function including hyaluronic acid (HA), type IV collagen (CIV), γ-glutamyl transferase (γ-GT), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined. Alpha smooth muscle actin (α-SMA) and proliferating cell nuclear antigen (PCNA) immunohistochemistry were also performed.

RESULTS: CCl_4 induction led to the damage of liver and development of fibrosis in Group B and Group C rats when compared to Group A rats. The treatment of WeiJia in Group C rats could reduce the fibrosis condition significantly compared to Group B rats. The effect could be observed after three weeks of treatment and was more obvious after eight weeks of treatment. Serum HA, CIV, ALT, AST and γ-GT levels after eight weeks of treatment for Group C rats were 58±22 μg/L (P<0.01), 57±21 μg/L (P<0.01), 47±10 U/L (P<0.01), 139±13 U/L (P<0.05) and 52±21 U/L (P>0.05) respectively, similar to normal control group (Group A), but significantly different from CCl_4 induced liver injury control group (Group B). An increase in PCNA and decrease in α-SMA expression level was also observed.

CONCLUSION: WeiJia could improve liver function and reduce liver fibrosis which might be through the inhibition of stellate cell activity.

Key words: WeiJia; Carbon tetrachloride; Liver fibrosis

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INTRODUCTION

Hepatic fibrosis is one of the processes that occur when the liver is damaged through viral activity, toxins, autoimmune diseases, metabolic disorder or genetic defects. It is a result of chronic liver injury that ultimately leads to cirrhosis and its complications of portal hypertension, liver failure and hepatocellular carcinoma. Millions of people die each year worldwide[1-5]. Efficient and well-tolerated antifibrotic drugs are lacking and current treatment of hepatic fibrosis is limited to withdrawal of the noxious agent[6].

Advance in pathophysiology, molecular biology, ge-

netically engineered animals, liver transplantation *etc*. has increased our understanding of the molecular mechanism in liver fibrogenesis. Hepatic stellate cell (HSC) was identified as the primary cell type to mediate fibrogenesis which is the major source of extracellular matrix deposition[7-9]. Antifibrotic drug development was focused on fibrogenic cells generating the scarring response in the past decade.

WeiJia is a protein peptide mixture extracted from neonate porcine liver. It is a Category-I new drug on the market approved by the State Food and Drug Administration (SFDA) for the treatment of severe hepatitis and shows promising results in clinical studies[10-12]. Studies also showed that WeiJia can act as a therapeutic agent in the treatment of cirrhosis[13, 14]. As progressive hepatic fibrosis would lead to cirrhosis, it is likely that WeiJia might also play a role in treatment of hepatic fibrosis. In this study, carbon tetrachloride (CCl_4) induced liver injury animal model was used to evaluate the potential of WeiJia as a therapeutic agent for hepatic fibrosis.

MATERIALS AND METHODS

Materials

Wistar rats, weighing 180-220g were bought from Experimental Animal Centre, Daping Hospital, Third Military Medical University, Chongqing. WeiJia was from LifeTec Enterprise Limited, Hong Kong; analytical grade CCl_4, alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transferase (γ-GT) testing kits and DAB were from Sigma (St. Louis, MO, USA); hyaluronic acid (HA) and type IV collagen (CIV) RIA kits were from Shanghai Naval Medical Research Institute; alpha smooth muscle actin (α-SMA) and proliferating cell nuclear antigen (PCNA) monoclonal antibodies were from Dako Ltd. (Glostrup, Denmark)

Animal treatment

Wistar rats were randomly divided into three groups: normal control group (Group A), CCl_4 induced liver injury control group (Group B) and CCl_4 induction with WeiJia treatment group (Group C). Each group consisted of 14 rats (8 male, 6 female). Liver damage and fibrosis was induced by subcutaneous injection with 40% CCl_4 in olive oil at 3 mL/kg body weight twice a week for eight weeks for Group B and C rats whereas olive oil alone was used for Group A rats. Rats were allowed to feed *ad libitum*. Starting from the third week, Group C rats also received daily intraperitoneal injection of WeiJia at a dose of 1.25 μg/kg body weight. All studies were conducted according to the guidelines described in the NIH Guide for the Care and Use of Laboratory Animals.

Sample collection

Half of the rats (4 male, 3 female) in each group were sacrificed at the fifth week and the other half were sacrificed at the eighth week. Amobarbital sodium (0.5%) was injected intraperitoneally into the rats at 4 mL/kg and blood was collected from the heart after anesthesia. Blood (5 mL) was collected and 1.2 mL serum was obtained after centrifugation and stored at 4 ℃ before analysis. Liver tissues (1 cm×1 cm×1 cm) from the right liver lobe were dissected and immobilized in 4% paraformaldehyde. Tissue was then embedded in paraffin wax and sectioned (4 μm thick) before analysis.

Serum chemistry

Liver fibrosis blood tests for HA and CIV were performed using competitive RIA method. Blood serum level of γ-GT, ALT and AST were measured by standard clinical chemical methods using an automatic analyzer type ALCYON 300i (Abbott Laboratories Ltd, USA).

Histological examination

Tissue was sectioned, haematoxylin and eosin (HE) staining, Van Gieson (VG) staining and immunohistochemistry were performed and examined under light microscope. All histological examinations were performed by experienced pathologist without prior knowledge of the animal treatment groups in the study. Images were acquired through Nikon Eclipse E400 (Nikon Corporation, Japan) and analyzed with analySIS 3.0 software.

Degree of fibrosis was measured on HE stained sections. Stage of liver fibrosis was graded with the METAVIR scale, which grades fibrosis on a five-point scale: F0 (no fibrosis), F1 (portal fibrosis without septa), F2 (portal fibrosis with a few septa), F3 (numerous septa without cirrhosis) and F4 (cirrhosis). METAVIR scale is a widely used scale that has excellent inter-observer reliability[15, 16].

Ballooning degeneration and steatosis for HE stained sections were graded according to a four point scale where Grade 0: negative, Grade (1): up to 33%, Grade (2): 33%-66% and Grade (3): > 66% cells show ballooning degeneration and steatosis[17].

The collagen content of the sections was determined by VG staining. Five random fields were chosen in each section and the amount of total collagen was detected as the area stained by VG and expressed as percentage relative to the total area.

α-SMA and PCNA immunohistochemistry were also performed. Sections were deparaffinized, rehydrated and incubated in 3% hydrogen peroxide at room temperature for 10 min to block endogenous peroxidase. After rinsing with distilled water, sections were incubated in phosphate buffered saline (PBS, 0.01 mol/L, pH 7.4) for 5 min and epitope retrieval was induced with heat in a microwave oven. Non-specific binding sites were blocked with 10% normal goat serum (NGS) / 10% bovine serum albumin (1 ∶ 1 dilution) for 30 min at room temperature followed by incubation with monoclonal mouse anti α-SMA or PCNA as primary antibodies at 1 ∶ 100 dilution in PBS containing 10% NGS and 0.3% Tween 20 overnight at 4 ℃. Sections were then washed with PBS for 3 times, each 5 min before applying the secondary antibody. Goat anti-mouse antibody conjugated with horseradish peroxidase (HRP) at 1 ∶ 200 dilution in PBS containing 10% NGS was applied and the sections were incubated for 30 min at 37 ℃. Sections were then washed with PBS for 3 times, each 5 min and stained with DAB for 1 to 5 min. Staining was stopped by washing with tap water. Sections were counterstained with haematoxylin, dried and visualized under light microscope.

Expression of α-SMA was determined according to four categories and each category was assigned a number.

Table 1 Liver fibrosis serum markers test for rats in different treatment group

Group	5th wk		8th wk	
	HA μg/L	CIV μg/L	HA μg/L	CIV μg/L
A	43±19	19±10	37±17	46±16
B[b]	324±92	206±70	130±52	136±28
C[a]	66±20[c]	74±19[c]	58±22[c]	57±21[1]

$n=7$ for each group, [a]$P<0.005$ *vs* Group B, [b]$P<0.001$ *vs* Group A, [c]$P<0.05$ *vs* Group A, [1]$P>0.1$ *vs* Group A.

Table 2 Degree of fibrosis for rats in different treatment group

Group	5th wk					8th wk				
	F0	F1	F2	F3	F4	F0	F1	F2	F3	F4
A	7					7				
B[b]		3	4					1	6	
C[a,d]		5	2				3	3	1	

$n=7$ for each group, [a]$P>0.05$ *vs* Group B at fifth week, [b]$P<0.01$ *vs* Group A, [d]$P<0.01$ *vs* Group B at eighth week.

Grade (1): no expression (-, 2^0=1); Grade (2): individual positive cells expressed in diseased area (+, 2^1=2); Grade (3): a few positive cells gathered together and expressed in the diseased area (++, 2^2=4) and Grade (4): wide spread of positive cells (+++, 2^3=8). Result is expressed in numbers according to different categories.

Expression of PCNA was determined using a double blind method. For each section, 5 random fields at high resolution were chosen and positive cells were recorded by two analysts. Result is expressed as the mean positive cells recorded by the two analysts.

Statistical analysis

Comparison of the degree of liver fibrosis between samples was performed by WILCOXON method. Other data were analyzed by SPSS11.0 software and reported as mean ± standard deviation using one-way ANOVA. Student's t-test was used for comparison between groups. P values of 0.05 or less are considered statistically significant.

RESULTS

WeiJia is an SFDA-approved Category-I new drug for the treatment of severe hepatitis. WeJia showed an overall efficacy of 88.9% in relieving symptoms and improving physical conditions of chronic hepatitis patients in a treatment period of six weeks in previous clinical study. Studies also showed that WeiJia can act as a therapeutic agent in the treatment of cirrhosis[13, 14]. As progressive hepatic fibrosis would lead to cirrhosis, it is likely that WeiJia might also play a role in the treatment of hepatic fibrosis. Thus the effect of WeiJia on liver fibrosis was investigated through an animal model in this study.

Rats were induced with CCl_4 followed by the treatment with WeiJia. Animals without CCl_4 induction or without WeiJia treatment were used as control for comparison. The effect of CCl_4 and WeiJia on rat liver fibrosis was determined through histological examination and serological markers test.

Biochemical tests of fibrosis

Serum levels of biochemical markers were determined to evaluate the severity of fibrosis. Levels of extracellular matrix constituents HA and CIV were measured which wer expected to increase as a result of remodelling and recurrent scarring in liver fibrogenesis. HA has correlation with stage 3 and 4 fibrogenesis. Together with CIV and other markers, differentiation of stage 1 and 2 fibrosis from stage 3 and 4 fibrosis can be obtained[18, 19]. HA and CIV serum levels for different treatment groups are shown in Table 1. Significant elevation of serum HA and CIV levels were observed upon CCl_4 induction ($P<0.001$ *vs* Group A). Their levels were significantly reduced upon treatment with WeiJia ($P<0.005$ *vs* Group B). Though there was still significant difference between the levels of Group C and Group A rats at the fifth week, there was no apparent difference for CIV level at the eighth week between the two groups, indicating prolonged treatment with WeiJia could alleviate the severity of fibrosis. Decrease of the CIV and HA levels were observed for Group B rats at the 8th wk when compared to their level at the 5th wk indicating some recovery processes took place. However, such recovery processes were not potent enough to revert or alleviate the severity of fibrosis as their enzyme levels were still significantly higher than that of Group A rats.

Histopathological study

The great elevation of HA and CIV levels upon CCl_4 induction indicated the successful generation of liver fibrosis animal model. The result was further confirmed by histopathology study. HE staining for sections of normal control group (Group A) showed structural integrity without necrosis, inflammation or fibrosis development. VG staining for collagen was negative too. However, CCl_4 induced liver injury control group (Group B) showed significant fibrosis ($P<0.01$) with the loss of structural integrity and formation of nodules that lacked a central vein. Steatosis and ballooning degeneration ($P<0.01$) were observed on the fifth week whereas increased collagen fibres and complete fibrous septa were observed on the eighth week. Nevertheless, CCl_4 induction with WeiJia treatment group (Group C) showed that WeiJia could significantly alleviate CCl_4 induced alterations as seen in Group B rats. No obvious changes for fibrosis were observed on the fifth week ($P>0.05$). However, the condition for ballooning degeneration and steatosis were significantly relieved at this stage ($P<0.05$). After prolonged treatment, a significant reduction in inflammation, steatosis ($P<0.05$), necrosis, fibrosis ($P<0.01$) and collagen fibres ($P<0.05$) were observed on the eighth week. Representative HE stained images of sample on the eighth week are shown in Figure 1. There was no apparent difference between male and female rats. The degree of fibrosis, ballooning degeneration and steatosis and collagen expression are summarized in Table 2, Table 3 and Table 4.

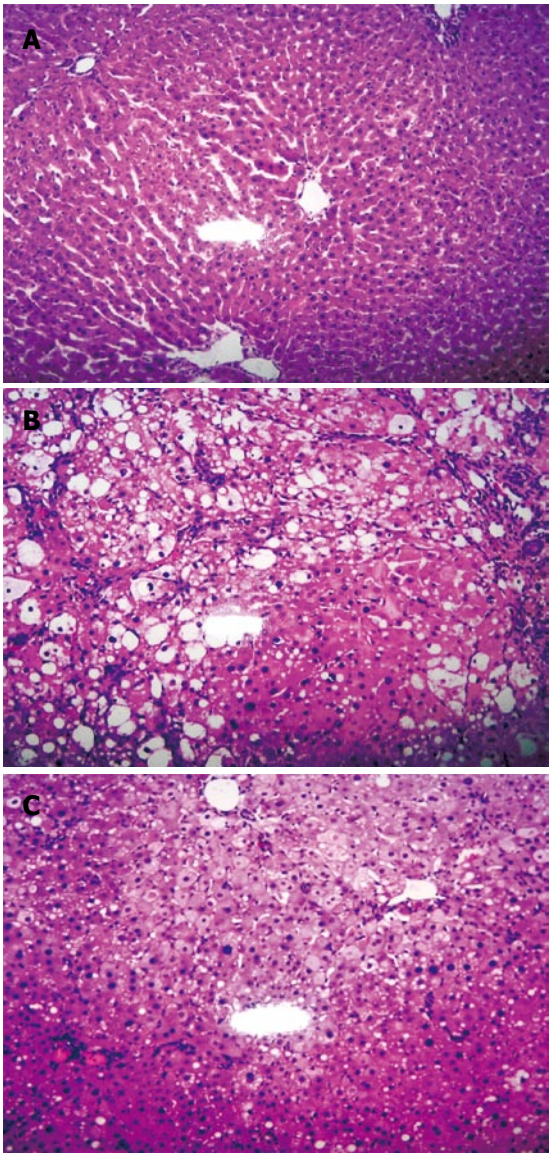

Figure 1 Effect of different treatments on rat liver fibrosis induced by CCl_4 (HE staining, ×100). **A**: Normal control Group A rats, treated with vehicle for eight weeks; **B**: CCl_4 induced control Group B rats, treated with CCl_4 for eight weeks; **C**: CCl_4 induced and WeiJia treatment Group C rats, treated with CCl_4 for two weeks followed by both CCl_4 and WeiJia treatment for six weeks.

Table 3 Ballooning degeneration and steatosis for rats in different treatment group

Group	5th wk				8th wk			
	0	1	2	3	0	1	2	3
A	7				7			
B[b]			3	4		1	2	4
C[a]		1	6			4	3	

$n = 7$ for each group, [a]$P < 0.05$ *vs* Group B, [b]$P < 0.01$ *vs* Group A.

Table 4 Percentage collagen content for rats in different treatment groups

Group	5th wk	8th wk
A	0	0
B[b]	5.2±1.6	7.3±1.6
C	3.8±0.9[a]	4.7±1.0[c]

$n = 7$ for each group, [a]$P > 0.05$ *vs* Group B, [b]$P < 0.01$ *vs* Group A, [c]$P < 0.05$ *vs* Group B.

Table 5 Liver function tests for rats in different treatment group

Group	5th wk			8th wk		
	ALT U/L	AST U/L	γ-GT U/L	ALT U/L	AST U/L	γ-GT U/L
A	34±12	99±12	13±7	32±9	100±19	15±12
B[a]	135±24	158±19	45±23	133±34	166±28	74±36
C	43±12[b,e]	118±10[b,f]	28±19[d,e]	47±10[b,g]	139±13[c,f]	52±21[d,f]

$n = 7$ for each group, [a]$P < 0.005$ *vs* Group A, [b]$P < 0.001$ *vs* Group B, [c]$P < 0.05$ *vs* Group B, [d]$P > 0.1$ *vs* Group B, [e]$P > 0.05$ *vs* Group A, [f]$P < 0.01$ *vs* Group A, [g]$P < 0.05$ *vs* Group A.

Liver function test

The effect of CCl_4 on liver and the role of WeiJia in treating liver fibrosis were also determined through liver function test. Serum levels of ALT, AST and γ-GT were measured and compared with control groups as shown in Table 5.

Significant increase in serum levels of ALT, AST and γ-GT upon CCl_4 induction were observed ($P<0.005$ Group B *vs* Group A). A significant decrease in the levels of ALT and AST were observed after WeiJia treatment ($P<0.05$ Group C *vs* Group B). However, the decrease was not significant for γ-GT ($P>0.1$ Group C *vs* Group B). This indicates that there was no strong correlation between the degrees of fibrosis and γ-GT as γ-GT is an indicator for primary or metastatic malignancy involving liver[20].

It has been known that ALT and AST are useful serum markers for inflammation and necrosis of the liver. ALT is especially useful in acute and cholestatic diseases whereas AST is more sensitive in chronic and infiltrative lesion of the liver. Though the levels of these enzymes decreased after WeiJia treatment, they were still significantly different when compared to Group A ($P<0.05$), except the levels of ALT and γ-GT at the fifth week. Studies have indicated that the ratio rather than the absolute values of the two enzymes provides high specificity in monitoring fibrosis[21-23]. By comparing the ratio of the two enzymes, there was no significant difference between rats treated with WeiJia and that from Group A. The results indicate that WeiJia could alleviate the adverse effect on liver function caused by liver injury.

Immunohistochemical study

As WeiJia treatment could alleviate the effects caused by CCl_4 induction, it is important to understand how WeiJia mediates its effect. It was suggested that the proliferative rate of regenerating hepatocytes may be an important pathogenetic factor in chronic liver disease[24]. Recent studies have also shown that HSC are the primary cell type in mediating fibrogenesis[7-9]. Thus the expression of PCNA and α-SMA were determined to evaluate the cell proliferation and HSC activation in liver injury respectively. Results are summarized in Table 6.

Table 6 Expression of PCNA and α-SMA of rats in different treatment group

Group	5th wk		8th wk	
	PCNA	α-SMA	PCNA	α-SMA
A	17.4±7.0	1.4±0.5	16.6±7.8	1.3±0.5
B	27.9±8.3[a]	4.9±2.3[b]	21.0±5.4[c]	4.6±1.5[b]
C[g]	43.4±14.0[e]	3.7±2.1[1]	31.0±10.0[e]	2.6±1.0[e]

$n=7$ for each group, [a]$P<0.05$ *vs* Group A, [b]$P<0.01$ *vs* Group A, [c]$P>0.05$ *vs* Group A, [1]$P>0.1$ *vs* Group B, [e]$P<0.05$ *vs* Group B, [g]$P<0.05$ *vs* Group A.

Normal hepatocytes are generally quiescent and replicate in a limited and regulated manner[25-27]. High proliferative rates were reported in hepatocellular carcinoma, cirrhosis and acute hepatic failure[28-32]. Nevertheless, recent evidences showed that the replicative activity of hepatocytes diminishes in advanced cirrhosis in humans and in chronic liver injury in mouse, reaching a state of replicative senescence[33-35].

Only limited expression of PCNA was found in normal control Group A whereas increased PCNA expression was observed upon CCl_4 induction in active hepatocytes nuclei. Significant difference was found at the fifth week ($P<0.05$, Group B *vs* Group A). However, expression of PCNA in Group B at the eighth week was not significantly different from that of Group A. PCNA expression increased further with WeiJia treatment ($P<0.05$ Group C *vs* Groups A and B).

In normal liver, HSCs are nonparenchymal quiescent cells for vitamin A storage. In pathological conditions as in liver fibrosis, HSCs lose retinoids and synthesize a large amount of extracellular matrix components including collagen, proteoglycan and adhesive glycoproteins. Morphology of these cells also changes from the star-shaped stellate cells to that of fibroblasts or myofibroblasts[36]. α-SMA is a good indicator for HSC activation.

Only limited α-SMA expression was observed in normal control group A. Upon CCl_4 induction, increased amount of α -SMA expression by activated HSC was observed ($P<0.01$ Group B *vs* Group A). The expression was reduced with WeiJia treatment. However, significant reduction was only observed at the eighth week ($P<0.05$ Group C *vs* Group B). Though WeiJia could reduce α -SMA expression, its level was still significantly different from that of Group A ($P<0.05$ Group C *vs* Group A). The results indicated that WeiJia could mediate the alleviation of CCl_4 induced injury through the proliferation of regenerating hepatocytes and the reduction of stellate cell activity.

DISCUSSION

WeiJia is an effective therapeutic agent for severe hepatitis. However, its action mechanism is not clear. WeiJia was also shown to be effective in cirrhosis treatment. Thus we hypothesize that it may also play a role in fibrosis treatment. In this study, its effect on liver fibrosis was evaluated through CCl_4 induced liver injury animal model. This study also provides some information for understanding the mechanism of WeiJia.

It was found that treatment of WeiJia could relieve CCl_4 induced liver necrosis, ballooning degeneration, steatosis and inflammation. The effect was significant at an early stage of treatment at the fifth week. An improved liver function was also observed at this stage of treatment. The results suggest that WeiJia protects liver cells from damage induced by CCl_4 and the therapeutic effects of WeiJia on severe hepatitis might be related to the protective effect of this medication.

Upon liver injury, the body will attempt to repair the damage through increasing the expression of hepatocyte growth factor (HGF), transforming growth factor beta (TGF-β) and other cytokines to enhance hepatocytes proliferation and initiate tissue repairing process. Replicative activity of hepatocytes diminishes in advanced cirrhosis in humans and in chronic liver injury in mouse [33-35]. PCNA expression in Group B was the response of hepatocyte to liver damage. The level of PCNA expression in Group C was significantly higher than that in Group B suggesting that liver cells of rats in Group C have stronger replicative activity. The increase in PCNA expression in WeiJia treated rats with CCl_4 induced liver damage demonstrated that WeiJia has protective effect on liver cells.

Uncontrollable remodelling and regeneration would lead to the development of fibrosis due to excessive deposition of extracellular matrix. The data from this investigation also showed that WeiJia was effective in fibrosis treatment. In addition to inflammation and necrosis, CCl_4 induction also resulted in collagen deposition and liver fibrosis as observed through histopathologic examination. Treatment with WeiJia reduced ballooning degeneration, steatosis and accumulation of collagen. The effect was significant at an early stage of treatment at the fifth week. An improved liver function was also observed at this stage of treatment with the increase in PCNA expression. Significant reduction of fibrosis was only observed after a longer period of treatment at the eighth week. Nevertheless, the results indicate that early treatment with WeiJia might prevent the progression of liver injury to fibrosis through increased liver regeneration and reduced liver necrosis.

It is believed that HSC activation is a critical step in hepatic fibrosis. Levels of serological markers (HA, CIV) and α-SMA expression clearly indicated that HSC was activated by CCl_4 induced liver injury. WeiJia treatment led to a significant reduction of theses proteins, indicating that the mechanism of WeiJia in reducing hepatic fibrosis may be through the inactivation of HSC.

In conclusion, WeiJia is shown to be an effective therapeutic agent that could alleviate liver fibrosis through the stimulation of liver regeneration and inhibition of HSC activation.

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BASIC RESEARCH

Effects of interleukin-10 on activation and apoptosis of hepatic stellate cells in fibrotic rat liver

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Abstract

AIM: To study the effects of interleukin-10 (IL-10) on the expression of α-smooth muscle actin (α-SMA), nuclear factor-κB(NF-κB) and Fas/Fas ligand (FasL) in hepatic stellate cells of experimental rats with hepatic fibrosis.

METHODS: Sixty clean SD rats were randomly divided into control group (group N), liver fibrotic group (group C) and IL-10 treatment group (group I). Control group received intraperitoneal injection of saline (2ml•kg^{-1}), twice a week. Fibrotic group was injected intraperitoneally with 50% carbon tetrachloride (CCl_4) (2 ml•kg^{-1}), twice a week. IL-10 treatment group was given IL-10 at a dose of 4 μg•kg^{-1} 20 minutes before CCl_4 administration from the third week. Hepatic stellate cells (HSCs) were isolated from these rats at the seventh and eleventh weeks during the course of liver fibrosis, respectively. The expression of α-SMA and NF-κB in HSCs was measured by S-P immunohistochemistry. The expression of Fas and FasL mRNA was measured by RT-PCR. Furthermore, liver tissues were harvested from three groups at the same time.

RESULTS: The CCl_4- induced experimental rat hepatic fibrosis model was established successfully. The purity of extracted hepatic stellate cells was about 95% and the yield of hepatic stellate cells was 1.2-2.3×10^6/g liver tissue averagely. The positive expression of α-SMA and NF-κB was 36.5% and 28.5% respectively in group N. The positive levels of α-SMA and NF-κB were increased significantly in group C compared to group N (P<0.01). The positive signals decreased significantly (P<0.05) in group I. In the 11th week, the HSCs of group I became round with visible pyknotic nuclei. The expression of NF-κB in group C was significantly increased in a time-dependentmanner (P<0.01), but there was no difference in the α-SMA expression (P>0.05). The mRNA of Fas and FasL in group C was significantly increased in a time-dependent manner compared to that in control group. After treated with IL-10, the expression level of Fas and FasL was higher in group I than in group C.

CONCLUSION: The positive expression of α-SMA and NF-κB in hepatic stellate cells is decreased by ectogenic IL-10 in liver fibrosis induced by CCl_4. The expression of Fas and FasL is increased in the course of liver fibrosis, and is further increased by IL-10. IL-10 could inhibit the activation of HSCs and cause apoptosis of activated HSCs.

Key words: Liver fibrosis; Hepatic stellate cell; Interleukin-10;α-smooth muscle actin; Nuclear factor-κB; Rat

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INTRODUCTION

Liver fibrosis is a model of wound-healing responses to chronic liver injury and is the excessive accumulation of extracellular matrix proteins in most types of chronic liver diseases[1]. The cellular and molecular mechanisms of liver fibrosis have greatly advanced since hepatic stellate cells (HSCs) were identified as the main collagen-producing cells in the liver. HSCs are the central event in hepatic fibrosis[2-3]. In normal liver, HSCs reside in the Disse's space and are the major storage sites of vitamin A. Following chronic injury, HSCs activate or transdifferentiate into myofibroblast-like cells, acquiring contractile and fibrogenic properties, secreting cytokines and expressing α-smooth muscle actin(α-SMA)[4-5]. The mechanisms involved in cytokine secretion by HSCs include activation of the transcription nuclear factor-κB (NF-κB). NF-κB, a key transcription factor induces genes involved in inflammation, responses to infection, and stress[6]. DNA binding activity of NF-κB is demonstrated in activated but not in quiescent HSCs, and activation of HSCs is associated with the nuclear translocation of

NF-κB. Inhibition of NF-κB by proteosome inhibitors or by adenovirus expressing the IkB superrepressor can markedly blunt cytokine secretion by activated HScs. Using differential display, investigators have shown that intercellular adhesion molecule 1 (ICAM-1) is expressed in HSCs activated in culture or *in vivo*, but not in quiescent HSCs[7]. The ICAM-1 gene contains a NF-κB binding site and its transcription is stimulated by NF-κB[8,9]. This observation provides functional support for a critical role of NF-κB in the activation of HSCs. As liver injury resolves, the number of activated stellate cells decreases through 1 of 2 potential pathways, namely spontaneous reversion or clearance by apoptosis[10,11]. To date, spontaneous reversion of myofibroblasts to quiescent cells has only been documented in culture, but not *in vivo*. In contrast, there is evidence that HSCs undergo apoptosis during resolution of liver injury *in vivo*. Some data demonstrate that apoptosis of HSCs in the course of activation is accompanied with increased expression of FasL by HSCs themselves[12]. Fas/FasL system is the key pathway for apoptosis of HSCs. Our work team found that ectogenic IL-10 could decrease liver fibrosis, but its mechanism still remains unclear. In the present study, α-SMA, NF-κB and Fas/FasL were selected as the targets to study the effects of interleukin-10 on activation and apoptosis of HSCs.

MATERIALS AND METHODS

Materials

Sixty clean male Sprague-Dawley rats, weighing 400-500g (provided by Shanghai Experimental Animal Center), were divided randomly into 3 groups. The control group (group N) included 8 rats, the fibrotic group (group C) included 28 rats and the IL-10 intervention group (group I) included 24 rats. All the rats were bred under clean conditions (room temperature: 22℃±2℃; humidity: 55%± 5%) in a 12 h light/dark cycle with free access to drinking water and food. High voltage disinfectant animal food was provided by BK Company in Shanghai, China.

Reagent

Recombined rat interleukin-10 was provided by Jingmei Biotechnology Corporation. Collagenase type IV and Nycodenz were provided by Sigma Corporation. Pronase E was provided by Merk Corporation. DNase I was provided by Sino-American Biotechnology Corporation. DMEM was provided by Gibco Corporation. Mouse anti-rat α-SMA and NF-κB monoclonal antibodies were provided by Santa Cruz Biotechnology Inc. SP immunocytochemistry kit was provided by American Zymed Company. RNA isolation kit was provided by Gentra Company (USA) and PCR kit was provided by Promega Company. The primers were synthesized by Beijing Sanbo Company.

Animal models

The rats of group N were injected intraperitoneally with saline (2ml·kg^{-1}), twice a week. The rats of groups C and I were intraperitoneally injected with 50% CCl_4 (dissolved in castor oil) (2ml·kg^{-1}), twice a week. From the third week, the rats of group I were injected intraperitoneally with IL-10 (4μg·kg^{-1}) (dissolved in saline) 20 minutes before they were injected with CCl_4[13]. All injections were performed on Monday and Thursday with the body weights determined before each injection. By the end of the experiment, 12 rats in group C and 8 in group I died. No animals died in group N. In the seventh and eleventh weeks, 3 rats in group N and 5 rats in the other two groups were sacrificed to collect their hepatic stellate cells. The liver tissue was collected from 1 rat in each group at this time point and fixed in 10% formalin and embedded with paraffin.

Isolation and culture of hepatic stellate cells

Nonparenchymal cells were isolated from rats of the experimental groups by sequential perfusion with collagenase and pronase E as described previously [14,15]. Buoyant HSCs were separated from the resulting cell suspension by elutriation over a Nycodenz gradient. In brief, the liver of rats was routinely perfused through a portal vein catheter with Ca^{2+}-free D-Hanks solution, then with pronase E and type IV collagenase dissolved in Ca^{2+} containing D-Hanks solution. The liver was homogenized and incubated with pronase E, type IV collagenase and DNase I dissolved in Ca^{2+} containing D-Hanks solution for 20 min at 37℃ with constant stiring. This suspension was centrifuged by 11% Nycodenz density gradient centrifugation for 15 minutes at 1500r/min after filtered. The cells were aspirated from the interface, washed twice in DMEM solution, and then resuspended in DMEM containing 20% calf serum. The HSCs were seeded onto plastic tissue culture bottle at 1×10^6 cells/mL and incubated at 37℃ with 50mL/L CO_2 in air.

Immunocytochemistry measurement

The HSCs cultured for 24 hours were fixed by 4% paraformaldehyde at 4℃ for 24 hours, then incubated in PBS containing 3%H_2O_2 to remove endogenous perxoidase activity and then in PBS containing 0.1mol/L citrate to saturate nonspecific binding sites. After incubation with 1∶100 mouse anti-rat α-SMA and NF-κB monoclonal antibody, the reaction was carried out with the instant S-P immunocytochemistry reagents. The primary antibody was replaced with PBS as blank contrast. The plates were incubated in a buffer containing 3,3-diaminobenzidine tetrahydrochloride (DAB) and H_2O_2 to produce a brown reaction product, then imaged under microscope.

Result assessment

Two hundred cells were calculated under the microscope. The reactions were graded and scored according to their intensities and percentage of the positive cells as follows: zero score for negative reaction, 1 score for pale yellowish staining, 2 scores for dense yellow staining and 3 scores for brown staining. The eventual result was produced by the two scores for staining intensity and positive cell percentage.

Reverse transcription- polymerase chain reaction (RT-PCR) for Fas and FasL

Total RNA was isolated from HSCs using Gentra

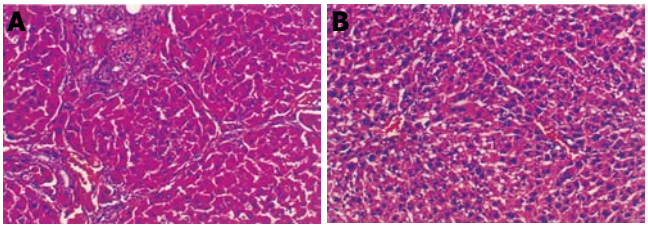

Figure 1 Liver of rats in groups C (**A**) and I (**B**) (week 11, H-E staining, ×100)

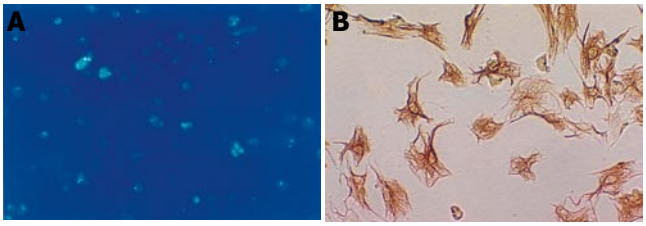

Figure 2 Autofluorescence (**A**) and desmin staining (**B**) of HSCs.

reagent (USA) according to the protocol provided by the manufacturer. The A260/A280 of total RNA was between 1.8 and 2.0. After treated with DNase-I, 1-2μg of total RNA was reversely transcribed into complementary DNA (cDNA) with oligo (dT) using cDNA synthesis kit. Then 2 μL of cDNA product was used as the template to amplify specific fragments in a 25μL reaction system. PCR was performed with an initial denaturation at 94℃ for 5 min, followed by 25 cycles at 94℃ for 45 s, annealing at 55℃ for 30 s (FasL at 58℃), at 72℃ for 60 s, with a final extension at 72℃ for 7 min. The primer sequences used are sense: 5' -GAATGCAAGGGACTGATAGC-3' and antisense: 5' -TGGTTCGTGTGCAAGGC TC-3' for Fas; sense: 5' -GGAATGGGAAGACACATATGGAACTGC-3' and antisense: 5'-CATATCTGGCCAGTAGTGCAGTAAT TC-3' for FasL; sense: 5'-GAGCTATGAGCTGCCTGA CG-3' and antisense: 5'-AGCACTTGCGGTCCAC GATG-3' for β-actin (660bp); sense: 5' -GAGCTATGAGCTGC CTGACG-3' and antisense: 5' -AGCACTTGCGGTCCACGATG-3' for β-actin (410bp).

Electrophoresis and semi-quantitative analysis

The PCR products were run on 2% agarose gel electrophoresis and visualized with ethidium bromide staining. The expected product sizes were Fas (414bp), FasL (239bp), β-actin (660bp and 410bp). Bioimagine system was used to detect the density of bands of PCR products. The values of Fas and FasL expression were calculated from percentage of the ratio of band density of PCR products and the band density of β-actin.

Statistical analysis

All data were expressed as mean ± SD. The significance for the difference between the groups was analyzed with SPSS10.0 by one-way ANOVA. $P<0.05$ was considered statistically significant.

RESULTS

Animal model

Liver fibrosis was remarkable during the treatment with CCl_4. In the seventh week, steatosis and ballooning degeneration of hepatocytes were obvious, plentiful inflammatory cells infiltrated into the Disse's space, collagen fibers increased and extended to the parenchyma. In the eleventh week, collagen fibers formed widely, complete fibrous septa were seen and pseudolobular structures were also present occasionally. In the IL-10 treatment group, the CCl_4-caused alterations as described above seemed to be markedly alleviated, less profound steatosis and necrosis were noted in the seventh week, and no distinct change was found in the eleventh week compared to the normal group (Figures 1A, 1B).

Hepatic stellate cell identification

HSCs were isolated successfully. The yield of HSCs was $1.2\text{-}2.3\times10^6$/g liver tissue. HSCs were identified according to their typical autofluorescence at 328-nm excitation wavelength (Figure 2A) and by immunohistochemistry with monoclonal antibody against desmin. Greater than 95% of the isolated HSCs were stellate cells (Figure 2B). The cells' viability was determined by trypan blue exclusion staining with viability over 90%.

Expression of α-SMA and NF-κB in HSCs

The granular positive products of α-SMA were localized in cytoplasm of HSCs, and the NF-κB was in cytoplasm and nuclei. In control group, the expression was weak and pale yellowish, the positive rate of α-SMA and NF-κB was 36.5% and 28.5%, respectively. In group C, the expression increased obviously with the development of liver fibrosis (positive rate was 100% and the granular positive products were dense yellow or brown). The distribution of NF-κB was found mainly in nuclei. In group I, the changes were less pronounced than in group C. Especially in the eleventh week, the HSCs became round with visible pyknotic nuclei, the vigour of cells was weak (Figures 3A-3E). Comparison of α-SMA and NF-κB positive expression levels among 3 groups is shown in Table 1 and in Figures 4A, 4B. In group I, IL-10 treatment resulted in decreased immunoreactivties of α-SMA and NF-κB ($P<0.05$). With the development of liver fibrosis, the intensities of NF-κB increased gradually, but that of α-SMA did not change significantly.

Expression of Fas and FasL in HSCs

The Fas and FasL mRNA could be measured in HSCs of control group. The mRNA of Fas and FasL in fibrotic group was increased in a time-dependent manner compared to that in control group. After treated with IL-10, the expression level of Fas and FasL mRNA was higher in fibrotic group than in control group. The expression of Fas and FasL mRNA was increased in the course of liver fibrosis and was further increased by IL-10 (Figures 4C, 4D). Comparison of Fas and FasL mRNA expression levels among 3 groups is shown in Table 2 and in Figures 4E, 4F.

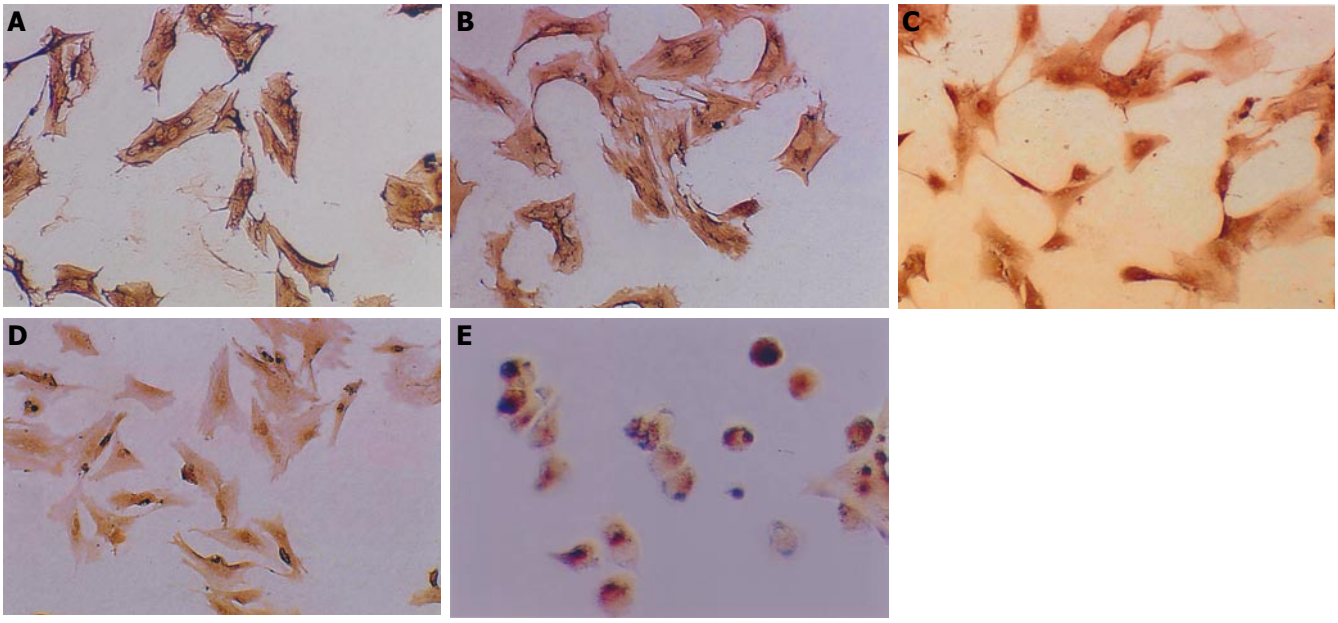

Figure 3 α-SMA in HSCs of group C (**A**) in week 11 and group I (**B**) in week 7; NF-κB in HSCs of group C (**C**) in week 11 and group I (**D**) in week 7; and changes of HSCs in group I (**E**) in week 11.

Table 1 Positive expression levels of α-SMA and NF-kB in HSCs of 3 groups (mean±SD)

	Group N		Group C		Group I	
	wk 7	wk 11	wk 7	wk 11	wk 7	wk 11
α-SMA	0.64±0.05	0.68±0.07[d]	2.43±0.03	2.47±0.14[b]	2.14±0.11	2.09±0.06[c]
NF-κB	0.43±0.08	0.42±0.05[d]	2.13±0.09	2.48±0.70[a]	1.92±0.42	1.62±0.10[a]

[a]P<0.05 *vs* week 7 of group N; [b]P<0.05 *vs* week 7 of group C: [c]P<0.05 *vs* group C; [d]P<0.05 *vs* week 7 of group N.

Table 2 Expression levels of Fas and FasL in HSCs of 3 groups (mean±SD)

group	Fas			FasL		
	group N	group C	group I	group N	group C	group I
7th week	0.36±0.02	0.66±0.02	0.74±0.02[a]	0.34±0.03	0.45±0.03	0.52±0.05[a]
11th week	0.40±0.01	0.72±0.02[a]	0.73±0.04[b]	0.36±0.05	0.62±0.04	0.83±0.04[a]

[a]P<0.05 *vs* groups C and N; [b]P<0.05 *vs* week 11 of group C.

DISCUSSION

Liver fibrosis results from the excessive secretion of matrix proteins by HSCs. In normal liver, HSCs are nonparenchymal, quiescent cells whose main function is to store vitamin A[16]. In response to liver injury, HSCs undergo an "activation" process in which they produce cytokines and chemokines, express receptors of cytokines and chemokines, and synthesize ECM[17]. Activation of HSCs is the central event of liver fibrosis, which consists of 2 major phases: initiation and perpetuation[18]. The earliest changes in stellate cells are likely to result from paracrine stimulation by all neighboring cell types, including Kupffer cells, sinusiodal endothelium, etc. Perpetuation of stellate cell activation involves several discrete changes in cell behavior, such as proliferation, chemotaxis, fibrogenesis, contractility, matrix degradation, of which contractility of HSCs may be a major determinant during liver fibrosis. The activated HSCs show common phenotypic features of smooth muscle cells and myofibroblasts, shape of well-developed stress fibers of actin cytoskeleton. The microfilament protein α-SMA has been explored as a marker for activated HSCs. Quiescent cells are negative *in vitro* or *in vivo* and activated HSCs are clearly positive[19]. This suggests a close relationship between α-SMA induction and liver fibrosis. Our data show that α-SMA is expressed in activated hepatic stellate cells in the course of liver fibrosis. After the treatment with IL-10, the expression of α-SMA decreased, indicating that ectogenic IL-10 may release activated HSCs.

NF-κB exists in cytoplasm as an inactive form associated with regulatory proteins called inhibitors of kB (IkB)[20]. Phosphorylation of IkB, an important step in NF-κB activation, is mediated by IkB kinase (IKK). Appropriate stimuli induce selective IkB phosphorylation, which is then degraded by the proteasome pathway. Free NF-κB migrates to nuclei by virtue of its nuclear localization signal and induces transcription of multiple kB-dependent genes. Newly synthesized IkB both in cytoplasm and in nuclei inactivates NF-κB. NF-κB regulates the transcription of a number of proinflammatory molecules involved in acute responses to injury and chronic liver inflammation, including TNF-a, IL-6, ICAM-1 etc[21]. The induction of NF-κB during liver regeneration after partial hepatectomy appears to be a required event to prevent apoptosis and allow for normal cell cycle progression. NF-κB is a key transcription factor involved in activation of HSCs[22,23]. Inhibition of the IKK/NF-κB pathway is sufficient to increase the rate at which activated hepatic stellate cells undergo

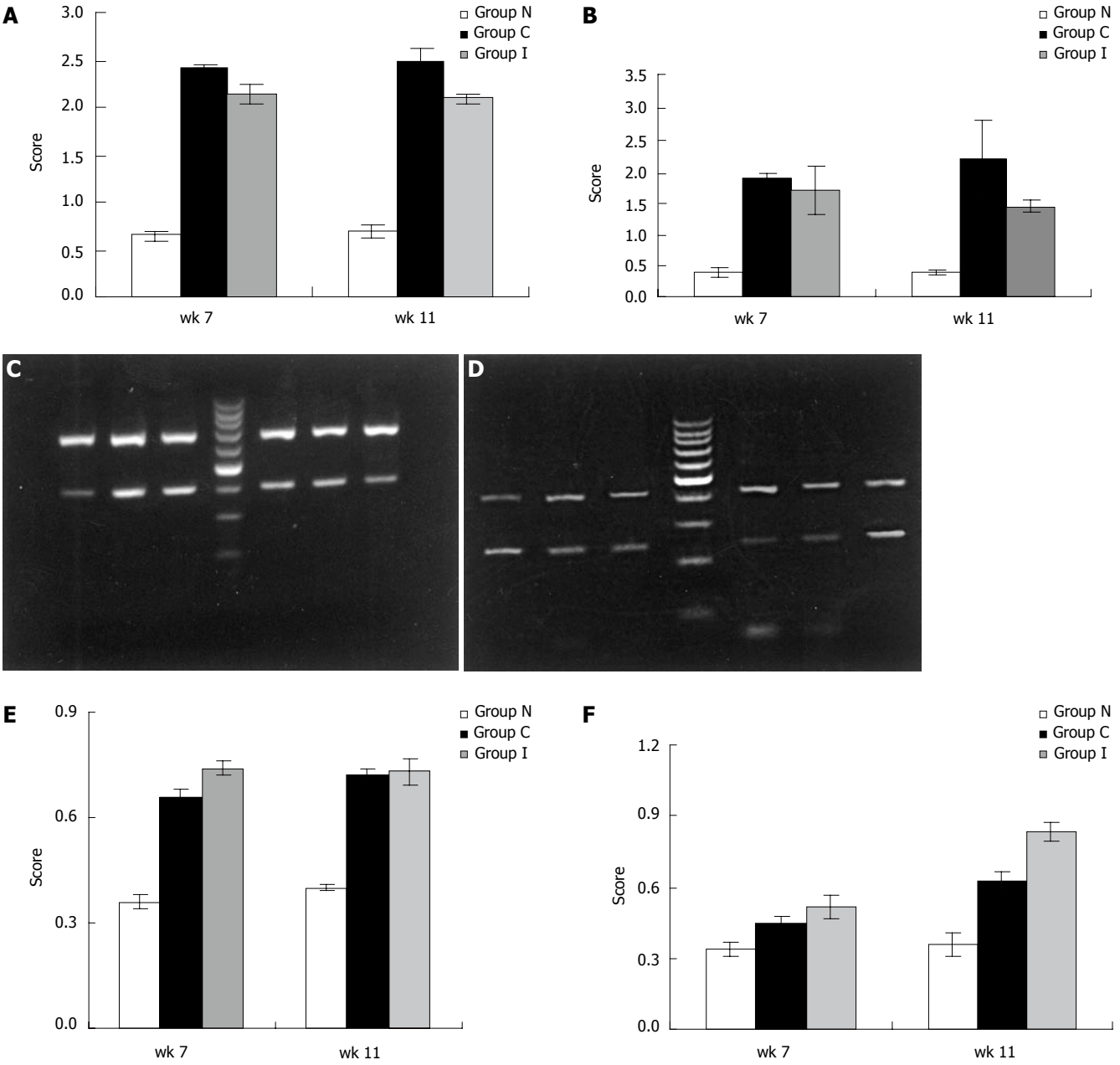

Figure 4 Expressions of α-SMA (**A**), NF-κB (**B**), Fas mRNA (**C**), FasL mRNA (**D**), Fas (**E**), and FasL (**F**) in HSCs.

apoptosis *in vitro* and *in vivo*. Drugs selectively targeting IKK have potential as antifibrotics[24]. Although in some studies IL-10 has been demonstrated to block NF-κB activation, our data suggest that IL-10 could decrease the NF-κB expression. The molecular target for IL-10-induced inhibition of NF-κB has not been established. There is evidence that IL-10 regulates NF-κB by dual mechanisms[25]. Firstly, IL-10 blocks IKK activity, thus inhibiting phosphorylation and degradation of IkBa. The preserved IkBa continues to bind to NF-κB in cytoplasm, prohibiting NF-κB nuclear translocation and NF-κB-dependent transcription. Secondly, IL-10 can directly block NF-κB DNA binding by a mechanism that is independent of NF-κB nuclear translocation.

Apoptosis associated with liver disease is increasingly viewed as a nexus through which many key pathways converge[26]. Pathologic apoptosis in liver may not only result from inflammation and fibrosis, but also in turn amplify these responses. In particular, HSCs contribute to apoptosis and inflammation. As the liver injury resolves, apoptosis of activated HSCs may be involved in the reversion of liver fibrosis[27]. Fas is known as an important mediator of apoptosis and acts as an inducer of apoptosis in Fas-expressing cells in response to ligand binding (FasL)[28]. Saile *et al*[29] reported that resting HSCs display no sign of apoptosis and spontaneous apoptosis becomes detectable in parallel with HSC activation, suggesting that apoptosis might represent an important mechanism terminating proliferation of activated HSCs. They also demonstrated that apoptosis of HSCs in the course of activation is accompanied with increased expression of FasL by the HSCs themselves. The activated HSCs possess more Fas and FasL, compared with HSCs in the resting and transitional phase. The apoptosis of HSCs could largely be

inhibited by blocking Fas, indicating that Fas/FasL system plays a major role in initiation of apoptosis. Thus, driving activated HSCs into apoptosis may be a way to resolve fibrosis. Our data show that with the development of liver fibrosis, the Fas/FasL system mRNA expression increases. In addition, IL-10 could promote the expression of Fas and FasL mRNA-activated HSCs, implying that IL-10 may promote activated HSCs into apoptosis through binding of FasL to Fas on the cell membranes of HSCs.

IL-10 is pleiotropic[30-31] and has multiple effects on diverse cell types. One of the most important properties of IL-10 is its anti-inflammatory action, which restrains the immune response under various stimuli, otherwise the individuals would have deleterious consequences. Evidence of *in vivo* function of IL-10 indicates that in the absence of IL-10 (in genetically IL-10 deficient animals), an exaggerated inflammatory response can lead to inflammatory states[32]. In our study, the ectogenic IL-10 could alleviate liver fibrosis induced by CCl_4 in rats. During the course of liver fibrosis, the positive expression of α-SMA in HSCs was decreased by IL-10, suggesting that activation of HSCs can be inhibited by IL-10. The mechanisms may involve blocking NF-κB activation. At the same time, the expression of Fas and FasL mRNA was further increased by IL-10, suggesting that IL-10 may induce apoptosis of HSCs. In brief, IL-10 could inhibit activation and promote apoptosis of HSCs, which may be related with its mechanism against fibrosis.

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CLINICAL RESEARCH

Molecular markers (*PECAM-1, ICAM-3, HLA-DR*) determine prognosis in primary non-Hodgkin's gastric lymphoma patients

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Abstract

AIM: To investigate the prognostic significance of *PECAM-1*, *ICAM-3* and *HLA-DR* antigens in patients with primary non-Hodgkin's gastric lymphoma.

METHODS: We immunohistochemically studied *PECAM-1*, *ICAM-3* and *HLA-DR* antigen expression in 36 B-cell MALT-type primary gastric lymphoma patients. Ten non-malignant and ten healthy gastric tissue specimens were used as controls. Clinicopathological and survival data were correlated with the staining results.

RESULTS: *HLA-DR* antigen expression was detected in 33 gastric lymphoma patients (91.7%) and 6 non-malignant patients (54.5%). *PECAM-1* stained tumor cells of 10 patients (27.8%), endothelial cells of 9 patients (25%) and inflammatory infiltrate of 4 patients (40%) with benign gastric disease. *ICAM-3* expression was observed on the tumor cells of 17 patients (47.2%), while 5 non-malignant patients (50%) were stained positive as well. None of the healthy controls was stained for any of the genes studied. In the multivariate analysis, *HLA-DR* antigen and *PECAM-1* were proved to be statistically significant independent prognostic factors associated with a favourable and an unfavourable prognosis respectively ($P = 0.009$ and $P = 0.003$). In the univariate analysis, *PECAM-1*(+)/*ICAM-3*(-) and *HLA-DR*(-)/*ICAM-3*(-) patients exhibited a significantly decreased overall survival compared to those with the exactly opposite gene expression patterns ($P = 0.0041$ and $P = 0.0091$, respectively). Those patients who were *HLA-DR*(+)/*ICAM-3*(+)/*PECAM-1*(-) ($n = 8$) had a significantly higher survival rate compared to the rest of the group ($n = 24$) ($P = 0.0289$).

CONCLUSION: *PECAM-1*, *ICAM-3* and *HLA-DR* are representative markers of tumor expansion potential and host immune surveillance respectively. Their combined use may help us to identify high-risk patients who could benefit from more aggressive therapeutic protocols.

Key words: *PECAM-1*; *ICAM-3*; *HLA-DR*; Non-Hodgkin's gastric lymphoma; Prognosis

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INTRODUCTION

Primary gastric B-cell non-Hodgkin's lymphomas are uncommon tumors, constituting less than 2% of all primary gastric malignancies[1]. Numerous clinicopathologic studies have identified stage and grade as the most important prognostic factors[1,2]. Still there are a number of patients with favourable stage and grade that exhibit an aggressive phenotype. A second line of molecular prognostic markers has been introduced lately to better describe this clinical entity.

Several different molecules (*bcl-2*, *p53*, *PCNA*, *c-fos*, *c-myc* and *Ki67*) and biologic pathways have been implicated in the initiation and progress of primary non-Hodgkin's gastric lymphoma[3]. Immunohistochemical tracing of these molecules in gastric lymphoma patients has already been employed not only for the diagnosis but for the determination of prognosis as well[4].

HLA-DR antigen is a class II MHC membrane-bound glycoprotein, which plays an important role in the regulation of the immune response[5]. Although it is normally expressed exclusively by antigen presenting cells of the immune system[6], it shows variable expression in malignancies[7,8]. During oncogenesis, it is modified to affect tumor

Table 1 Demographics and clinicopathological characteristics of 36 patients with primary gastric lymphoma

Characteristics	Frequency	Percentage (%)
Age of disease presentation (yr)	58.39 ± 15.39 (median: 59, range: 33-82)	
Gender		
Male	21	58.4
Female	15	41.6
Type of Surgery		
Total gastrectomy	10	27
Subtotal gastrectomy	20	55.5
Unknown	6	17.5
Microscopic Margins		
Positive margins	5	13.9
Negative Margins	31	86.1
Stage		
I	17	47.2
II	7	19.4
III	6	16.7
IV	6	16.7
Histologic Grade		
Low grade	15	41.7
Intermediate grade	7	19.4
High grade	14	38.9
Tumor Surface (cm^2)	50.04 ± 47.9 (median 29.15, range 1.5-180)	
Tumor Thickness (mm)	7.05 ± 4.07 (median: 6, range:1.5- 15)	
Tumor Diameter (cm)	6.66 ± 3.8 (median: 5.75, range: 1-15)	
Adjuvant Chemotherapy		
Yes	25	69.4
No	8	22.3
Unknown	3	8.3

cell behavior by decreasing or enhancing specific anti-tumor immune mechanisms[9].

Cell adhesion molecules are membrane glycoproteins that play a major role in neoplastic disease by interfering with cell-matrix and cell-cell interactions. They are also believed to participate in host immune surveillance by providing antigen non-specific recognition between Th-cells, APCs, Tc-cells, NK-cells and their potential targets. *ICAM-3* and *PECAM-1* are considered to be representative members of the immunoglobulin Ig superfamily of cell adhesion molecules (*CAMs*). *ICAM-3* is constitutively highly expressed by leukocytes especially in lymphomas and myelomas[10]. It is postulated that it may constitute the critical ligand for the initiation of lymphocyte immune responses [11] with possible antitumoral properties. Tumor expansion is angiogenesis dependent[12,13], a function which is orchestrated by a constant interaction between tumor cells and host cells. This "cross talk" can lead either to cell cycle arrest and tumor regression or to tumor progression[14,15]. *PECAM-1* (platelet/endothelial cell adhesion molecule) is considered to be an accurate measure for the assessment of vascular proliferation on tumor sections[12] determining prognosis in a variety of tumors[16,17]. Moreover, *PECAM's-1* expression by immunocompetent cells[11] as well as its regulatory role in the extravasation of leukocytes[18], suggests a potential pathway through which lymphoid tumors may escape immune surveillance.

Although the above molecules appear to represent interacting pathways responsible for the antitumoral response and tumor progression, up to day very little information is available on their role in the pathogenesis and prognosis of primary gastric lymphoma[19-21]. The aim of this study was to analyze the immunohistochemical expression of *ICAM-3* (*CD50*), *PECAM-1* (*CD31*) and *HLA-DR* antigens in a group of patients operated for primary non-Hodgkin's gastric lymphoma with respect to their clinicopathological characteristics and clinical outcome.

MATERIALS AND METHODS

We retrospectively recruited 36 B-cell MALT-type non-Hodgkin's primary gastric lymphoma patients (76.6%). All of them were diagnosed and treated during the period from 1991 to 1997 at the First Department of Propaedeutic Surgery of the Hippokration Hospital, Athens Medical School. Follow-up time measurements were specifically interrupted by death of the affected individuals from any cause. In this regard, follow-up time ranged between 15 d and 135.5 mo, with a mean of 60.5 ± 38.8 mo and a median of 68.5 mo. Favorable treatment outcome was defined as undetectable disease at the most recent follow-up. Unfavorable treatment outcome was defined as tumor recurrence, either locally or distantly, or death due to the tumor. Four patients had inadequate 5-year survival data and were excluded from the survival analysis. All reported deaths were attributed to the underlying disease, thus the overall survival corresponded to tumor-associated survival. Our study also included 10 gastric tissue specimens of non-malignant origin (Analytically this group consisted of 4 patients with gastritis and 6 patients with non malignant ulcer of the stomach, none of whom was submitted to any kind of surgery) and 10 healthy control tissue specimens. The characteristics of gastric lymphoma patients as well as information regarding adjuvant therapy are summarized in Table 1.

All patients were diagnosed by endoscopic biopsy and preoperatively evaluated by bone marrow biopsy, chest radiography, abdominal CT and indirect laryngoscopy. The mean age of disease presentation was 58.39 ± 15.39 years (median: 59 years).

Staging was performed according to the Japanese Classification of Gastric Carcinoma by the Japanese Gastric Cancer Association[22]. Seventeen patients (47.2%) were stage I, 7 (19.4%) stage II, 6 (16.7%) stage III and 6 (16.7%) stage IV. Histopathologic examination was undertaken according to the working formulation[23]. Fifteen (41.7%) were found to be low grade lymphomas (grade I), 7 (19.4%) intermediate grade lymphomas (grade II) and 14 (38.9%) high grade lymphomas (grade III). The presence of *H pylori*-associated MALT-type lymphoma was not determined in the majority of cases and this information was not included in our statistical analysis. Ten patients (27.7%) received a total gastrectomy and 20 (55.5%) a subtotal gastrectomy, while there was no information regarding the type of surgery for 6 (16.8%) patients. All patients had macroscopically clear margins, while microscopically

involved resection margins were detected in 5 patients (13.9%). Intraoperative staging consisted of biopsies of the liver and any enlarged abdominal lymph nodes. Splenectomy was performed only if the spleen was directly invaded.

Adjuvant chemotherapy was administered to 25 patients (69.4%), 3 of whom (8.3%) received a combination of adjuvant chemo/radiotherapy. Eight patients (22.3%) did not receive any kind of supplemental therapy, while no data were retrieved for three more (Table 1).

Immunohistochemistry

Immunohistochemical studies were performed on formalin-fixed and paraffin-embedded sections using the streptavidin- biotin- peroxidase method (Novostain Super ABC Novocastra laboratories Ltd, Newcaste, UK) with monoclonal antibodies specific for *HLA-DR* (DAKO, Glostrup, Denmark, dilution 1:70) and *PECAM-1* (Oncogene Research Products, Calbiochem, Boston, dilution 1:50) and *ICAM-3* (Oncogene Research Products, Calbiochem, Boston, dilution 1:1000). The assignment was performed on surgical specimens obtained during the surgical excision of the tumors.

The staining technique used was the same as previously described[24, 25]. The sections were deparaffinized in xylene and rehydrated in graded ethanol. Antigenic determinants masked from the formalin-fixation and parafin-embedding were exposed to saponin. Briefly, the endogenous peroxidase activity was blocked using a hydrogen peroxide solution. The primary antibody was then applied overnight at 4℃ followed by incubation with a polyvalent antibody for 30 min and then a streptavidin-peroxidase reagent for 30 min at room temperature. Diaminobenzidine tetrahydrochloride (DAKO) was used as the chromogen, and hematoxylin for counterstaining. Appropriate positive and negative controls were used.

All slides were evaluated by two independent reviewers. The percentage of *HLA-DR* (+) tumor cells was estimated, compared to the total area covered by the tumor in 10 randomly selected low power fields (x40). The proportion of *HLA-DR* (+) stromal cells close to and far from the tumor (macrophages, leukocytes, activated T-cells, necrotic tissue) was also assessed, as well as *HLA-DR* expression in normal gastric mucosa and stroma, for all the slides containing benign gastric tissue. HLA-DR antigen expression was located mainly in the cytoplasm but on some occasions membrane staining was seen. According to our initial estimation paraffin sections expressing *HLA-DR* in less than 5% of the tumor were considered negative. Tumor sections in which most of the tumor was found positive for *HLA-DR* expression in 10 randomly selected low power fields, were characterized as >75% positive.

A semi-quantitative grading system for *ICAM-3* and *PECAM-1* was used with the following criteria:

Negative (-): no immunoreaction or < 5% of tumor cells stained; (+) : 5-10% of tumor cells stained; (+ +) :10-50% of tumor cells stained; (+ + +) : >50% of tumor cells stained.

Tumor vascularity was assessed using the method described by Horak *et al*[12] and penfold *et al*[26]. Microvessels identified with PECAM-1 staining were counted on three 400 × fields (A=0.302 mm^2) within areas of maximum vascularity and the mean microvessel count was calculated for each area.

Statistical analysis

Data were expressed as mean ±SD. Survival analysis was performed using the Kaplan-Meier method with the log rank test. To determine independent prognostic factors we used the Cox proportional hazards model, which estimates the odds ratio in 95% confidence intervals. Univariate analyses comparing subgroups of patients were performed with the chi-square test (Pearson, Mantel-Haenzel test for linear association). The results of the morphometric examination were studied in various levels of the variables examined by the Student's *t*-test or the one-way ANOVA. The Levene test for homogeneity of variance was performed before the application of *t*-test or ANOVA. Non-parametric tests such as the Mann-Whitney test and the Kruskal –Wallis one-way ANOVA were applied in every case where the requirements of the t-test or the one-way ANOVA were not met. For all statistical tests, $P < 0.05$ was considered statistically significant.

RESULTS

PECAM-1 was immunohistochemically expressed on the tumor cells of 10 gastric lymphoma patients (27.8%) (mean expression = 26.5% ± 8.8% for *PECAM-1* positive cells) (Figure 1A). *PECAM-1* (+) endothelial cells were found in the vessels of 9 gastric lymphomas (25%) (Figure 1B). The tumor stromal *PECAM-1* (+) vessel counts/mm^2 varied between 1.987/mm^2 and 8.609/mm^2 (mean ± SD: 4.6358 ± 2.0679) and were significantly higher ($P = 0.027$) than *PECAM-1* (+) vessel counts located at the tumor margin (mean ± SD: 2.1341 ± 2.7460, range: 0 - 8.609). Similarly, the number of centrally located *PECAM-1* (+) vessels/mm^2 was significantly higher in gastric lymphoma patients with lymph node involvement (7.285 *vs* 3.753, $P = 0.002$). Four gastric lymphoma patients (11.11%) expressed *PECAM-1* on their tumoral and neovascular endothelial cells. *PECAM-1* was also expressed by the inflammatory infiltrate of 2 (50%) gastritis and 2 (33.33%) non-malignant ulcer patients. Nevertheless, its immunohistochemical expression was not detected on the endothelial cells of any of the above patients. *PECAM-1* upregulation did not correlate with patients' age, gender, tumor stage and grade or type of surgical treatment, as well as tumoral diameter, surface or thickness.

ICAM-3 positive staining was observed in 17 gastric lymphoma patients (47.2%) (mean expression = 34.118% ± 14.5% for *ICAM-3* positive lesions) (Figure 2A). *ICAM-3* was also stained in 1 (25%) gastritis and 4 (66.66%) non-malignant ulcer specimens. It was mainly expressed in patients with more advanced local disease as demonstrated by the increased tumor surface (70.074 cm^2 *vs* 29.7 cm^2, $P = 0.033$) and thickness (8.786 mm *vs* 5.531 mm, $P = 0.034$) (Table 2). Furthermore, *ICAM-3* expression level differed significantly in gastric lymphomas with different histologic grade ($F = 4.833$, $P = 0.014$) (Figure 2B and Table 3). *ICAM-3* expression level correlated with

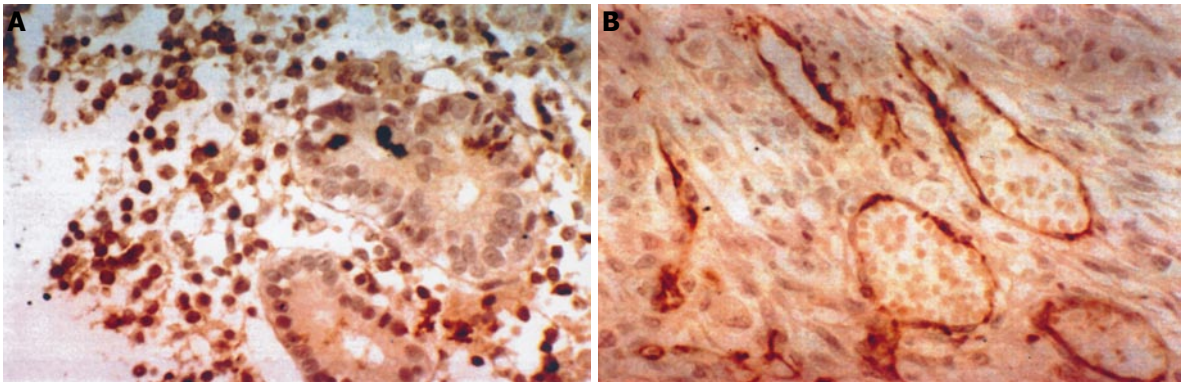

Figure 1 Positive membrane immunostaining (**A**) and endothelial staining (**B**) for *PECAM-1* in diffusely growing lymphocytes (magnification 1X400). Gastric glands were identified as negatively stained.

Table 2 Differences in tumor thickness, surface and diameter according to *ICAM-3* expression status in patients with non-Hodgkin primary gastric lymphoma (mean ± SD)

	ICAM-3 Tumor Expression		
	ICAM-3 (+) (*n* = 14)	*ICAM-3* (-) (*n* = 16)	*P*
Mean tumor thickness (mm)	8.79 (± 4.67)	5.53 (± 2.81)	<0.05
Mean tumor surface (cm^2)	70.07 (± 9.18)	29.70 (± 28.96)	<0.05
Mean tumor diameter (cm)	7.89 (± 7.89)	5.59 (± 2.78)	NS[1]

[1] NS: Non-significant.

Table 3 *ICAM-3, PECAM-1* and *HLA-DR* antigen quantitative expressions according to tumor grade (mean ± SD)

Gene expression (%)	Grade I (*n* = 15)	Grade II (*n* = 7)	Grade III (*n* = 14)	*P*
ICAM-3 (+) tumor cells	26.67 ±22.09	3.57 ±9.45	11.07± 5.95	< 0.05
PECAM-1 (+) tumor cells	6 ±10.55	10.71±13.36	7.14± 5.28	NS[1]
HLA-DR (+) tumor cells	40 ±25.98	55.71 ±26.21	31.43± 6.63	NS[1]

[1] NS: Non-significant

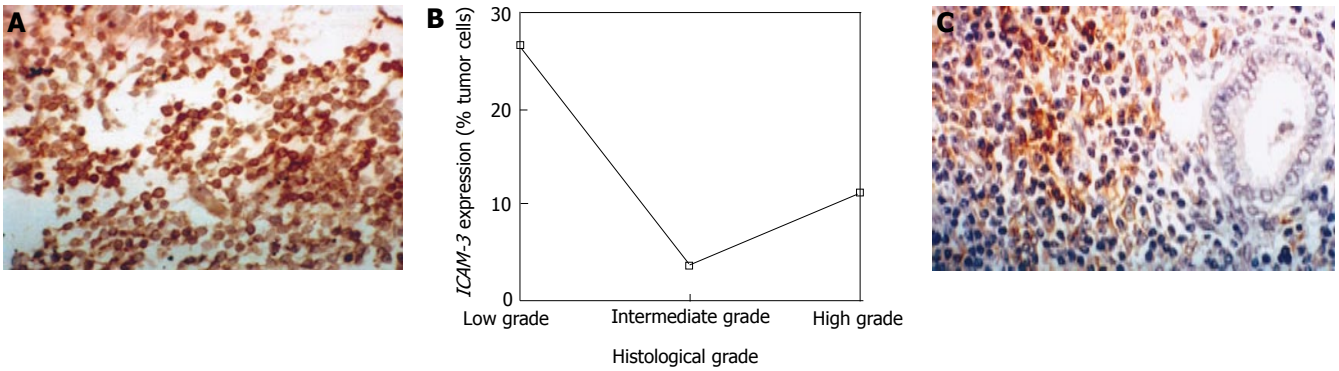

Figure 2 Positive membrane immunostaining for *ICAM-3* in diffusely growing lymphocytes (magnification 1X400) (**A**) and fluctuations of the mean *ICAM-3* expression level according to tumor grade (**B**) and positive intracytoplasm immunostaining for *HLA-DR* antigen in gastric lymphoma lymphocytes (magnification 1X200) (**C**) with gastric glands indicated as negatively stained.

increased tumor diameter ($r = 0.400$, $P = 0.028$), tumor surface ($r = 0.462$, $P = 0.012$), tumor thickness ($r = 0.526$, $P = 0.003$) and higher tumor grade ($r = -0.362$, $P = 0.030$), but not with age, gender, stage or type of surgical treatment.

HLA-DR antigen expression on tumor and peritumoral stromal cells was observed in 91.7%($n = 33$) of gastric lymphoma patients (Figure 2C).The proportion of *HLA-DR* positive tumor and peritumoral stromal cells was equally distributed in gastric lymphoma patients with different stage, grade, gender and type of surgery. *HLA-DR* was also expressed on stromal cells in 2 (50%) gastritis and 4 (66.6%) non malignant gastric ulcer control cases.

Several patients co-expressed two or more of the markers studied. The patterns of different protein co-expression are demonstrated in Table 4. None of the 10 healthy controls expressed any of the molecules studied.

Survival analysis

Univariate analysis showed that *ICAM-3* expression was associated with improved 5-year survival rate (78.6% *vs* 55.6%, $P = 0.1701$). *PECAM-1* expression on gastric lymphoma tumor cells was associated with significantly decreased 5-year survival rate (28.6% *vs* 76%, $P = 0.0078$) (Figure 3A). Although the presence of *PECAM-1* (+) vessels within the tumor was associated with decreased overall

Table 4 *ICAM-3*, *PECAM-1* and *HLA-DR* antigen expressions in gastric lymphoma tumor cells [1]

Gene expression	Frequency	Percentage (%)
ICAM-3(+)/*PECAM-1*(+)/*HLA-DR* tumor(+)	4	11.1
ICAM-3(+)/*PECAM-1*(+)/*HLA-DR* tumor(-)	1	2.8
ICAM-3(+)/*PECAM-1*(-)/*HLA-DR* tumor(-)	4	11.1
ICAM-3(+)/*PECAM-1*(-)/*HLA-DR* tumor(+)	8	22.2
ICAM-3(-)/*PECAM-1*(+)/*HLA-DR* tumor(+)	4	11.1
ICAM-3(-)/*PECAM-1*(+)/*HLA-DR* tumor(-)	1	2.8
ICAM-3(-)/*PECAM-1*(-)/*HLA-DR* tumor(+)	10	27.8
ICAM-3(-)/*PECAM-1*(-)/*HLA-DR* tumor(-)	4	11.1

[1]While *PECAM-1* and *ICAM-3* were positive when more than 5% of the tumor cells were stained positive, *HLA-DR* antigen positivity depicted in Table 4 represents the 15% cut-off level used in our survival analysis.

Table 5 Five-year survival rate according to patients´ gender, tumor stage, histologic grade and microscopic resection margins

Characteristics	Number of cases	5-yr survival rate (%)	*P*
Gender			NS[1]
Male	20	65	
Female	12	66.7	
Stage			NS
I	16	62.5	
II	9	66.7	
III	1	100	
IV	6	66.7	
Histologic grade			NS
Low	13	76.9	
Intermediate	7	71.4	
High	12	50	
Microscopic margins			NS
Positive	5	60	
Negative	27	66.7	

[1] NS: Non-significant.

survival rate (55.6% *vs* 69.6%), this difference was not proved to be statistically significant ($P=0.4067$). Nevertheless, patients who showed *PECAM-1* expression both on their endothelial cells ($n=4$), presented a decreased overall survival rate compared to the rest of the group (25% *vs* 71.4%, $P=0.0403$).

Different levels of proportional *HLA-DR* antigen expression were sequentially evaluated for their prognostic value. *HLA-DR* antigen expression in more than 15% of the tumor cells ($n=26$, 72.2%) was associated with an increased 5-year survival rate (75% *vs* 37.5%, $P=0.0469$) (Figure 3B). A corresponding though non-significant increase in the overall survival rate was also observed in those gastric lymphoma patients expressing *HLA-DR* antigen in more than 15% of their peritumoral stromal cells ($n=19$, 52.8%) (66.67 % *vs* 64.29 %, $P=0.8593$).

A univariate analysis was also performed to identify high-risk groups of patients with regard to gene co-expression. For the needs of this analysis, the 15% *HLA-DR* antigen expression level was considered a measure of *HLA-DR* positivity between tumor cells. Those patients who were *PECAM-1*(+)/*ICAM-3*(-) ($n=5$) presented a significantly decreased overall survival rate compared to those who were *PECAM-1*(-)/*ICAM-3*(+) ($n=12$) (20% *vs* 83.3%, $P=0.0041$) (Figure 4A). Furthermore, gastric lymphoma patients who were *HLA-DR* (+)/*ICAM-3*(+) ($n=10$) presented a significantly improved overall survival rate compared to those who were *HLA-DR*(-)/ *ICAM-3*(-) ($n=4$) (90% *vs* 25%, $P=0.0091$) (Figure 4B). When all three genes were studied together, all *HLA-DR*(+)/*ICAM-3*(+)/*PECAM-1*(-) gastric lymphoma patients ($n=8$) were alive 5 years postoperatively (100% 5-year survival rate), compared to a 54.2% survival rate for the rest of the group ($n=24$) ($P=0.0289$) (Figure 5).

Univariate analysis revealed that patients´ gender, tumor stage, histologic grade and marginal status were not associated with their overall survival rate as demonstrated in Table 5.

To identify the independent prognostic factors that would predict survival, multivariate analysis was performed. The analysis included *HLA-DR* antigen and *PECAM-1* upregulation (which was proved to be statistically significant prognostic markers in univariate analysis), age of the patients, tumor stage and tumor grade. Four patients were excluded from the process due to inadequate 5-year survival data. Multivariate Cox regression analysis revealed that *PECAM* and *HLA-DR* antigen expressions were the only statistically significant independent prognostic variables in the group of gastric lymphoma patients ($P=0.005$ and $P=0.016$, respectively). Similar results were obtained when *PECAM-1* and *HLA-DR* antigen expressions were the only covariates submitted to multivariate analysis ($P=0.003$ and $P=0.009$, respectively). The results of multivariate analysis are depicted in Table 6 and Table 7.

DISCUSSION

Primary non-Hodgkin's gastric lymphoma represents a rare malignant tumor comprising 2-5% of all cases of malignant gastric tumor[27, 28]. The 5-year survival rate ranges between 57%[29] and 96% for IE and IIE patients[30], while it has been considered as low as 25% when all stages are grouped together. Several molecular markers like *P27*, *cyclin E*[31] and *bcl-6*[32] have been recently assessed for their prognostic value. Still, most of them have no independent prognostic value.

In malignant lymphoma patients, clinical outcome and prognosis appear to depend largely on host immune response and vascular invasion. The current study attempted to clarify the prognostic value of *HLA-DR* antigen, *ICAM-3* and *PECAM-1* since they represent specific markers of regional immune reactions, cell-cell interactions and transendothelial migration.

HLA-DR antigen expression is a marker of host immune response in human malignant neoplasms. Its expression in tumor cells has been reported to be related with a favorable prognosis in patients with different types of cancer such as breast cancer[33] and squamous cell laryngeal carcinoma[34]. In our multivariate analysis, *HLA-DR* antigen expression was found to be a statistically significant independent prognostic factor associated

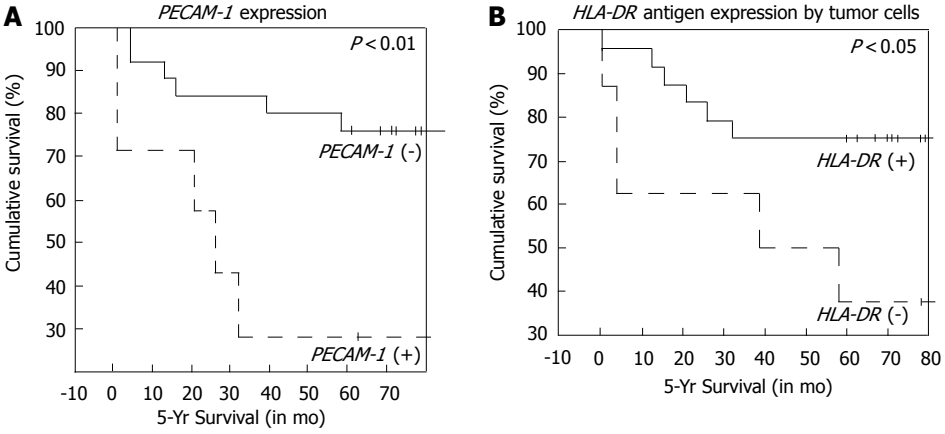

Figure 3 Five- year survival according to *PECAM-1* (**A**) and *HLA-DR* antigen (**B**) expression (*HLA-DR* positive patients had expression of *HLA-DR* antigen in more than 15% of their tumor cells).

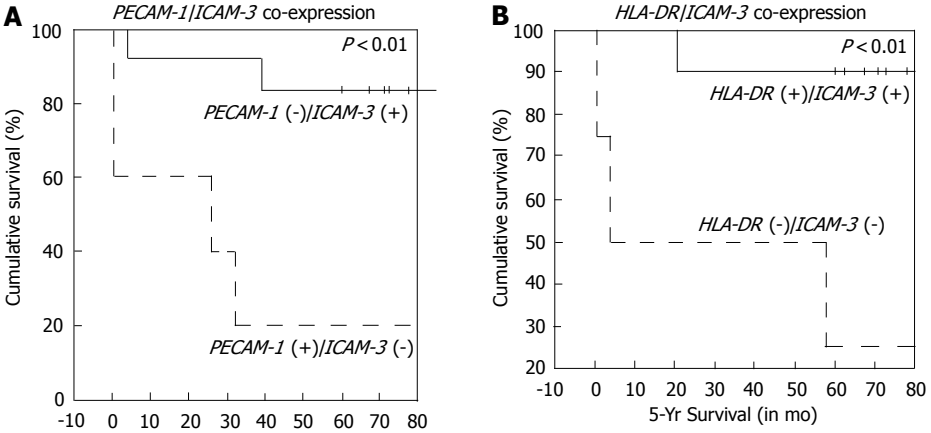

Figure 4 Decreased 5 –year survival rate in *PECAM-1*(+)/*ICAM-3*(-) (**A**), and increased 5-year survival rate in *HLA-DR*(+)/*ICAM-3*(+) (**B**) gastric lymphoma patients.

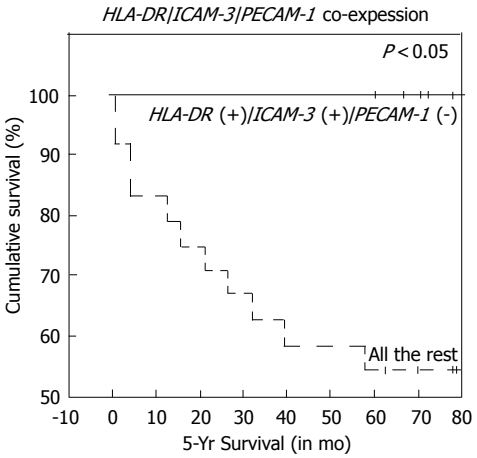

Figure 5 One hundred percent 5-year survival rate in *HLA-DR*(+)/*ICAM-3*(+)/*PECAM-1*(-) gastric lymphoma patients.

with a favorable clinical outcome ($P = 0.0199$). This is in agreement with previous reports[33, 34]. The expression of HLA-DR antigen in tumor cells has a modulating effect on the host immune response, possibly by helping in the presentation of tumor associated antigens to T-lymphocytes[35], thus enabling the immune system to inhibit cancer growth. Regarding peritumoral stroma cells, the presence of tumor infiltrating leukocytes (TIL) invading the site of malignancy, suggests that the immune system is capable of reacting with tumors. The presence of such immune infiltrates has been documented in numerous reports and is often associated with improved prognosis[36, 37]. In our primary gastric lymphoma group of patients, *HLA-DR* antigen expression in peritumoral stromal cells was associated with an increased (though non-significant) survival rate.

Cell adhesion molecules are membrane glycoproteins that play a major role in neoplastic disease. Four primary groups of cell adhesion molecules have been described including the integrin receptor family, the immunoglobin superfamily, selectins and cadherins[38]. *PECAM-1* constitutes a significant representative of the immunoglobulin superfamily and has been applied with considerable accuracy as an angiogenesis marker in several kinds of human neoplasia[16, 17, 39, 40].

A significant number of gastric lymphoma patients expressed *PECAM-1* in their tumor cells ($n = 10$, 27.8%). Since *PECAM-1* is constitutively expressed not only on the surface of endothelial cells, but on platelets, leukocytes, monocytes, neutrophils and selected T cell subsets as well[11], our findings can be attributed to the lymphoid origin of the malignancy. *PECAM-1* positive staining on gastric lymphoma tumor cells was found to be a statistically significant independent prognostic factor, associated with unfavorable prognosis ($P = 0.0029$). Although there are several reports[17, 39] correlating increased angiogenesis with an unfavorable prognosis, this is the first report

Table 6 Multivariate Cox regression analysis I (Age, grade, stage, *PECAM-1* and *HLA-DR* antigen expression were included)

Variable	*P* value	Exp (B)	95% CI for Exp (B)
Age	NS	1.0085	0.9541-1.0660
Stage	NS	2.0919	0.2317-18.8828
Grade	NS	0.2272	0.0475-1.0855
PECAM-1 tumor expression	$P<0.01$	19.9490	2.7865-142.8178
HLA-DR tumor expression	$P< 0.05$	0.1373	0.0258-0.7299

Table 7 Multivariate Cox regression analysis II (Only *PECAM-1* and *HLA-DR* antigen expressions were included, since they were the only statistically significant factors found in the univariate analysis)

Variable	*P* value	Exp (B)	95% CI for Exp (B)
PECAM-1 tumor expression	$P<0.01$	10.3520	2.2196-48.2812
HLA-DR tumor expression	$P< 0.01$	0.1308	0.0286-0.5989

on the clinical outcome of gastric lymphoma patients based on *PECAM-1* expression by tumor cells. As a key participant in cell adhesion cascade, *PECAM-1* can lead to the extravasation of leukocytes[18], constituting a possible regulator of the metastatic process in lymphoid tumors. Its presence on the surface of tumor cells can be therefore associated with altered cellular adhesivity, enabling malignant cells to dissociate from their primary sites, leading to tumor growth and metastasis[41-43].

The detection of *PECAM-1* (+) vessels within the tumor was related to an unfavorable, though non-significant clinical outcome. This difference reached statistical significance only for gastric lymphoma patients ($n=4$) who showed *PECAM-1* expression both in their tumor cells and in their endothelial cells ($P=0.0403$). Although the fact that *PECAM-1* expression in tumor endothelial cells has been associated with unfavorable prognosis in several tumors[2, 16, 17, 40, 44-46], tumors with *PECAM-1* (+) endothelia exhibit an extended[47] overall survival rate.

Vascular density at the tumor margins is significantly lower than that within tumor stroma[12]. Additionally, gastric lymphoma patients with lymph node involvement present a significantly higher number of *PECAM-1* (+) stromal vessel counts (7.285 *vs* 3.753, $P=0.002$). It appears that the density of *PECAM-1* (+) stromal microvessels/mm^2 correlates with the lymphatic metastatic pathway. Several studies have reported that *PECAM-1* is a non-specific angiogenesis marker, which stains both lymphatic (weaker staining) and blood vessel endothelial cells[48, 49]. New proliferating capillaries in the tumor stroma have fragmented basement membranes[50], while endothelial cells at the tips of growing capillaries secrete collagenases and plasminogen activators [51]. These properties facilitate the metastatic process and may explain the association between tumor stromal vessel counts and lymph node metastasis as observed in our study.

Antigen-dependent and/or independent interactions between target cells and lymphocytes are required for the initiation of a specific immune response. In addition to antigen-specific interactions, "accessory" cell-surface molecules, ICAMs[52,53], mediate an antigen-independent, non-specific adhesion between the reactant cells, which is considered the primary step in activation of lymphocytes[54]. These events often take place in a host against malignant cells. Recent work has indicated that resting leukocytes express a third ligand, ICAM-3, which appears to be the major ligand for *LFA-1* in initiating phases of immune response[10,55]. Although it is poorly investigated, *ICAM-3* upregulation has been well documented in lymphoid tumors[56,57]. In our gastric lymphoma group of patients, *ICAM-3* upregulation was associated with a non- significantly improved 5-year survival rate. The fact that *ICAM-3* expression level is associated with increased tumor surface and thickness provides evidence that its regulation is directly proportional to the tumor burden.

Furthermore, *ICAM-3* expression when studied in combination with *PECAM-1* and/or *HLA-DR*, improved their prognostic accuracy. From our univariate analysis, three distinct gene expression patterns were distinguished. Two of them [*PECAM-1*(+)/*ICAM-3*(-) and *HLA-DR*(-)/*ICAM-3*(-)] were associated with a significantly decreased 5-year survival rate, when compared to the exactly opposite gene expression patterns [*PECAM-1*(-)/*ICAM-3*(+) with $P=0.0041$ and *HLA-DR*(+)/*ICAM-3*(+) with $P=0.0091$, respectively]. Furthermore, gastric lymphoma patients who were *HLA-DR* (+) / *ICAM-3* (+) / *PECAM-1* (-) had 100% 5-year survival rate. Similar results have been reported by Hosch *et al*[58], who observed that the co-expression of *HLA* class *I* molecules and *ICAM-1* is a significant predictor of increased disease-free survival in patients with primary esophageal carcinomas. It appears that the lack of expression for both *HLA-DR* and *ICAM-3* [*HLA-DR*(-)/*ICAM-3*(-)] in gastric lymphoma patients is associated with a compromised host immune response against the tumor as well as with an unfavourable prognosis compared to those who were *HLA-DR*(+)/*ICAM-3*(+) ($n=10$, $P=0.0091$). On the contrary, gastric lymphomas expressing both *HLA-DR* and *ICAM-3* but not *PECAM-1* have not only a more potent host immune surveillance, but a negative expansion potential and a growth disadvantage as well.

In our study, *PECAM-1* and *HLA-DR* antigen expressions were proved to be statistically significant independent prognostic factors in a group of patients with primary B-cell MALT-type non-Hodgkin's gastric lymphoma, suggesting that upregulation of both *PECAM-1* and *HLA-DR* antigen is closely related to the clinical phenotype exhibited by the affected cases. Furthermore, it appears that *ICAM-3*, *PECAM-1* and *HLA-DR* represent complementary biologic pathways associating host immune surveillance with non-specific intercellular interactions and endothelial transmigration. Their combined study in gastric lymphoma patients may amplify their prognostic accuracy and provide a better description of the biologic behaviour of these tumors.

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CLINICAL RESEARCH

Ileocecal masses in patients with amebic liver abscess: Etiology and management

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Abstract

AIM: To assess the causes of ileocecal mass in patients with amebic liver abscess.

METHODS: Patients with amebic liver abscess and ileocecal mass were carefully examined and investigated by contrast-enhanced CT scan followed by colonoscopy and histological examination of biopsy materials from lesions during colonoscopy.

RESULTS: Ileocecal masses were found in seventeen patients with amebic liver abscess. The cause of the mass was ameboma in 14 patients, cecal tuberculosis in 2 patients and adenocarcinoma of the cecum in 1 patient. Colonic ulcers were noted in five of the six (83%) patients with active diarrhea at presentation. The ileocecal mass in all these patients was ameboma. Ulcers were seen in only one of the 11 (9%) patients without diarrhea. The difference was statistically significant from the group with diarrhea ($P < 0.005$).

CONCLUSION: Ileocecal mass is not an uncommon finding in patients with amebic liver abscess. Although, the ileocecal mass is due to ameboma formation in most cases, it should not be assumed that this is the case in all patients. Colonoscopy and histological examination of the target biopsies are mandatory to avoid missing a more sinister lesion.

Key words: Amebiasis; Ameboma; Colon; Colonoscopy; Diagnosis

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INTRODUCTION

Intestinal amebiasis occurs throughout the world[1]. It is estimated that out of all people infected with *E. histolytica*, 40 million develop colitis or extra-intestinal abscesses and of these 40 000 die annually[1]. Extra-intestinal amebiasis is also not uncommon and the liver is the most common extra-intestinal organ to be involved[1-3]. A post-mortem study reported that the liver is involved in 37% of cases[4]. Involvement of the colon is also common in patients with amebic liver abscess (ALA)[5, 6]. A recent study showed that colonic ulcers are observed in 55% of patients with ALA. These ulcers are more common in patients with active diarrhea or history of diarrhea in the recent past[6]. Some of the patients with ALA also have a palpable ileocecal mass on abdominal examination. The cause of the ileocecal mass in these patients still remains unclear.

Formation of an ameboma has been noted to be an uncommon complication of amebiasis[7-18]. It has been estimated that ameboma occurs in only about 1.5% of all cases[8]. Although there have been recent reports dealing with the problem of ameboma[14, 17], in regions where ALA is not very common, ameboma has been confused with cancer of the colon[9, 13, 14, 18]. However, in regions where ALA is common, the reverse may happen and if diagnostic facilities are not widely available as in the case of several developing countries, cancer of the colon or tuberculosis may be confused with ameboma, especially in patients with concurrent ALA and its clinical signs and symptoms of high fever, local pain and tenderness. We therefore report our findings in patients with amebic liver abscess(es) and associated cecal mass. We believe that this is the largest collection of patients with ameboma.

MATERIALS AND METHODS

Consecutive patients with ALA having a palpable ileocecal mass were studied. The diagnosis of ALA was made on the basis of the clinical features of fever, right upper abdominal pain, tender hepatomegaly with a typical ultrasonic finding of a single or multiple hypoechoeic lesion(s) in the liver and antiamebic antibody titer >160 in serum (immunofluorescent assay).

A detailed clinical history was obtained from all patients and recorded. Patients were asked specifically about loose stools (three or more in a day for more than 48 h), with or without blood during the last eight weeks. Whole stool samples were observed and three fresh samples were examined for the presence of trophozoites and cysts of *E. histolytica*.

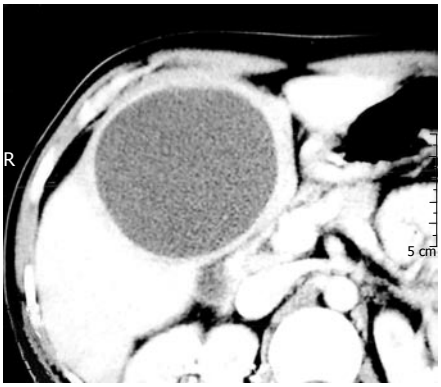

Figure 1 Hypodense round lesions in the liver due to amebic liver abscess.

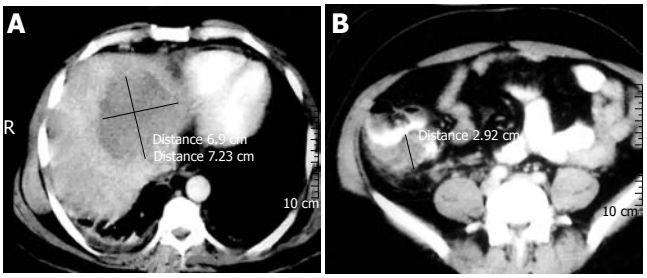

Figure 3 Contrast-enhanced CT scan of the abdomen with the presence of ALA in the liver (**A**) and ileocecal mass (**B**).

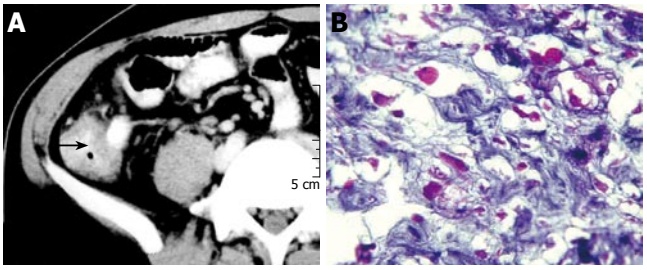

Figure 2 Contrast-enhanced CT scan of the abdomen showing the ileocecal mass with thickening of the cecal wall (**A**) and histological examination of the biopsies showing trophozoites of E. *histolytica* (**B**) (H & E X 800).

Contrast-enhanced CT scan was performed for all patients and apart from the abscess in the liver, the ileocecal area was carefully studied.

Colonoscopy was carried out in all but one patient within two days of instituting anti-amebic treatment after stool samples were obtained for microscopic examination. In one patient colonoscopy could be performed on the fifth day of instituting anti-amebic therapy.

Colonic preparation was done for all patients using a polyethylene glycol-electrolyte-based solution (*Peglec*, Tablets India Ltd, Chennai). The procedure was performed under conscious sedation with intravenous diazepam (5-10 mg) and pentazocine (25-50 mg). The findings observed during colonoscopy were recorded. The site, size, number and presence of inflammation around the ulcers were recorded if ulcers were noted. Multiple biopsies were obtained from the ulcer margins for histopathological examination. When the cecum was reached, the area was carefully inspected and multiple biopsies were obtained from any lesion that was encountered.

Patients who were too sick, those who did not give consent, those with ruptured ALA, those requiring urgent needle or catheter drainage and those with severe comorbid illnesses, were excluded from the study. Patients who received anti-amebic treatment during the preceding eight weeks were also excluded.

The protocol for the study was cleared by the Ethical Committee of the Hospital and a detailed informed consent was obtained from all participating patients.

RESULTS

A total of 17 patients with ALA and ileocecal mass were studied. There were 16 males and one female. The mean (SD) age of these patients was 37.9 (9.9) years (range 24 to 62 years). Two patients presented with active lower gastrointestinal bleeding.

Contrast-enhanced CT scan demonstrated rounded hypodense lesions in the liver due to ALA (Figure 1). In all the patients, thickening of the cecal wall was noted (Figure 2A). Lymphnodal enlargement was not evident and a definite diagnosis could be made on the basis of the CT findings in none of the patients.

Diarrhea was present in six patients. The other 11 patients did not complain of diarrhea. Ulcers were noted in the colon of five of the six (83%) patients with active diarrhea. The ileocecal mass in all these patients was due to ameboma formation (Figure 2B). Ulcers were seen in only one of the 11 (9%) patients without diarrhea. The difference between the two groups was statistically significant ($P < 0.005$, Fisher's exact test).

At colonoscopy, sixteen patients showed mass lesions in the cecum. The patient who underwent colonoscopy on the fifth day of starting anti-amebic therapy showed evidence of amebic liver abscess (Figure 3A) and thickening of the cecal wall on contrast-enhanced CT scan (Figure 3B), but colonoscopy showed multiple round and oval, deep ulcers of varying sizes confined to the cecum and the adjoining ascending colon (Figure 4A). Colonic biopsies obtained from the ulcers showed trophozoites of *E. histolytica* (Figure 4B).

The colonoscopic appearance in all patients, except for one who underwent delayed examination, showed mass lesions in the cecum and the colonoscopic appearance was similar in all of them. The final diagnosis of ileocecal mass was made after histological examination of the tissue obtained at colonoscopy. The final diagnosis was ameboma in 14 patients, ileocecal tuberculosis in two patients (Figure 4C) and adenocarcinoma of the cecum in one patient.

The patient with cancer of the colon was a 32-year-old man who was admitted due to massive lower GI bleeding. At the time of hospitalization, the pulse rate was 160/min. The systolic blood pressure was 70 mm Hg. The hemoglobin was 5 g/dL. The patient was in shock and managed with intravenous fluids and blood transfusion. After haemodynamic stability was achieved, emergency colonoscopy was performed, which showed that the bleeding originated from the ascending colon. The bleeding stopped spontaneously on the next day. An ultrasound examination of the abdomen showed a large hypoechoic

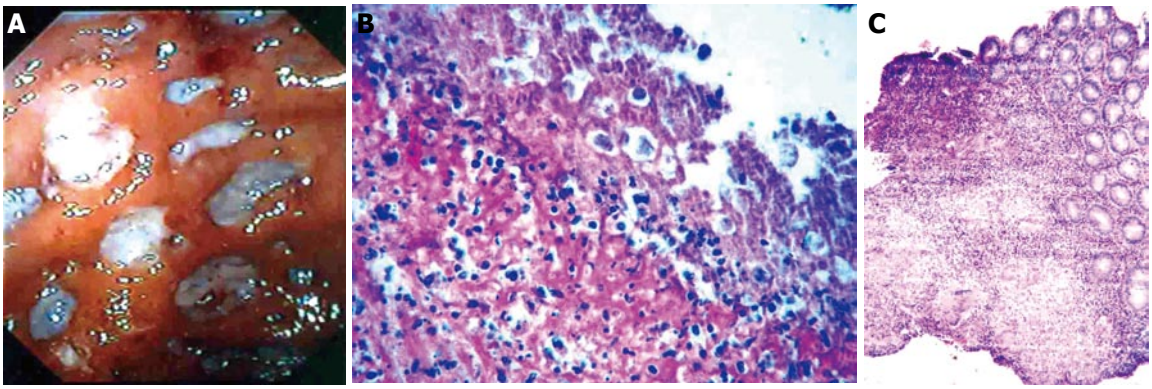

Figure 4 Colonoscopy findings in a patient showing deep round and oval ulcers in the cecum (**A**), histological examination of the biopsies obtained from ulcers in the cecum showing the presence of trophozoites of E. *histolytica* (**B**) and from a patient with amebic liver abscess and cecal mass showing the presence of multiple epithelioid cell granulomas and Langhans giant cells (**C**) (H & E X 40).

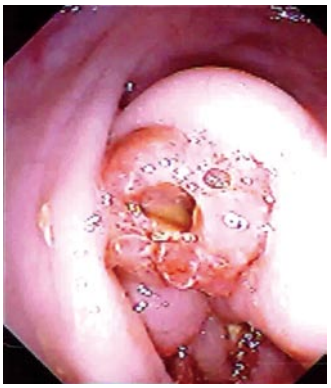

Figure 5 Colonoscopic appearance of the mass lesion in a patient with cecal cancer.

lesion in the left lobe of the liver, consistent with the diagnosis of amebic liver abscess. CT scan showed similar findings. In addition to the lesion in the liver, there was thickening of the wall of the cecum. No other abnormality was detected. Aspiration of the liver abscess yielded an anchovy sauce-like material. Cytological examination of the aspirate revealed acute inflammatory cells. No amebic trophozoites or malignant cells were seen. Indirect hemagglutination test for presence of antibody against *E. histolytica* was positive in titres of 1:160.

Repeat colonoscopy following bowel preparation showed irregular, nodular friable growth in the cecum (Figure 5). Multiple biopsies were obtained from the growth. Histological examination of the biopsies revealed adenocarcinoma. The patient was advised to undergo right hemi-colectomy but did not consent to be operated. The amebic liver abscess responded to needle aspiration and metronidazole.

A 62- year old lady who was diagnosed to have ALA five months ago received anti-amebic therapy at another hospital and was reported to have been cured of the disease. She however, visited our hospital with diarrhea, high fever and a mass in the right lower quadrant. Ultrasonography and CT scan of the abdomen showed features of ALA. CT scan also showed thickening of the cecal wall. Colonoscopy showed a friable nodular mass in the cecum. A provisional diagnosis of cancer of the cecum was made. Histological examination of biopsies obtained from the cecal mass during colonoscopy, however, showed trophozoites of *E. histolytica* and the mass was finally diagnosed as an ameboma.

All the patients received metronidazole. Nine patients required needle aspiration of ALA. All patients were cured of ALA. The ileocecal masses due to ameboma formation disappeared after treatment with metronidazole in all the patients. Repeat colonoscopy did not reveal any abnormality in 12 patients and mild hyperemia in the remaining two patients with ameboma. The two patients with ileocecal tuberculosis received anti-tuberculous treatment and were well during follow-up. The cecal masses were no longer palpable after follow-up of 6 and 7 months respectively.

DISCUSSION

Although ALA is rare in developed countries, it is not an infrequent disease in developing countries. However, the occurrence of ileocecal mass due to ALA is rare, except for a case report[11]. If an ileocecal mass is detected in a patient with ALA, an ameboma should be diagnosed.

It has been estimated that out of all cases with amebiasis, ameboma formation occurs in only about 1.5% of patients[8]. However, it is important to note that in regions where ALA is not very common, ameboma has been confused with cancer of the colon[9, 13, 14, 18]. More importantly, in regions where ALA is common, the reverse may happen and cancer of the colon or tuberculosis may be confused with ameboma, especially in patients with concurrent ALA and its clinical signs and symptoms of high fever, local pain and tenderness. This was evident in one patient in this series who had carcinoma of the cecum but was clinically thought to have an ameboma due to the young age and concurrent occurrence of ALA. Two other patients in this series were clinically thought to have ameboma of the cecum on the basis of the colonoscopic findings, but showed tuberculosis of the cecum on histological examination of colonoscopic biopsies. In an elderly patient, a clinical diagnosis of cecal cancer was considered and it was only on histological examination of the biopsies that the diagnosis of ameboma could be made.

It is generally considered that ameboma formation occurs due to untreated or partially treated amebic colitis[1,7]. However, in the present series, only one patient

had a history of anti-amebic treatment five months ago for ALA. None of the other patients received any anti-amebic treatment in the recent past. Moreover, all of them had evidence of ALA at the time when they were found to have an ileocecal mass, suggesting that ameboma formation can occur even in the acute or subacute phase.

CT findings of cecal ameboma with pericolonic lessions have been reported recently[17]. The ileocecal valve shows a large abscess within the mesentry causing compression of the adjacent terminal ileum and cecum. Even then a differential diagnosis of lymphoma, neoplasm, enterocolitis and inflammatory bowel disease are considered. It was reported that due to concentric thickening of the cecal wall and mass like appearance, a clinical diagnosis of ameboma could only be established after histopathological examination of colonoscopic biopsies[14]. It is therefore evident that all patients with ileocecal masses, even with concurrent ALA, should undergo colonoscopy and biopsies should be obtained from any suspicious lesions in order not to miss tuberculosis or cancer.

Although in the present series, we encountered only amebomas, cancer of the cecum and tuberculosis as the cause for the ileocecal mass, lymphomas as well as enterocolitis, acute appendicitis, Crohn's disease, abscess and fungal infections should also be considered as a probable diagnosis.

The simultaneous occurrence of ALA and colonic tuberculosis in this part of the world is not surprising because both diseases are commonly encountered in this region[6, 19-21]. This situation may hold true for most developing countries and the two diseases may also occur simultaneously even in developed countries, especially in immigrants, people living in confined areas and patients with immunodeficiency virus infection. The ileocecal mass in patients with cecal tuberculosis is due to proliferative lesions in the cecum resulting in a tumor-like presentation[19]. The cause of the lesions could not be therefore diagnosed on the basis of CT or colonoscopic findings.

As noted in an earlier study[6], the frequency of colonic ulcers is higher in patients with active diarrhea at presentation as opposed to those who had no diarrhea at the time of admission. What was interesting to note is that, in all patients with active diarrhea, the ileocecal mass is due to ameboma formation. Whether active diarrhea in such patients signifies ameboma can only be ascertained if a larger number of patients with similar presentation are studied.

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RAPID COMMUNICATION

Up-regulation of NAD(P)H quinone oxidoreductase 1 during human liver injury

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Abstract

AIM: To investigate the expression and activity of NAD(P)H quinone oxidoreductase 1 (NQO1) in human liver specimens obtained from patients with liver damage due to acetaminophen (APAP) overdose or primary biliary cirrhosis (PBC).

METHODS: NQO1 activity was determined in cytosol from normal, APAP and PBC liver specimens. Western blot and immunohistochemical staining were used to determine patterns of NQO1 expression using a specific antibody against NQO1.

RESULTS: NQO1 protein was very low in normal human livers. In both APAP and PBC livers, there was strong induction of NQO1 protein levels on Western blot. Correspondingly, significant up-regulation of enzyme activity (16- and 22-fold, $P < 0.05$) was also observed in APAP and PBC livers, respectively. Immunohistochemical analysis highlighted injury-specific patterns of NQO1 staining in both APAP and PBC livers.

CONCLUSION: These data demonstrate that NQO1 protein and activity are markedly induced in human livers during both APAP overdose and PBC. Up-regulation of this cytoprotective enzyme may represent an adaptive stress response to limit further disease progression by detoxifying reactive species.

Key words: NQO1; Quinone oxidoreductase; Acetaminophen; APAP; Primary biliary cirrhosis; PBC; Nrf2; Diaphorase

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INTRODUCTION

Although NAD(P)H quinone oxidoreductase 1 (NQO1) has been historically associated with generation of hydroquinones from reactive quinones, additional substrates including nitrosoamine, nitro and azo chemical moieties have been identified. NQO1 is also capable of scavenging superoxide anions generated during oxidative stress and regenerating reduced forms of protective endogenous antioxidant compounds.

There is very low expression of NQO1 mRNA and protein in normal human livers, with slightly greater mRNA levels observed in biliary epithelial cells[1, 2]. NAD(P)H quinone oxidoreductase 2 (NQO2) mRNA is greater in hepatocytes compared to NQO1 mRNA and is in turn thought to play a more critical role in maintaining low levels of quinones in hepatocytes[2]. Consequently, it has been suggested that human NQO1 does not play a major role in hepatic xenobiotic metabolism under normal conditions[3].

Instead, NQO1 may be more important during periods of hepatic oxidative stress and damage. NQO1 mRNA protein and activity are markedly increased in mouse liver following bile duct ligation, a model of obstructive cholestasis (José Manautou, unpublished observations). Similar elevations in rodent NQO1 mRNA also occur after exposure to centrilobular hepatotoxicants such as acetaminophen (APAP), carbon tetrachloride and bromobenzene[4, 5]. Although different regions of the liver lobule are injured in cholestasis and drug-induced hepatocellular necrosis, oxidative stress contributes to the pathogenesis of both diseases. We hypothesize that up-regulation of NQO1 may represent a common response to liver injury with an oxidative stress component.

The present study was undertaken to determine if NQO1 expression and activity are similarly altered in two types of human liver disease, injury by APAP overdose and primary biliary cirrhosis (PBC). The results showed that protein levels and activity of human hepatic NQO1 were elevated during APAP overdose and PBC. Immunohistochemical analysis highlighted injury-specific patterns of NQO1 staining. APAP livers showed loss of

Table 1 Characteristics of patients

Patient	Gender	Age	Diagnosis
1	F	54	Normal liver
2[2]	F	adult	Normal liver
3	M	21	Normal liver
4	M	20	Normal liver
5	M	18	Normal liver
6[1]	F	33	Fulminant hepatic-failure (APAP-induced)
7[1]	F	35	Fulminant hepatic-failure (APAP-induced)
8[1]	M	15	Fulminant hepatic-failure (APAP-induced)
9[1]	F	55	Primary biliary cirrhosis
10[1]	F	62	Primary biliary cirrhosis
11[1]	F	63	Primary biliary cirrhosis

[1] Specimen collected at transplantation
[2] Age not provided

centrilobular cells and NQO1 staining in midzonal and periportal hepatocytes. Conversely, PBC livers exhibited prominent staining of hyperplastic biliary epithelium and hepatocytes at the periphery of cirrhotic nodules.

MATERIALS AND METHODS

Chemicals

2,6-dichlorophenol-indophenol (DCPIP), dicumarol, sucrose, Tris-hydrochloride, nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) were all obtained from Sigma Chemical Co. (St. Louis, MO).

Human liver specimens

Archival samples of frozen and formalin-fixed, paraffin-embedded adult livers (normal, primary biliary cirrhosis and APAP overdose) were obtained from the Liver Tissue Procurement and Distribution System at the University of Minnesota which was funded by NIH Contract #N01-DK-9-2310. Diagnosis was first established by the University of Minnesota and confirmed by histological examination of tissue sections at the University of Connecticut in a blinded fashion. Patient characteristics are provided in Table 1.

NQO1 protein expression

Livers were homogenized in sucrose-Tris buffer (0.25 mol/L sucrose, 10 mmol/L Tris–HCl, pH 7.4) and centrifuged at 105 000 r/min for 60 min at 4°C. The resulting supernatant contained the cytosolic fraction. Protein concentrations were determined by the method of Lowry using Bio-Rad protein assay reagents (Bio-Rad Laboratories, Hercules, CA). Anti-NQO1 monoclonal antibodies (clones A180 and B771) were kindly provided by David Ross (University of Colorado, Health Science Center, Denver, CO). Cytosolic proteins (40 μg protein/lane) were electrophoretically resolved using polyacrylamide gels (12% resolving, 4% stacking) and transblotted overnight at 4ºC onto PVDF-Plus membrane (Micron Separations, Westboro, MA). Immunostaining was performed as previously described[1]. NQO1 protein-antibody complexes were detected using an ECL chemiluminescent kit (Amersham Life Science, Arlington Heights, IL) and exposed to Fuji medical X-ray film (Fisher Scientific, Springfield, NJ). Equal protein loading was confirmed by Coomassie blue staining of blots (data not shown). Antibody specificity was confirmed using 2.5 μg recombinant human NQO1 (rNQO1)(Sigma Chemical Co. St. Louis, MO) as a positive control.

NQO1 activity assay

NQO1 activity was calculated by measuring the colorimetric oxidation of NADPH to NADP+ using DCPIP as substrate. The disappearance of NADPH was measured at 600 nm for over 1 min as described by Ernster with modifications by Benson[6, 7]. NQO1 activity was measured in 1-mL reactions (27°C) containing liver cytosol, 200 μmol/L NADPH, 40 μmol/L DCPIP and Tris-HCl buffer (25 mmol/L Tris-HCl, pH 7.4, 0.7 mg/mL bovine serum albumin). Parallel reactions were performed in the presence of 20 μmol/L dicumarol. The rate of dicumarol-sensitive NQO1 activity was determined as the difference between the uninhibited and dicumarol-inhibited rates and was normalized to total cytosolic protein as previously described[6].

NQO1 immunohistochemistry

Immunohistochemistry was performed on tissue sections cut from archival paraffin blocks as previously described[1]. Negative control staining was performed by incubating the sections without primary antibody. The sections were photographed on an Olympus BX50 microscope equipped with a QImaging MicroPublisher 3.3 RTV camera. Images were acquired with QCapture Pro software.

Statistical analysis

NQO1 activity was presented as mean nmol reduced DCPIP/min/mg protein ± SE (n = 3-5). Differences in NQO1 activity between normal livers and either APAP or PBC livers were determined using Student's t test. $P < 0.05$ compared to normal livers was considered statistically significant.

RESULTS

NQO1 expression and activity in normal human liver were very low as previously described[1]. Immunoblot analysis demonstrated marked induction of NQO1 protein in liver cytosol isolated from APAP and PBC specimens (Figure 1A). This increase in protein corresponded to a significant up-regulation of NQO1 activity from 3 ± 2 nmol DCPIP/min/mg protein in normal livers to 45 ± 16 nmol DCPIP/min/mg protein ($P < 0.05$) and 62 ± 32 nmol DCPIP/min/mg protein ($P < 0.05$) in APAP and PBC livers, respectively (Figure 1B).

Immunohistochemical staining demonstrated minimal NQO1 expression in hepatocytes and biliary epithelium of normal liver tissue (Figures 2A and 2B). Histology of APAP livers showed centrilobular necrosis and cell loss with lymphocytic accumulations and minimal biliary hyperplasia (Figures 2C and 2D). NQO1 staining was cytosolic and concentrated in midzonal and portal hepatocytes surrounding regions of centrilobular cell loss.

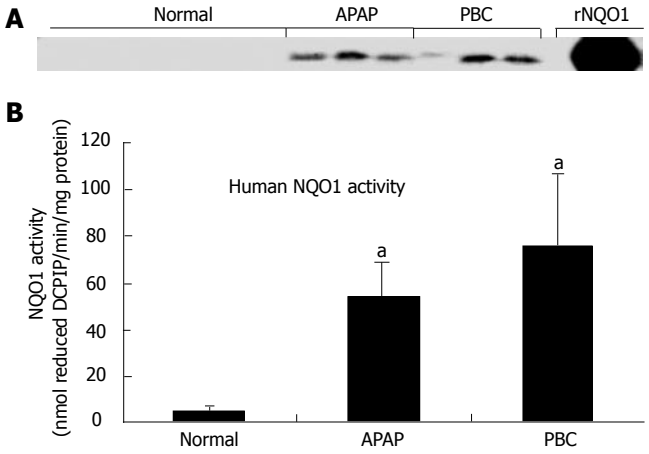

Figure 1 NQO1 protein (**A**) and activity (**B**) in cytosolic fractions from normal, APAP and PBC human liver specimens. The data are presented as nmol reduced DCPIP/min/mg protein ± SE (n = 3-5, $^{a}P < 0.05$ *vs* normal liver specimens).

Conversely, sections from PBC livers exhibited necrosis, fibrosis and nodular regeneration with lymphocytic-histiocytic infiltration and mild bile stasis (Figures 2E and 2F). Hepatocytes on the periphery of nodules were stained positive for cytosolic NQO1 protein. Positive staining was also observed in hyperplastic biliary epithelium in PBC livers (Figures 2G and 2H). No signal was detected in negative control sections (data not shown).

DISCUSSION

This is the first report documenting strong induction of NQO1 protein and activity in livers from individuals with two very different types of liver injury; exposure to toxic amounts of APAP or chronic PBC. Drug-induced liver injury is the most common cause of acute liver failure, with APAP exposure accounting for more than 40% of cases[8]. Ingestion of supratherapeutic amounts of APAP results in degeneration and necrosis of centrilobular hepatocytes. Conversely, PBC is a progressive disease primarily attributed to autoimmunity. Chronic inflammation and destruction of bile ducts during PBC cause biliary hyperplasia, marked fibrosis, and cirrhosis in later stages. The specificity of NQO1 up-regulation during liver damage is unknown. Increased NQO1 expression in APAP and PBC livers is similar to that in non-viral human hepatocellular carcinoma[9]. Conversely, livers from patients with type B or C hepatitis-induced hepatocellular carcinoma, focal nodular hyperplasia or cholangiocarcinoma do not demonstrate altered NQO1 levels[2, 10]. Together, these and previously reported data suggest differential regulation of NQO1 in human liver disease of varying etiologies.

Transcription factor NFE-2-related factor 2 (Nrf2) is the key regulatory pathway for NQO1 expression. Oxidative stress causes translocation of Nrf2 to the nucleus and binding to antioxidant response elements in the promoter region of numerous detoxification genes, including NQO1[11]. Subsequent gene activation leads to production of NQO1 and other proteins involved in cytoprotection. With the use of Nrf2-null mice, NQO1

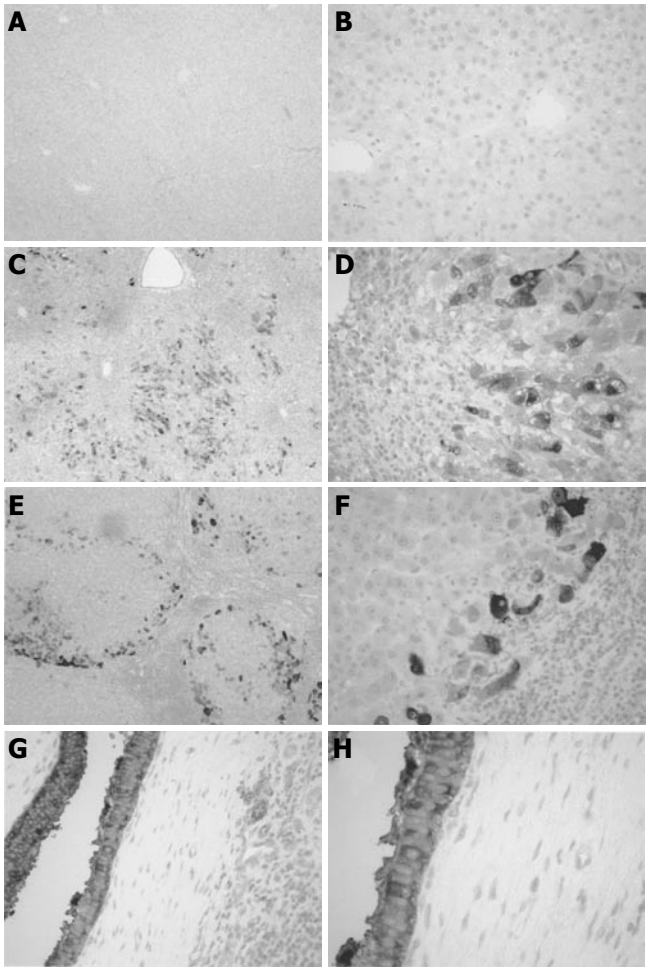

Figure 2 Immunoperoxidase staining of NQO1 in normal (**A**, **B**), APAP (**C**, **D**), and PBC (**E**, **F**) human liver specimens as well as in PBC hyperplastic biliary epithelium (**G**, **H**).

expression and induction have been shown to be Nrf2-dependent[12]. Activation of the Nrf2 signaling pathway during different types of human liver disease may be responsible for NQO1 up-regulation in drug-induced damage, cirrhosis and carcinoma.

As an Nrf2 target gene, NQO1 plays a role in cellular antioxidant defense beyond general drug metabolism, which includes dismutation of superoxide[13]. Therefore, NQO1 may be a scavenger of superoxide anions that are generated during both APAP toxicity and PBC[14]. Similarly, NQO1 also limits oxidative stress and lipid peroxidation by converting endogenous antioxidants (such as vitamin E) back to their more active, reduced forms[13, 15].

Loss of NQO1 function heightens susceptibility of rodents and humans to a number of diseases as demonstrated in transgenic mice lacking NQO1 and humans with a naturally-occurring NQO1 polymorphism. NQO1-null mice exhibit increased susceptibility to menadione-induced hemolytic anemia while patients possessing a C → T substitution at residue 609 of NQO1 have lower levels of NQO1 protein and exhibit an increased risk of benzene toxicity, urothelial tumors, acute myelogenous leukemia, and numerous additional cancers[13, 16, 17].

Since the pathogenesis of APAP hepatotoxicity is

mediated by an electrophilic quinone, NQO1 might possess the ability to convert the APAP metabolite back to parent compound[18]. Limited data suggest that inhibition of NQO1 by dicumarol potentiates APAP toxicity in mice[19]. Conversely, administration of Nrf2 activating compounds, such as oltipraz, protects hamsters against APAP hepatic damage by inducing detoxification pathways including NQO1[20, 21].

The relative constitutive level of hepatic NQO1 among rodents and humans may also explain in part the different susceptibility to APAP hepatotoxicity across species. NQO1 activity is markedly higher in rat liver compared to mouse and human livers. Along the same line, rats exhibit a greater resistance to APAP-induced liver damage compared to mice and humans. Although additional variations in APAP metabolism between rodents and humans are known, differences in basal NQO1 activity among these species may also be a determinant of susceptibility to APAP.

In conclusion, NQO1 is upregulated in APAP–induced and cholestatic human hepatic injury, suggesting that this Nrf2 gene may limite the progression of these liver diseases.

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RAPID COMMUNICATION

Giardia lamblia infection in patients with irritable bowel syndrome and dyspepsia: A prospective study

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Abstract

AIM: To evaluate the prevalence of *Giardia lamblia* (*G. lamblia*) infection in patients with irritable bowel syndrome (IBS) and dyspepsia and to establish which is the most accurate test to diagnose the infection in this setting.

METHODS: One hundred and thirty-seven patients who consecutively attended the Outpatient Gastroenterology Clinic for the first time between January 2002 and December 2003 due to symptoms of IBS and/or dyspepsia were recruited. All patients underwent clinical evaluation, first-step haematology and chemistry tests, serologic assays for celiac disease, lactose-H_2 breath test, abdominal ultrasonography, and esophagogastroduodenoscopy. *Helicobacter pylori* status was evaluated. In patients with symptoms of IBS older than 45 years, colonoscopy was also performed. In all patients, duodenal biopsies and stool samples were examined for trophozoites and cysts of *G. lamblia* by several methods.

RESULTS: *G. lamblia* was identified in 9 patients. The following diagnoses were also made: IBS (100/137, 73%), functional dyspepsia (62/137, 45%), organic dyspepsia (33/137, 24%), and lactose intolerance (75/137, 55%). A significant association was found between giardiasis and *H pylori* infection ($\chi^2 = 6.632$, OR = 12.4, CI = 1.5-68.1). There were no symptoms that reliably allowed the recognition of giardiasis. Direct search of the parasite in duodenal biopsy and stool sample examinations gave concordant results in all cases while histological examination of duodenal biopsies displayed a low sensitivity (e.g., 22.2%).

CONCLUSION: In this consecutive series, diagnosis of *G. lamblia* infection accounted for 6.5% of patients with IBS and dyspepsia. Duodenal biopsies for diagnosis of giardiasis may be unnecessary if stool sample examination is performed.

Key words: Dyspepsia; Giardiasis; *H pylori*; Irritable bowel syndrome

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INTRODUCTION

Giardia lamblia is a cosmopolitan parasite with worldwide distribution and the most common protozoan isolated from gastrointestinal tract[1]. The prevalence of infection varies from 2%-7% in industrialized countries to 40% in tropical/subtropical regions with poor sanitation and hygienic conditions[2-5].

Giardiasis typically occurs following the ingestion of water or foods contaminated with fecal material containing cysts, and the infective dose may be as low as 10 cysts. The clinical aspects of giardiasis are largely nonspecific. The infection can be asymptomatic or patients may present with extraintestinal symptoms, such as fever, maculopapular rashes, pulmonary infiltrates, lymphadenopathy, polyarthritis and urticaria. However, the most common symptoms are diarrhea, abdominal pain, bloating, flatulence and weight loss resulting from malabsorption[6]. Since these symptoms may overlap those of patients with other gastrointestinal disorders[7-9], it is not clear when the infection should be suspected. Classically, diagnosis of giardiasis is performed by microscopic examination of stool samples and/or duodenal biopsies and further methods include immunocromathography and immunofluorescence on stool samples. There are

few studies comparing the diagnostic proficiency of the methods using either stool or duodenal biopsy samples.

The aim of this study was to evaluate the prevalence of *G. lamblia* infection in patients with irritable bowel syndrome (IBS) and dyspepsia and to establish the most accurate test for its diagnosis in this setting.

MATERIALS AND METHODS

Patient recruitment

Four hundred and thirty-five consecutive patients attending our hospital for the first time between January 2002 and December 2003 on two days of the week (Monday and Wednesday) due to symptoms of IBS who satisfied Rome II criteria[10] and/or dyspepsia were considered for the study. Patients with alarm features (such as weight loss, dysphagia, vomiting, bleeding), familiarity for gastrointestinal neoplasia and inflammatory bowel disease were excluded as well as patients who took nonsteroidal anti-inflammatory drugs and proton pump inhibitors in the last 15 days and antibiotics in the last 30 days. Patients with symptoms lasting more than one year were also excluded. Finally, 137 patients (48 M, 89 F; median age 39 years, range 19-77 years) were enrolled.

All patients underwent routine blood investigations, including erythrocyte sedimentation rate, C-reactive protein, thyroid hormones, antigliadin, antiendomysial and antitransglutaminase antibodies. Furthermore, urinalysis, microscopic examination of stools, esophagogastroduodenoscopy with duodenal biopsy, evaluation of *Helicobacter pylori* (*H pylori*) infection, abdominal ultrasonography, and lactose-H_2 breath test were performed. Patients with symptoms of IBS older than 45 years ($n=46$) underwent also colonoscopy.

Laboratory tests for G. lamblia infection

The presence of the parasite was evaluated by three different approaches: direct search of the parasite in the duodenal biopsy samples with or without use of fixation and dyes, histological examinations of other aliquots of the above bioptic samples, and parasitological evaluations of stool samples.

Search of the parasite in duodenal biopsy samples Duodenal biopsies were obtained during the upper gastrointestinal endoscopy, immediately submitted to microbiology laboratory where they were put into a sterile plastic container filled with 10 mL of sterile saline. The samples were processed within 2 h of arrival in the laboratory. Wet mount preparations were obtained by cutting the original biopsy into 0.5 mm pieces on a glass slide, with the help of a razor blade and a plastic pipette tip. Then such pieces were suspended in 2 drops of sterile saline and a cover glass was put on them. Microscopic examination for motile organisms was carried out using low and high dry power (× 200 and × 400). Permanent stains were prepared as reported above. However the biopsy pieces were air dried, fixed and the giemsa (Giemsa Plus, Trend Scientific) or trichrome staining (Trichrome Stain, Scientific Device Laboratory Inc., Des Plaines, IL, USA) were carried out following the manufacturer's instructions. Also the vital staining with acridine orange was used on unfixed specimens examined by confocal microscopy to help identify the parasite and address both the mobility and some details of its cytostructure.

Histology of bioptic samples Duodenal biopsies obtained during the upper gastrointestinal endoscopy were also submitted to the Pathology laboratory, where the specimens where paraffin-included, stained with hematoxylin-eosin and examined for Giardia trophozoites as well as for inflammatory infiltrate particularly of plasma cellular type.

Stool parasitology Liquid or semi liquid fecal specimens were immediately submitted to the microbiology laboratory, a wet mount with saline and Lugol's iodine (Dobell reactive) was prepared and microscopic examination for motile organisms and Giardia cysts was carried out using low and high dry power (× 200 and × 400). Moreover, both liquid and solid fecal samples were processed using the formalin/ether enrichment method followed by microscopy for Giardia cysts. Coproantigens were also evaluated in the stool specimens by immunochromatographic technique (Xpect, Remel Europe, Dartford Kent, UK), an EIA method (Prospect, Remel Europe, Dartford Kent, UK), and commercial direct fluorescent antibody assay (Merifluor, Meridian Bioscience Inc., Cincinnati, USA). Laboratory tests for Giardia coproantigens were carried out and the results were evaluated following the manufacturer's instructions.

The microbiologist (GM) and pathologist (AA) were blinded to the clinical and laboratory data.

H pylori status

Patients were classified as *H pylori* positive when the urease quick test (Yamanouchi Pharma, Milan, Italy) was positive. Patients underwent C-13 urea breath test (Cortex, Milan, Italy) when the urease quick test was negative and were considered free of the infection when the two tests were both negative for *H pylori*.

Statistical analysis

The relation between presence of giardiasis and *H pylori* infection was evaluated with χ^2 test and odds ratio (OR). Data were given together with 95% confidence interval (CI).

RESULTS

G. lamblia infection was found in 9 of the 137 patients (6.5%). According to the clinical and laboratory evaluations, the following diagnoses were also made: IBS, lactose intolerance, functional and organic dyspepsia, colon diverticulosis, celiac disease, inflammatory bowel disease, gastric cancer, colon cancer, and collagenous colitis (Figure 1). All patients with giardiasis (7 M, 2 F; median age: 38 years, range 26-61 years) had nonspecific (mostly mild) symptoms largely overlapping those complained by patients without giardiasis (Table 1). Indeed, according to the clinical and laboratory findings, these patients were considered affected also by other functional and organic disorders (Table 1). In patients with giardiasis, blood examination was normal with only one patient

Table 1 Characteristics of 9 patients with *G. lamblia* infection among the 137 complaining of symptoms of irritable bowel syndrome (IBS) and/or dyspepsia (FD: functional dyspepsia; OD: organic dyspepsia) or displaying lactose intolerance (LI)

Patient	Age (yr)	Sex	Symptoms	IBS	LI	FD	OD
1	61	M	Abdominal pain, constipation/diarrhea	+	-	+	-
2	38	F	Abdominal pain, diarrhea, pirosis	+	+	-	+
3	33	M	Abdominal pain, diarrhea	+	-	-	-
4	26	F	Abdominal pain and bloating, constipation	+	+	-	-
5	48	M	Abdominal pain, diarrhea	+	+	-	-
6	47	M	Abdominal pain, diarrhea, pirosis	+	-	+	-
7	37	M	Abdominal pain and bloating, diarrhea	+	-	-	+
8	26	M	Abdominal pain and bloating, diarrhea	+	+	+	-
9	39	M	Abdominal pain, diarrhea	+	-	-	-

Table 2 Comparison of the laboratory tests for *G. lamblia* infection in the study population (n = 137)

Patient	Age (yr)	Sex	Direct search in duodenal biopsy	Stool sample examination	Histology of duodenal biopsy
1	61	M	+	+	-
2	38	F	+	+	-
3	33	M	+	+	+
4	26	F	+	+	-
5	48	M	+	+	-
6	47	M	+	+	-
7	37	M	+	+	+
8	26	M	+	+	-
9	39	M	+	+	-

In the remaining 128 patients, all the three tests were negative for the presence of *G lamblia*.

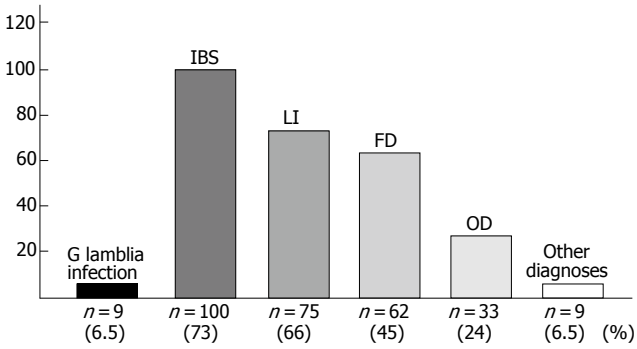

Figure 1 Diagnostic categories in the 137 patients complaining of symptoms of irritable bowel syndrome (IBS) and/or dyspepsia (FD: functional dyspepsia; OD: organic dyspepsia). LI: lactose intolerance; Other diagnoses included: colon diverticulosis (n = 3), celiac disease (n = 2), inflammatory bowel disease (n = 1), gastric cancer (n = 1), colon cancer (n = 1), and collagenous colitis (n = 1).

showing a moderate eosinophilia (1.190/μL; normal range: 0-500/μL). Direct search of the parasite in duodenal biopsy (Figures 2A, 2B) and stool sample (microscopic) examination (Figures 2C, 2D) were concordantly positive (9 of 137 patients) and negative (128 of 137 patients) for *G. lamblia*, while histological evaluation of duodenal biopsy was positive in only two cases (2 of 137 patients) and negative in the remaining (135 of 137 patients) (Table 2). Using as reference the former two tests, the sensitivity and specificity of the histological examination of duodenal biopsy for the diagnosis of giardiasis were 22.2% and 100%, respectively.

Fifty-eight patients (42%) were infected with *H pylori* (18 M, 40 F; median age 40 years, range 20-65 years). A significant association was found between giardiasis and *H pylori* infection. Eight of 58 patients (14%) with *H pylori* infection had giardiasis while 1 of 79 (1%) without *H pylori* infection had giardiasis (χ^2 = 6.632, OR = 12.4, CI = 1.5-68.1).

DISCUSSION

This study aimed at investigating the impact of *G. lamblia* infection on patients who complained of symptoms referring to IBS and dyspepsia. In order to prevent information and observational bias, we properly excluded patients who have had a previous gastroenterological consultation, those with symptoms lasting more than one year and those who had been recently taking drugs that might interfere with complaints and laboratory investigations. Furthermore, to minimize the presence of serious illness, patients complaining of alarm symptoms were excluded. In the consecutive series of 137 patients with symptoms of IBS and/or dyspepsia, the prevalence of giardiasis was 6.5% (9/137). Possibly due to the selection of a symptomatic study population, this figure is slightly higher than the average in the general population in a Western country[2-5]. Nevertheless, no comparisons can be made with the incidence of giardiasis in the asymptomatic population coming from our geographical area.

Furthermore, an important finding of this prospective study is that no symptoms could reliably discriminate patients with giardiasis from those without the parasite. Giardiasis may be present in patients with other gastrointestinal disorders as observed in this study, it seems that symptoms of one disease may overcome/overlap those of the other. Nevertheless, the prevalence of giardiasis in patients suffering from IBS and/or dyspepsia can be considered as the cause of 1BS and/or dyspepsia.

Another important finding of this study involves the diagnostic procedures to be taken into account when dealing with *G. lamblia* infection. The data showed that histological examination of duodenal biopsies for *G. lamblia* was unsuitable due to an unacceptable rate of false negative results (e.g., 22.2% sensitivity). At the same time, it has been shown that stool examination is as accurate as the direct search of the parasite in the duodenal samples.

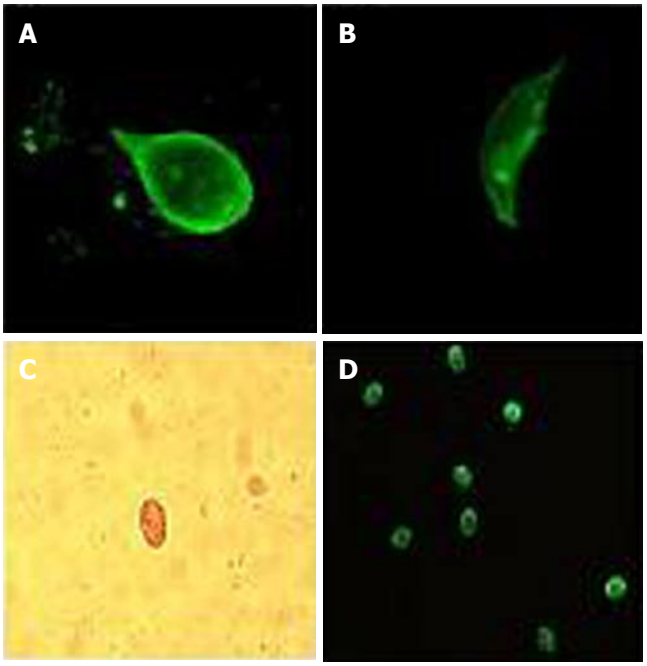

Figure 2 Confocal microscopy images (**A** and **B**) of *G. lamblia* trophozoite (x1000) in duodenal bioptic sample, using acridine orange as a vital stain. **A:** a dorsal projection showing the peculiar tear-shaped cell with flagella. **B:** an unusual flank projection shows the nuclei on the back of the ventral disk. **C:** *G. lamblia* cysts (x200) microscopic images after a wet mount with Lugol's iodine of fecal specimens. **D:** *G. lamblia* cysts (x200) in stool sample following formalin/ether enrichment of filtered samples and staining with a direct fluorescent antibody.

Consequently, when a skilled microbiologist and appropriate techniques are available, duodenal biopsy seems unnecessary in accurate identification of *G. lamblia*, thus invasive and expensive tools such as upper gastrointestinal endoscopy could be avoided.

This study also showed that giardiasis was significantly associated with *H pylori* infection, possibly reflecting that the two infections share a number of risk factors[11]. This association may have several clinical implications in regard to the transmission mode[12,13], the possibility of a synergy in metronidazole resistance[14], and the experimental evidence of a common pathogenesis scenario, leading to gastrointestinal metaplasia[15].

In conclusion, the prevalence of giardiasis in patients suffering from symptoms of IBS and dyspepsia is non-negligible, and duodenal biopsies for the parasite may be unnecessary if stool examination is performed.

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RAPID COMMUNICATION

Clinical characteristics of a group of adults with nodular lymphoid hyperplasia: A single center experience

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Abstract

AIM: To describe the clinical and histological characteristics of a group of adults with small-bowel nodular lymphoid hyperplasia (NLH).

METHODS: Patients were searched for five years in pathology records of our institution. The biopsy material was reassessed using strict histopathological criteria. Clinical data were obtained from medical records.

RESULTS: Small-bowel NLH was diagnosed in 18 cases. The female: male ratio was 2 : 1. The most frequent symptoms were diarrhea (72%), involuntary weight loss (72%) and abdominal pain (61%). Nine patients (50%) had immunodeficiency. Small-bowel bacterial overgrowth was found in three (17%) cases. At small-bowel NLH diagnosis, three (17%) had associated lymphoma: two intestinal and one extra-intestinal lymphomas. In two patients with villous atrophy and anti-endomysial antibodies the diagnosis of celiac disease was established. *Giardia lamblia* infection was found in only one patient with hypogammaglobulinemia (Herman's syndrome).

CONCLUSIONS: NLH is uncommon in adult patients. Associated diseases are immunodeficiency and lymphoid tissue malignancies.

Key words: Nodular lymphoid hyperplasia; Hypogammaglobulinemia; Lymphoma

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INTRODUCTION

Nodular lymphoid hyperplasia (NLH) of the gastrointestinal (G1) tract is characterized by markedly hyperplasic, mitotically active germinal centers with well-defined lymphocyte mantles[1]. The nodules are found in mucosa and/or submucosa anywhere in the gastrointestinal tract and may resemble small adenomatous polyps on gross examination[2,3]. Approximately 20% of adults with common variable immunodeficiency (CVI) are found to have NLH. It has been suggested that NLH is a risk factor for both intestinal and extra intestinal lymphoma[4]. It has also been reported in patients with human immunodeficiency virus infection[5]. In children, NLH tends to have a benign course and usually regresses spontaneously but in adults it is rare and poorly described.

In a previous report by Canto *et al*[6] NLH was diagnosed in 11 Mexican subjects. In five of them a diagnosis of Herman's syndrome was established. Patients were collected from the pathology database of our institute from 1973 to 1988. Since this time, an increased number of duodenal biopsies have been taken for the approach of patients with chronic diarrhea who were referred to our institution from different places. In this work we described the clinical characteristics of 18 new patients with NLH seen at the Instituto Nacional de Ciencias Médicas y Nutrición "Salvador Zubirán", a National Institute of Health located in Mexico City, which is a referral center for gastrointestinal diseases.

MATERIALS AND METHODS

Patients

From January 1998 to December 2002, patients with the histological diagnosis of NLH were identified from the pathology database. Medical records were reviewed and a pathologist (STH) reassessed slides or paraffin-embedded biopsies. NLH was considered when the following histological criteria were observed: hyperplasic lymphoid follicles, mitotically active germinal centers with well-defined lymphocytes mantles, and lymphoid follicles localized at mucosa and/or submucosa.

Histology

The original duodenal mucosal biopsy specimens were

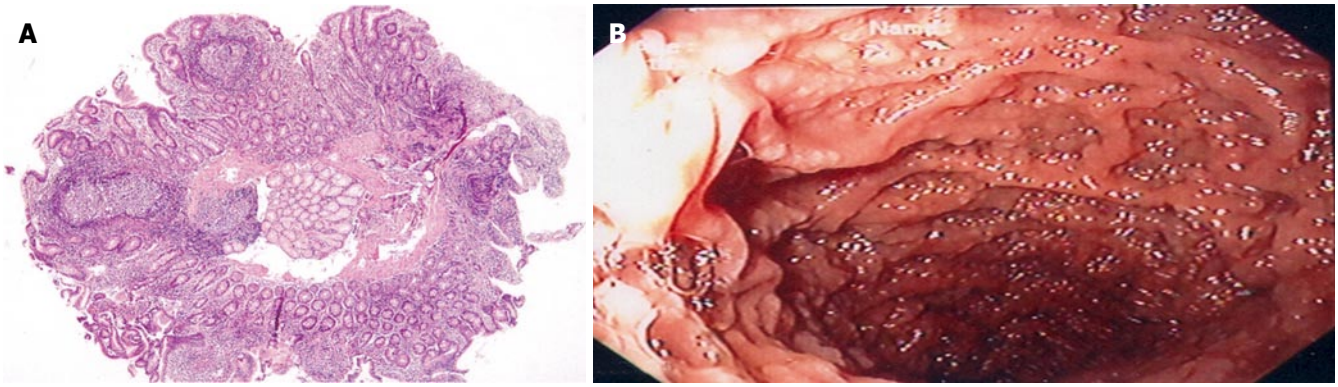

Figure 1 Histological (**A**) and endoscopic (**B**) characteristics of NLH. Hyperplasic follicles with active germinal centers localized at *mucosa* and *submucosa*, Brunner's glands at center and a pseudo-polypoid pattern of duodenum mucosa were observed.

used for reassessment. The histopathological evaluation was performed on hematoxylin-eosin-stained samples, including counting and localization of lymphoid follicles, type and severity of inflammation in *lamina propria*, and presence of villous atrophy. If the quality of the original sections was too poor for the reassessment, new sections from the original paraffin wax-embedded biopsy blocks were made.

Clinical and laboratory data

Age, duration and type of symptoms, body mass index (BMI) and associated diseases were evaluated. The following tests were recorded at the time of diagnosis: hemoglobin, mean cell volume, total proteins, albumin, globulins, β-carotene, electrolyte profile, prothrombin time, partial thromboplastin time, human immunodeficiency virus antibody, liver enzymes, stool analysis for ova and parasites, microscopic examination and culture of sterile collected duodenal fluid.

Statistical analysis

Clinical and histopathological data were expressed as absolute and/or relative frequencies and mean±SD. The Mann-Whitney U or χ^2 tests were used to compare groups. $P<0.05$ was considered statistically significant. SPSS software (version 10) was used.

RESULTS

Patients

Eighteen out of 550 duodenal biopsies had the histological diagnosis of NLH. After re-evaluation of the histological material all of them were included in the analysis. Twelve were women and 6 were men, yielding a female: male ratio of 2:1. Their median age at diagnosis was 41 years (18 to 76 years). Duration of symptoms from the onset to diagnosis varied from 1 to 48 mo (median = 18 mo). The median body mass index at diagnosis was 19 kg/m^2 (11 to 26 kg/m^2).

Clinical data

Diarrhea and weight loss were the predominant symptoms in 13 (72%) patients. A mean of 6 ± 4 bowel movements per day and a weight loss of 11.6 ± 6.9 kg in the last six months were presented. Eleven (61%) had abdominal pain (Table 1).

Table 1 Symptoms and signs of patients with NLH

Symptom or sign	*n* (%)
Diarrhea	13 (72)
Involuntary weight loss	13 (72)
Abdominal pain	11 (61)
Lymphadenopathy	4 (22)
Pallor	4 (22)
Chronic cough	2 (11)
Edema	2 (11)
Arthralgia	1 (6)
Neuropathy	1 (6)

Histopathology

A mean of 2.5 (range 2-6) hyperplasic lymphoid follicles was observed in a single field 4 × magnification with light microscopy. Hyperplasic lymphoid follicles were localized in mucosa of 13 cases (69%) and in mucosa and submucosa of five cases (31%) (Figure 1).

Patients with 3 or more hyperplasic follicles had lower β-carotene levels (46.7 ± 25 mg/dL *vs* 108 ± 42.6 mg/dL, $P<0.05$) and mixed localization of follicles (mucosa and submucosa) (8 *vs* 1, $P<0.05$) than patients with ⩽2 follicles. Subjects with mixed localized lymphoid follicles showed a statistically significant lower hemoglobin (11.9 ± 1.4 g/L *vs* 14 ± 13 g/L, $P=0.03$) and serum β-carotene (44 ± 21.9 mg/dL *vs* 111.2 ± 38.7 mg/dL, $P<0.02$) as well as a tendency to prolong thromboplastin time (17.3 ± 7.2 *vs* 11.5 ± 2.5, $P<0.07$) than those with exclusively mucosa-localized follicles.

Villous atrophy (partial or total) was observed in seven cases (38%) and it was associated with weight loss (7 *vs* 2, $P<0.02$), lientery (4 *vs* 0, $P<0.07$) and low globulin level (2.3 ± 0.87 g/L *vs* 3.2 ± 5.2 g/L, $P<0.06$). This group had also lower hemoglobin (12.3 ± 1.3 g/L *vs* 14.2 ± 14 g/L, $P<0.03$) and cholesterol serum level (170 ± 38.1 mg *vs* 200 ± 17.1 mg, $P<0.03$) than those without atrophy (Table 2).

Associated diseases

NLH was associated with hypogammaglobulinemia in 9 (50%) patients, CVI in 8 cases and selective IgA deficiency

Table 2 Findings in duodenal mucosa biopsy specimens from patients with NLH

Abnormality	*n* (%)
Localization of lymphoid follicles	
• Mucosa	13 (69)
• Mucosa *and submucosa*	5 (31)
Inflammatory changes in *lamina propria*	
Chronic	18 (100)
• Lymphocytes-plasma cells	10 (53)
• Plasma cells	7 (38)
• Lymphocytes	1 (9)
Acute and chronic	2 (15)
Villous atrophy	7 (38)
• Partial	5 (23)
• Total	2 (15)
Increased intraepithelial lymphocytes	2 (15)
Crypt hyperplasia	1 (9)

in 1 case. In this group the mean level of globulins was 1.6 ± 3.5 g/L which was quite different from that in subjects with normal globulins (3.2 ± 4.5 g/L, $P < 0.001$).

Giardia lamblia infection was found in only one case. *E. coli*, *Streptococcus sp,* and *Klebsiella sp* were respectively isolated from duodenal fluid in three (17%) (10^6 bacterial colonies per milliliter). These patients showed a markedly prolonged prothrombin time (18.5 ± 7.4 s *vs* 11.4 ± 1.9 s, $P < 0.014$). Anti-endomysium antibodies were tested in 16 subjects and two (12.5%) were positive. Both had villous atrophy thus the diagnosis of celiac sprue was established.

Lymphoma was found in three (17%) cases: one with nodular sclerosing-Hodgkin´s disease on the mediastinum, one with jejunum non-Hodgkin's lymphoma (diffuse large B-cell lymphoma) and one with gastric lymphoma. Patients with lymphoma were older (58 ± 10 years old) and only one of them presented diarrhea as a predominant symptom. No AIDS-associated NLH was found in this series (Table 3).

Table 3 Disorders found in patients with NLH

Disorder	*n*
Hypogammaglobulinemia	9
Lymphoma	3
Intestinal bacterial overgrowth	3
Celiac sprue	2
Giardiasis	1
Graves disease	1
MNGIE	1
Chronic pancreatitis	1
Pancreas divisum	1

MNGIE: mitochondrial neurogastrointestinal encephalomyopathy.

DISCUSSION

Nodular lymphoid hyperplasia (NLH) is a well-characterized condition frequently associated with increased risk of gastrointestinal tumors, mainly gastrointestinal lymphoma. As a pathological entity it seems to be rare. It has been described in immunocompromised and immunocompetent subjects, normal children and adults and patients with AIDS[1-5].

This series represent the whole experience of the National Institute of Health in Mexico, which is a tertiary referral center for gastrointestinal diseases. In previous works Canto *et al*[6] and Castañeda *et al*[7] have described some cases with NLH seen at the same institution. In these two papers 12 patients are included in a period of 18 years contrasting with the 18 cases found by us in only a 5-year period. This difference could be explained by the selection criteria used. While Canto *et al*[6] selected the cases based on the radiological and histological findings. We included only the cases who met the strict histological criteria from a large database of subjects with duodenal biopsy. On the other hand, upper GI endoscopy with biopsy and duodenal fluid aspirate was performed in each patient with chronic diarrhea. NLH was found in 18 cases (4%). This low frequency can be explained by our selection criteria. Again we only included subjects with a duodenal biopsy with or without small bowel radiology. It was reported that NLH is most frequently located in jejunum-ileum[1-3].

The pathogenesis of NLH is unknown but is likely the result of an accumulation of plasma-cell precursors due to a maturational defect in the development of B-lymphocytes in order to compensate for functionally inadequate intestinal lymphoid tissue[8]. The role of functionally inadequate intestinal lymphoid tissue is suggested by the common occurrence of hypogammaglobulinemia and recurrent infections (particularly by *Giardia lamblia*)[9]. Nine patients had disgammaglobulinemia, but we were able to demonstrate *Giardia lamblia* infection only in one. It should be noted that we only used stool analysis for ova and parasites and microscopic duodenal fluid examination. It is possible that some cases could be missed because we did not perform fecal ELISA for *giardia-specific* antigen, which is the most sensitive and specific method for *giardia* detection[10]. Another plausible explanation is the high frequency of auto-medication used in our patients. Mexican subjects with chronic diarrhea are empirically treated with antibiotics including metronidazole.

Histological diagnosis of NLH can demonstrate the hyperplasic lymphoid follicles with mitotically active germinal centers at mucosa and/or submucosa. The number of follicles is not a diagnostic criterion[1,2]. We found a mean of 2.5 hyperplasic lymphoid follicles in a single 4× magnification field. Patients with three or more localized hyperplasic follicles in mucosa or submucosa showed markers of poor intestinal absorption such as low serum hemoglobin and β-carotene and a marked prolonged prothrombin time, suggesting that fat malabsorption may in turn lead to deficiencies of fat-soluble vitamins. These patients also had chronic diarrhea and a marked weight loss.

The association between NLH and other malignant and benign diseases has been clearly described[9,12-15]. In our series, seven cases had CVI and one had selective IgA deficiency. CVI is a heterogeneous form of immunodeficiency associated with decreased serum immunoglobulin levels, recurrent sinopulmonary infections, gastrointestinal disorders, and increased frequency of malignancies[12,13]. The association between NLH, hypogammaglobulinemia and *Giardia lamblia* infection is known as Herman's syndrome[8]. We only found one case with disgammaglobulinemia, NLH and *Giardia lamblia* infection. The only real difference with

Canto's work was the low detection of giardiasis in our series, a feature which is needed to establish the diagnosis of Herman's syndrome.

The risk of malignancy has been well recognized in subjects with NLH[14]. Three of our cases had lymphoma. Lymphoma has been reported in patients with and without immunodeficiency[15,16]. The link between the extra intestinal lymphoma and NLH is less clear. We found one patient with Hodgkin's disease localized on the mediastinum. Jonsson *et al*[4] have reported a case of extra intestinal lymphoma associated with NLH. Hyperplasic tissue completely disappeared after chemotherapy with remission of lymphoma and then reappeared at relapse.

To the best of our knowledge, no relationship between NLH and celiac disease has been reported. In our series, intestinal atrophy, increased intraepithelial lymphocytes and anti-endomysium antibodies were found in two patients. One of them was asymptomatic on gluten-free diet, and complete resolution of anatomical changes was found in duodenal-specimens taken two months later. In the other case, no clinical response was found six weeks after implementation of a gluten-free diet. This patient lost her follow-up and we do not know her current histological status. Moderate to severe intestinal atrophy and increased intraepithelial lymphocytes have been reported in patients with CVI known as "pseudo-celiac" pattern[17]. In contrast to the typical changes seen in patients with celiac disease, CVI cases showed mild inflammatory infiltrate at the *lamina propria* with normal enterocyte maturation. We identified partial villous atrophy in five patients with negative anti-endomysium antibodies. Perhaps these 5 patients correspond to the pseudo-celiac pattern previously described.

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RAPID COMMUNICATION

Hyperlactatemia in patients with non-acetaminophen-related acute liver failure

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Abstract

AIM: To characterize hyperlactatemia in patients with non-acetaminophen acute liver failure (ALF) in an attempt to clarify the mechanisms implicated and the role as a prognosis factor.

METHODS: In the setting of liver transplantation, 63 consecutive patients with non-acetaminophen acute liver failure were studied in relation to tissue oxygenation, hemodynamic and metabolic parameters. Before and after transplantation, the number of infected patients and outcome were registered.

RESULTS: Acute ALF showed higher levels of lactate than subacute ALF (5.4 ± 1 mmol/L versus 2.2 ± 0.6 mmol/L, $P = 0.01$). Oxygenation parameters were within the normal range. Lactate levels showed good correlation with respiratory quotient ($r = 0.759$, $P < 0.005$), mean glucose administration ($r = 0.664$, $P = 0.01$) and encephalopathy ($r = 0.698$, $P = 0.02$), but not with splanchnic arteriovenous difference in PCO2, pH and the presence of infection ($P = 0.1$). Portal vein lactate was higher ($P < 0.05$) than arterial and mixed venous lactate, suggesting its production of hyperlactatemia in the intestine and spleen. The presence of infection was an independent predictor of survival.

CONCLUSION: Hyperlactatemia is not a prognosis factor due to byproduct of the overall acceleration in glycolysis.

INTRODUCTION

Up to now hyperlactatemia, a common finding in the setting of acute liver failure (ALF), has been attributed mainly to severe circulatory disturbance, abnormal vasomotor tone, plugging of small vessels by platelets and/or interstitial edema[1] and it has been proposed as a bad prognosis factor. Several studies suggest that splanchnic circulation abnormalities during ALF may result in inadequate distribution of blood flow and occlusion of the microvasculature, leading to tissue hypoperfusion, although oxygen delivery is increased in a macro circulation[2,3]. Inadequate balance between splanchnic oxygen delivery and demand results in splanchnic hypoxia of tissues that consume oxygen avidly[4]. In this condition, as cellular oxygen concentrations decrease, ATP concentrations cannot be maintained by oxidative phosphorylation. In an attempt to maintain cellular function, the cells shift over to anaerobic glycolysis with accumulation of lactic acid and hydrogen ions as ATP is hydrolyzed.

However, other mechanisms may be implicated in hyperlactatemia in the context of ALF. Although lactate is the end-product of anaerobic glycolysis and blood concentrations rise in response to hypoxia, well-oxygenated tissues may also produce lactate due to aerobic glycolysis[5]. On the other hand, since the liver is the principal organ responsible for removal of whole body lactate clearance, hyperlactatemia may be the result of a deficit of lactate clearance by the insufficient liver.

We undertook the present study to characterize hyperlactatemia in patients with non-acetaminophen ALF who were considered candidates for liver transplantation in an attempt to clarify the mechanisms implicated in high

lactate levels as well as the role as a prognosis factor.

MATERIALS AND METHODS

Patients

A total of 63 patients with diagnosis of non-acetaminophen ALF were admitted to the Liver Intensive Therapy Unit in our hospital and submitted to liver transplantation. All patients were prospectively evaluated and included in the study. Four of them were considered hyperacute, 39 acute while 20 subacute hepatic failure based on the criteria of the Kings College Hospital Group[6].

This prospective study was approved by the Clinic Hospital Research Ethic Committee. Informed consent for the study was obtained from each patient or patient's family.

General management

All patients were managed in the Liver Intensive Therapy Unit, in a standard manner[7]. They were maintained on 10%-20% glucose solution infusion (keeping blood glucose levels between 90 and 120 mg/dl). Prophylaxis for upper gastrointestinal bleeding and close microbiological surveillance (all patients received norfloxacin and nystatin) were performed. Coagulation profile was corrected only in the presence of overt bleeding. Those in grade III and IV coma (43 patients) were treated with standard sedation and mechanical ventilation. An epidural transducer (Ladd Research Laboratories, Burlington, VT) was inserted into the epidural space to enable early recognition and treatment of intracranial hypertension (>25 mmHg), mannitol (0.5 to 1 mg/kg) for over 30 min and pentobarbital infusion as a following step were used to control intracranial pressure (ICP). Systemic arterial hypotension (systolic <100 mmHg) was managed by dopamine infusion according to the hemodynamic conditions in an attempt to maintain cerebral perfusion pressure (CPP) (>60 mmHg). No artificial liver support techniques were used and no patient was treated with N-acetylcysteine or epoprostenol.

Anesthesia and hemodynamic management

In 20 patients who remained awake (encephalopathy grades I and II), anesthesia was induced following our standard policy. All patients were mechanically ventilated (Servo 900C, Siemens) and the fraction of inspired oxygen was adjusted to achieve an arterial PaO_2 of 180 - 220 mmHg. Ventilatory parameters were regulated to maintain the end tidal CO_2 around 32 mmHg and P_aCO_2 below 35 mmHg. In all patients vasopressor dopamine at 3 μg/kg was started at the beginning of the surgical procedure and maintained through the transplant and increased to 8 μg/kg if necessary to achieve a mean arterial pressure greater than 75 mmHg. After graft reperfusion, arterial hypotension was treated by intravenous bolus of 10 μg of epinephrine. Fiberoptic pulmonary artery floatation catheter (7.5 French, Edwards Laboratories, Irvine, Calif.) was introduced through the right internal jugular vein and an arterial catheter (18 gauge, Arrow, Reading, PA) was placed via the left radial artery. At the beginning of the surgical procedure portal vein catheter (Certofix ® Mono S330 16G Braun) was placed by introducing it through a branch of the superior mesenteric vein and advanced to the portal vein until it was felt, in order to register portal pressure and to obtain blood samples for oxygen and metabolic parameters in splanchnic area. Not all patients received portal vein cannulation because it was not considered technically possible by the surgeon in 11 patients (4 with subacute ALF and 7 with acute ALF).

Variables analyzed in the setting of LTx

Before the surgical transplant procedure was started, the following parameters were measured. Mean arterial pressure (MAP, mmHg), cardiac index (CI, L/min/m^2) and systemic vascular resistance index (SVRI, dyn.sec.cm^{-5}.m^2) were obtained.

Systemic parameters including oxygen content difference [D(a-v)O_2, ml/dl], oxygen delivery (DO_2, mL/min/m^2), oxygen consumption (VO_2, mL/min/m^2), oxygen extraction ratio (VO_2/DO_2, %), mixed venous/arterial gradient of PCO_2 (VACO2, mmHg), arterial/mixed venous gradient of pH (AVpH, U) as well as respiratory quotient (RQ) were calculated.

Splanchnic parameters including arterial/portal venous oxygen content difference [D(a-v)O2, ml/dl], oxygen extraction ratio (VO_2/DO_2), portal veno/arterial gradient of PCO2 (VACO2, mmHg), and arterial/portal venous gradient of pH (AVpH, U) were obtained. All these variables were calculated following standard formulas.

Plasma lactate levels were measured with three blood samples simultaneously drawn from arterial catheter (La), the distal part of pulmonary catheter (Lv) and portal vein catheter (Lp). Blood lactate level was determined using a Kodak Ektachem 700XR (Rochester, NY. USA) analyzer[8].

The need of catecholamine administration and the mean glucose administration in the last 48 hours before transplant were recorded. The need of vasopressor drugs administered during the transplant procedure was also registered. The degree of encephalopathy and intracranial pressure were recorded.

Explanted liver pathology

Weight and structural characteristics of all explanted livers were studied.

Infectious complications

Diagnosis of infection was made by the presence of the white blood cell count greater than 12×10^9/L or less than 4×10^9/L, the presence of immature neutrophils, temperature higher than 38 ℃ or lower than 36 ℃ and microbiological confirmation. Also, chest infection was confirmed by radiology. All these parameters were recorded daily during the ICU admission before transplantation and the ten following days. The number of infected patients and episodes and pathogens microbiologically confirmed was registered, respectively.

ICU stay and immediate outcome

The mean stay in ICU during the first admission of patients after transplantation and immediate outcome (first admission in the hospital) were recorded.

Table 1 Clinical and demographic characteristics of the patients

Age (yr)	32.7±11
Sex (M/F)	26/37
Etiology (*n*, %)	
Viral hepatitis	18 (30)
Cryptogenic (non-A, non-B, non-C)	32 (52)
Drug toxicity	7 (11)
MAOi	3
Rifampin+Isoniacid	2
Isoflurane	1
α metil-dopa	1
Metabolic disease	4 (6)
Encephalopathy	
I-II (subacute form: 20 p)	20 (31.7)
III-IV (acute form:39 p) (hyperacute form: 4 p.)	43 (68)

Statistical analysis

Statistical analysis was performed using two-sided paired *t*-test for comparison of paired data and two-sided unpaired *t*-test for comparison of groups. The Bonferroni correction test was applied as appropriate. Categorical data were compared with the chi-square test for relationship between encephalopathy and infection. Correlation between lactate and the hemodynamic and oxygenation variables was assessed by linear regression analysis. Variables reaching significance in the univariate analysis between survivors and non-survivors were included in the multivariate analysis. Multivariate analysis was carried out by stepwise logistic regression analysis to determine discriminants of survival. All values were expressed as mean±SD. $P<0.05$ was considered statistically significant.

RESULTS

Demographic and clinical characteristics of the patients are shown in Table 1. No patient needed blood transfusion before the transplant.

Systemic hemodynamic and oxygenation parameters

Systemic hemodynamic parameters showed a hyperdynamic circulatory status with high CI (4.53±1.4 L/min/m^2) and low SVRI (1029±420 dyn.sec.cm^{-5}.m^2). Oxygenation parameters remained between normal ranges (DO_2 623±36 mL/min/m^2, VO_2 96.4±21 mL/min/m^2 and VO_2/DO_2 18.4±3.1%).

Blood lactate levels

The production of lactate in the intestine or in the spleen (Table 2), in accordance with Murphy *et al*[9], was suggested by the increased difference between portal, arterial and mixed venous lactate ($P<0.05$). Acute ALF showed significant higher levels (acute: 5.4±1 mmol/L, subacute: 2.2±0.6 mmol/L, $P=0.01$).

Lactate levels did not correlate with any of the hemodynamic or oxygenation parameters studied, except for the respiratory quotient (Figure 1 A). The grade of encephalopathy and the amount of glucose administered 48 hours before transplant (Figure 1 B and Figure 1 C) correlated well with blood lactate levels.

Table 2 Plasma lactate levels at different sites (mean±SD)

	Total (*n* = 52)	Acute (*n* = 36)	Subacute (*n* = 16)	*P*
La (mmol/L)	4.1±1.8 (1.1-15.2)	5.4±1	2.2±0.6	0.01
Lv (mmol/L)	4.3±2 (1.4-16.3)	4.7±2	2.4±1.4	0.03
Lp (mmol/L)	5.3±1.1a (2.1-17.6)	6.8±1.8a	2.9±0.9	<0.01
pH (units)	7.36±0.07 (7.28-7.42)	7.33±0.02	7.36±0.04	NS
VA_{CO2}(mmHg)	12.4±7 (8.3-13.4)	13.8±5	9.6±9	NS
AvpH (units)	0.06±0.03 (0.02-0.07)	0.08±0.02	0.07±0.04	NS

La: arterial lactate; Lv: mixed venous lactate; Lp: portal vein lactate; L(a-p): transplanchnic arterial/portal difference of lactate; $V_{A\ CO2}$: portal/arterial gradient of CO^2; AV_{pH}: arterial/portal gradient of pH; $^aP<0.05$ *vs* La, Lv and Lp.

Splanchnic oxygenation parameters

The splanchnic VA_{CO2} and AV_{pH} levels were in normal range and did not correlate with plasma lactate levels ($r=0.203$ and $r=0.164$, respectively). No differences were found (Table 2) when we compared patients with high (acute ALF) and low lactate levels (subacute ALF).

In order to maintain hemodynamic stability, only five patients with acute ALF needed administration of dopamine prior to the transplant. Only one of the patients demonstrated a high level of lactate (8.2 mmol/L). There was no significant correlation between lactate levels and the dosages of dopamine and epinephrine ($r=0.020$ and $r=0.13$ respectively) through the transplant. The total dose of epinephrine administered after graft reperfusion in patients with low (subacute ALF) and high lactate level (acute ALF) was similar in both groups (42.6±12 μg and 54.1±18 μg, respectively).

Explanted liver pathology

No relationship was found between lactate levels and liver weight. Interestingly, although the rate of massive necrosis was similar, liver weight of subacute ALF patients was significantly lower than that of acute ALF patients (762±22 g and 932±38 g, $P<0.05$).

Infectious complications

Twenty-one patients (33.3%) were infected in the perioperative period. Bacterial infection was found in 16 patients (25.4%), fungal infection in 5 patients (7.9%) and viral infection in 2 patients (3.1%). The incidence of infectious episodes of acute (13 patients, 30.2%) and subacute (8 patients, 40%) hepatic failure showed no difference. The level of lactic acidosis (Figure 2) no correlated with the presence of infection ($P=0.1$). Sixteen patients were infected before the transplant, 11 of them requiring mechanical ventilation because of pulmonary infection (confirmed on chest radiograph and microbiologically), showed significantly higher arterial lactate levels compared with mixed venous lactate level (5.2±1.1 and 4.5±0.8 mmol/L, $P=0.03$), suggesting lactate production within the lungs.

ICU stay and immediate outcome of patients with ALF

During the stay in ICU, 13 patients died (8 patients with acute and 5 subacute liver failure). Bleeding was not controlled in 2 patients. Furthermore, 3 patients needed

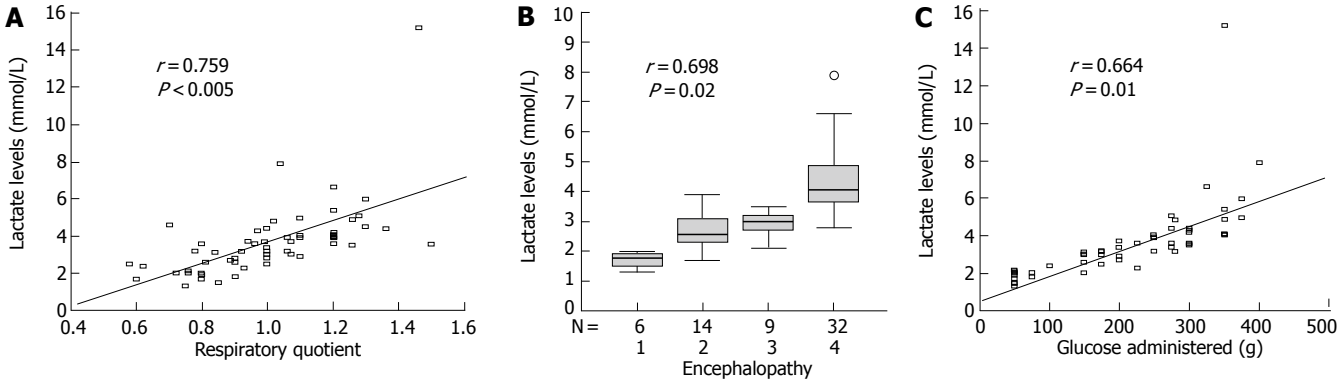

Figure 1 Correlation between arterial lactate level (nmol/L) and respiratory quotient ($n = 61$) (**A**), arterial lactate level (nmol/L) in patients with different grade of encephalopathy ($n = 61$) (**B**), correlation between arterial lactate level (nmol/L) and total amount of glucose (g) administered 48 hours before the transplant (**C**).

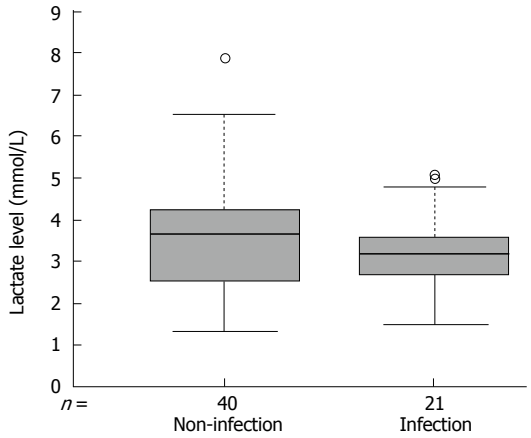

Figure 2 Arterial lactate levels in 21 infected and 40 non-infected patients. Box plot indicates that there is no significant difference between the infected and non-infected patients.

retransplantation because the primary graft did not function and one of them died after retransplantation because of infection complications.

The mean stay in ICU was 14.6 days (range 4 - 38 d). The ICU stay correlated well with infection complications ($P < 0.01$) but not with plasma lactate levels. The mean plasma lactate level was similar in nonsurvivors and survivors (4.3 ± 0.7 mmol/L and 3.9 ± 10 mmol/L, respectively, ($P = 0.69$). Univariate analysis showed that infectious complications (71.4% *vs* 22.4%, $P = 0.02$) and subacute ALF (30% *vs* 18.6%, $P = 0.08$) were the potential risk of non-survivors compared to survivors. Multiple logistic regression revealed that the presence of infection was the only discriminator of survival. Encephalopathy grade and splanchnic VACO2 and AVpH did not significantly improve the prediction of immediate survival.

DISCUSSION

Hyperlactatemia observed in the context of non-acetaminophen ALF seemed to be related to aerobic glycolysis but not to tissue hypoperfusion. Additionally, the incidence of infection in these patients, which appeared as an independent predictor of survival, did not correlate with the degree of hyperlactatemia.

It is important to emphasize that the foremost cause of ALF in our series was acute viral hepatitis that differs substantially in other series. Since acetaminophen exerts a direct toxic effect on cellular respiration, this type of cytotoxicity may be different from other types of ALF.

Currently, the presence of hyperlactatemia in patients with ALF is assumed as the consequence of underlying overt tissue hypoxia. In these patients, oxygen delivery may be impaired. Combined measurement of VACO2 and AVpH may serve as a good indicator of tissue hypoxia with a closer relation to cardiac output than blood lactate, because it is less affected by changes in fuel substrate utilization or enzymatic alterations [10-12]. In our study no differences were found in splanchnic VACO2 and AVpH between patients with high (acute ALF) and low lactate levels (subacute ALF).

If hyperlactatemia is attributable to tissue hypoperfusion, the increase in oxygen delivery should reduce lactate levels. However, several reports[13-15] have failed to identify this evidence in patients with ALF due to acetaminophen overdose.

In our patients selected to receive liver transplantation, plasma lactate levels did not correlate with hemodynamic or oxygenation parameters, except for RQ and the amount of glucose administered, suggesting that aerobic glycolysis is responsible of lactate hyperproduction. Since patients with ALF may exhibit hyperinsulinemia due to pancreatic hypersecretion and/or decreased hepatic clearance of insulin, they need glucose infusion in order to maintain blood glucose level. Previous studies[16-18] showed that infusion of glucose results in a dose-dependent rise in splanchnic lactate levels. Moreover, peripheral tissues of cirrhotic patients produce an exaggerated lactate production in response to glucose administration[19]. In our study, 7 patients who did not require glucose administration (subacute liver failure), showed normal lactate levels (<1.3 mmol/L). A complete round of the Cori cycle is proton-neutral because the H+ produced by lactate from glucose is subsequently removed during synthesis of glucose from lactate in the liver. In the context of liver failure, the possibility to handle lactate to glucose (gluconeogenesis) is handicapped and consequently the possibility to develop "lactic acidosis" without the presence of high muscular lactate overproduction is not significant. In our patients the arterial blood pH stayed within normal ranges (7.28 to 7.41). If hyperlactatemia is not related

to the mortality, infection, or operative hemodynamic management difficulty, the usage of dichloroacetate (which acts by stimulating pyruvate dehydrogenase) is not a priority unless its benefit to avoid postoperative severe alkalosis (lactate metabolism by the graft consume H^+) is considered. However, it seems that this treatment fails to attenuate metabolic alkalosis after transplant[20].

Clemmesen *et al*[5] have suggested a hypermetabolic condition secondary to an excessively high glycolysis in relation to the small liver mass, but we have not shown any correlation between liver weight and splanchnic lactate level in patients with ALF. Wendon *et al*[21] demonstrated that there is a significant correlation between arterial lactate and survival in acetaminophen related ALF, but in our study no relationship was found between plasma lactate and outcome. Inadequate splanchnic blood flow and tissue hypoperfusion could contribute to bacterial translocation and sepsis, however in these patients several factors can predispose to infection such as comatose state, steroid therapy, mechanical ventilation and intravenous catheters. Patients with ALF are susceptible to infection as a result of multiple immunologic defects (excessive cytokine production from cells, such as monocytes and macrophages in response to a number of stimuli including bacterial lipopolysaccharide)[22]. In our patients the Gram-positive organisms were predominant. The presence of Gram-negative organisms such as pseudomonas was not uncommon, showing a high incidence in this group of patients. These results suggest that selective bowel decontamination can be used in the treatment of such patients[23]. We did not find any correlation between lactate levels and infection incidence although there is evidence that cytokines may promote augmented production of lactate[24] by several organs, enhancing cellular glucose uptake and glycolytic metabolism. However, in agreement with other studies[25], arterial lactate is higher than venous mixed lactate in patients with pulmonary infection.

In summary, the presence of high plasma lactate levels in patients with non-acetaminophen ALF, is not related to tissue hypoperfusion and is not a prognostic factor in the treatment of ALF.

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RAPID COMMUNICATION

Clinical features of hepatopulmonary syndrome in cirrhotic patients

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Abstract

AIM: To evaluate the frequency, clinical and paraclinical features of hepatopulmonary syndrome (HPS) and to determine their predictive values in diagnosis of this syndrome in patients in Iran.

METHODS: Fifty four cirrhotic patients underwent contrast enhanced echocardiography to detect intrapulmonary and intracardiac shunts by two cardiologists. Arterial blood oxygen, O_2 gradient (A-a) and orthodoxy were measured by arterial blood gas (ABG) test. The patients positive for diagnostic criteria of HPS were defined as clinical HPS cases and those manifesting the intrapulmonary arterial dilation but no other criteria (arterial blood hypoxemia) were defined as lHPS cases. HPS frequency, sensitivity, positive and negative predictive values of clinical and paraclinical features were studied.

RESULTS: Ten (18.5%) and seven (13%) cases had clinical and subclinical HPS, respectively. The most common etiology was hepatitis B. Dyspnea (100%) and cyanosis (90%) were the most prevalent clinical features. Dyspnea and clubbing were the most sensitive and specific clinical features respectively. No significant relationship was found between HPS and splenomegaly, ascites, edema, jaundice, oliguria, and collateral veins. HPS was more prevalent in hepatitis B. $PaO_2 < 70$ and arterial-alveolar gradient had the highest sensitivity in HPS patients. Orthodoxy specificity was 100%.

CONCLUSION: Clubbing with positive predictive value (PPV) of 75% and dyspnea with negative predictive value (NPV) of 75% are the best clinical factors in diagnosis of HPS syndrome. $PaO_2 < 70$ and P (A-a) $O_2 > 30$ and their sum, are the most valuable negative and positive predictive values in HPS patients.

Key words: Hepatopulmonary syndrome; Cirrhosis; Contrast enhanced echocardiography

Mohammad Alizadeh AH, Fatemi SR, Mirzaee V, Khoshbaten M, Talebipour B, Sharifian A, Khoram Z, Haj-sheikh-oleslami F, Gholamreza-shirazi M, Zali MR. Clinical features of hepatopulmonary syndrome in cirrhotic patients. *World J Gastroenterol* 2006; 12(12): 1954-1956

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INTRODUCTION

Ascites by elevating the diaphragm and confounding the ventilation/perfusion might lead to mild hypoxemia in most patients due to chronic hepatic involvement, not regarding the etiology. When cirrhotic patients have no sign of any cardiovascular diseases, severe hypoxemia($PO_2 < 60$mmHg) strongly recommends hepatopulmonary syndrome[1-3]. Hepatopulmonary syndrome is one of the pulmonary complications of cirrhosis which affects the treatment and disease prognosis and is a factor for arterial blood oxygen reduction. The diagnosis of this syndrome is confirmed by presence of cirrhosis in liver biopsy, absence of cardiovascular diseases, arterial blood oxygen reduction found in arterial blood gas (ABG) tests and pulmonary vein dilation in imaging[4]. Many studies have been performed on evaluating the prevalence, etiology, clinical features, early diagnosis, treatment and prognosis of this syndrome worldwide. The aim of this study was to evaluate the clinical and paraclinical characteristics and their predictive values in diagnosis of this syndrome.

MATERIALS AND METHODS

This study was performed in 54 randomly chosen cirrhotic patients referred to Gasteroenterology Department of Taleghani Hospital in 2004. In the patients who entered the study, cirrhosis was confirmed by biopsy, clinical and paraclinical evaluations. Echocardiography and pulmonary function tests were done for all patients and plain chest x-ray was taken. Those with cardiovascular and known respiratory diseases were excluded from this study. The patients with ascites underwent large volume paracentesis. These 54 patients underwent contrast enhanced echocardiography performed by two cardiologists from Cardiovascular Department of Talaghani Hospital. The procedure was performed by injecting agitated saline into patient's right hand cubital vein. Left and right sides of the heart were evaluated by echo after 5 beats. Presence of opacity after 5 beats in the left heart was determined as intrapulmonary shunt. If opacity was present immediately after injection, it was a sign of intracardiac shunt. ABG test was performed in patients at supine. After one hour in vertical position, the oxygenation saturation, arterial blood oxygen, (A-a) O_2 gradient and orthodoxy were evaluated. Physical examination was performed to detect clinical features including clubbing in fingers and toes, central and peripheral cyanosis, presence of spider angioma, telangiectasia, jaundice, collateral veins in abdomen, ascites, consciousness, splenomegaly, dyspnea, peripheral edema, palmar erythema, oliguria or anuria and pleural effusion for the underlying etiology. All patients were tested for hepatitis B, hepatitis C, biliary, autoimmune, metabolic, cardiac, alcoholic and idiopathic etiologies. Complete blood count (CBC), liver function test (LFT), creatinine, prothrombin time (PT), partial thromboplastin time (PTT), albumin and other routine tests were measured in all patients. Ascitic fluid was tested for protein, albumin and white blood cells.

The patients presenting the three diagnostic criteria of hepatopulmonary syndrome, including hepatic cirrhosis, arterial blood deoxygenation ($PO_2 < 80$ mmHg) and intrapulmonary arterial dilation were defined as clinical hepatopulmonary cases. Those presenting intrapulmonary arterial dilation but no other two criteria (arterial blood hypoxemia) were defined as subclinical hepatopulmonary cases. The study was carried out in accordance with the Helsinki Declaration and approved by the Ethics Committee of the Research Center for Gastroenterology and Liver Disease, Shaheed Beheshti University of Medical Sciences. The data were presented by descriptive statistics. The variables were compared by χ^2 test. Sensitivity, specificity, positive and negative predictive values of clinical and paraclinical features in diagnosis of hepatopulmonary syndrome were evaluated. $P < 0.05$ was considered statistically significant.

RESULTS

Among the 54 patients who participated in the study, 10(18.5%) met the clinical hepatopulmonary syndrome criteria and 7(13%) with intrapulmonary arterial dilation (but no other criteria) were defined as subclinical hepatopulmonary cases. Most frequent age group was 71-80 years. Figure 1 shows the age distribution. The most common etiology was HBV, but there was no significant relation. Figure 2 shows the prevalence of HPS etiologies.

Table 1 Characteristics and diagnostic values of signs and symptoms in hepatopulmonary syndrome

Symptoms and signs	Frequency in HPS (%)		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	*P*
	Pos	Neg					
Cyanosis	90	15.9	90	80.1	60	97	<0.0001
Clubbing	80	9.1	80	90.9	75	95	<0.0001
Dyspnea	100	25	100	75	50	100	<0.0001
Palmar erythema	80	29.5	80	70.5	38	94	0.003
Spider angioma	80	29.5	80	70.5	38	94	0.005

HPS: hepatopulmonary syndrome, PPV: positive predictive value, NPV: negative predictive value.

Table 2 Child class in hepatopulmonary syndrome and subclinical patients

Child classification	HPS positive	HPS negative	Total
Class A	0	5	5
Class B	2	27	29
Class C	8	12	20
Total	10	44	54

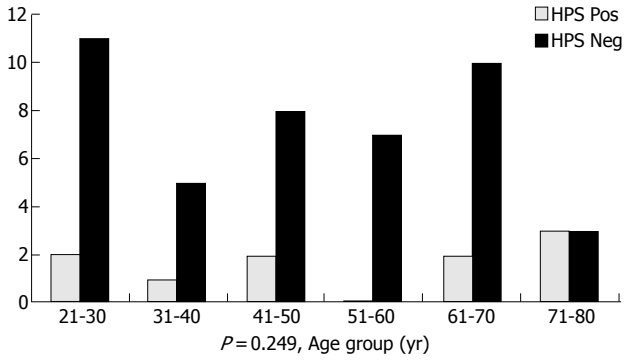

Figure 1 Age distribution in patients with hepatopulmonary syndrome.

By paying attention to the frequency of clinical features in HPS patients, dyspnea (100%) and cyanosis (90%) were the most prevalent. Dyspnea and clubbing were the most sensitive and specific clinical features. Table 1 presents the characteristics and diagnostic values of signs and symptoms in HPS patients.

No significant relation was found between splenomegaly, ascites, edema, jaundice, oliguria, collateral veins and hepatopulmonary syndrome. Table 2 presents the patients with or without hepatopulmonary syndrome. HPS was more common in class C. Table 3 presents the diagnostic value of arterial blood gas in HPS. PaO_2 and arterial–alveolar oxygen gradients were most sensitive in diagnosis of HPS. Orthodoxy specificity was 100%.

DISCUSSION

Hepatopulmonary syndrome includes the triad of liver

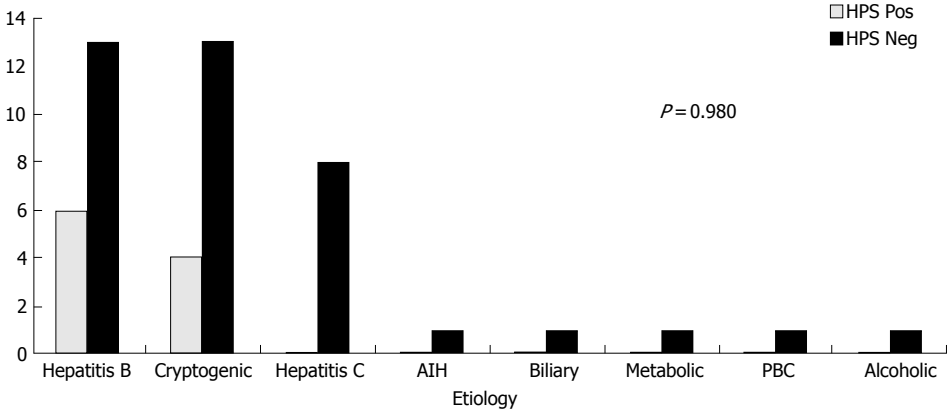

Figure 2 Causes of liver disease in patients with hepatopulmonary syndrome.

Table 3 Diagnostic value of arterial blood gases in hepatopulmonary syndrome

Laboratory results	Frequency (%)		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	*P*
	Pos	Neg					
$Pa0_2$<70 and (A-a)0_2>30	100	4.5	100	92	85	100	<0.0001
$Pa0_2$<70	100	4.5	100	95	83	100	<0.0001
$a0_2$<65	70	4.5	70	98	77	95	<0.0001
$Pa0_2$<60	30	4.5	30	92	66	87	0.039
(A-a)0_2>15	100	75	100	25	23	10	0.1
(A-a)0_2>20	100	63.6	100	36	26	100	0.24
(A-a)0_2>30	100	31.8	100	70	88	89	0.001
(A-a)0_2>40	90	11.4	90	88	65	75	<0.0001
Orthodoxy>10	60	0	60	100	15	93	<0.001

disease, arterial blood deoxygenation and pulmonary vein dilation. Although the mortality of this syndrome is high, its influence on patient survival is unknown. In our study, the prevalence of hepatopulmonary syndrome was 18.5% and the prevalence of pulmonary vein dilation was 13%. Our findings are compatible with those of other studies in this field[1-5].

In our study, age and Child class C were significantly associated with HPS, suggesting that this syndrome is related with development of cirrhosis. Studies have shown a relation between HPS and cyanosis, clubbing and orthodoxy, although spider angioma is significantly related to intrapulmonary vein dilation[1]. The same results were observed in our study also. Cyanosis, clubbing and orthodoxy had positive and negative predictive values of 75% and 100% in hepatopulmonary syndrome.

Previous studies showed that the most common underlying etiologies of HPS were cryptogenic cirrhosis and cirrhosis due to hepatitis B[3,4]. The reported positive predictive values were 37% and 53% for (A-a) O_2 gradients and 93% and 94% for PaO_2[3,4]. Orthodoxy has been reported to be 88%, but in our study it was 66% and no significant statistical relation was found[6-7]. These clinical results strongly lead to diagnosis of HPS and the above tests can be used in screening patients.

In conclusion, hepatopulmonary syndrome and intrapulmonary vein dilation are relatively frequent in patients with portal hypertension. Clubbing with the highest positive predictive value (75%) and dyspnea with the highest negative predictive value (100%) are the best clinical features in HPS patients. Further studies are needed to confirm our results.

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RAPID COMMUNICATION

Seroprevalence of *Helicobacter pylori* in dyspeptic patients and its relationship with HIV infection, ABO blood groups and life style in a university hospital, Northwest Ethiopia

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Abstract

AIM: To determine the prevalence of *Helicobacter pylori* (*H pylori*) among dyspeptic patients and to assess the relationship between *H pylori* infection, blood group, HIV infection and life style of the patients.

METHODS: In a hospital-based cross-sectional study, patients attending Outpatient Department of University of Gondar Hospital were enrolled. Socio-demographic information was collected using questionnaires. Serum was analyzed for anti-*H pylori* IgG antibodies using a commercial kit. HIV serostatus was determined by enzyme-linked immunosorbent assay (ELISA). Blood grouping was performed by slide agglutination tests.

RESULTS: A total of 215 dyspeptic patients were included in the study. One hundred and sixteen patients (54%) were females and 99 (46%) were males. Anti-*H pylori* IgG antibodies were detected in sera of 184 (85.6%) patients. The prevalence was significantly higher in patients aged 50 years and above. Twenty point five percent of the patients were found to be seropositive for HIV. No significant association was found between sex, ABO blood groups, consumption of spicy diets, socio-economic status and seropositivity for *H pylori*. However, alcohol consumption was significantly associated with *H pylori* serology.

CONCLUSION: The prevalence of *H pylori* infection is associated with a history of alcohol intake and older age. The effect of different diet, alcohol and socioeconomic status as risk factors for *H pylori* infection needs further study.

Key words: *H pylori*; Blood group; HIV; Life style

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INTRODUCTION

Helicobacter pylori (*H pylori*), a flagellate Gram negative rod, infects over half of the world's population[1] and plays a pivotal role in the etiology of a number of gastroduodenal diseases and development of gastric malignancy[2-4]. *H pylori* has been recognized as grade one carcinogen[5] and the National Institute of Health Consensus Development Conference Statement recommended that all patients who are found to have gastric or duodenal ulceration and concurrent *H pylori* infection should receive treatment aimed at eradicating the bacterium[6].

Low socio-economic status, low level of education, consumption of alcohol, gender and occupation are the risk factors for *H pylori* infection[7]. In addition, blood group antigens, which mediate attachment of *H pylori* to gastric mucosa, are associated with a risk of developing peptic ulcer and gastric cancer[1]. About 95% of patients with duodenal ulcers and perhaps 80% of patients with peptic ulcers are infected with this bacterium and its eradication greatly diminishes the recurrence of these ulcers[8]. However, there have been very few studies on the prevalence of *H pylori* in Ethiopian patients[9,10]. Therefore,

Table 1 Association between socio-demographic characteristics and seropositivity for antibodies against H. pylori infection in dyspeptic patients (Northwest Ethiopia, 2003)

Characteristics	*n*	Serum anti-HP+ [*n* (%)]	Serum anti-HP- [*n* (%)]	OR (95% CI)	*P* value
Sex					0.2
Male	99	88 (88.9)	11 (11.1)	1	
Female	116	96 (82.8)	20 (17.2)	0.6 (0.272,1.32)	
Age group (yr)					0.2
<20	22	18 (81.8)	4 (18.2)	1	
20-29	62	51 (82.3)	11 (17.7)	1.03 (0.29, 3.65)	
30-39	58	47 (81.0)	11 (19.0)	0.95 (0.27, 3.37)	
40-49	32	27 (84.4)	5 (15.6)	1.20 (0.28, 5.08)	
50-59	27	27 (100.0)	0 (0.0)	2205.4 (0.0, 4.7E+19)	
60-69	10	10 (100.0)	0 (0.0)	2205.4 (0.0, 1.5E+3)	
70-79	4	4 (100.0)	0 (0.0)	2205.4 (0.0, 5.6E+45)	
Occupation					0.69
Farmer	114	97 (85.1)	17 (14.9)	1	
House wife	14	12 (85.7)	2 (14.3)	1.05 (0.22,5.12)	
Govt employee	20	18 (90)	2 (10.0)	1.57 (0.34, 7.43)	
Student	29	24 (82.8)	5 (17.2)	0.84 (0.28, 2.51)	
Unemployed	14	14 (100.0)	0 (0.0)	639.67 (0.0, 3.6E+39)	
Merchant	6	5 (83.3)	1 (16.7)	0.87 (0.09, 7.97)	
Soldier	2	2 (100.0)	0 (0.0)	639.67 (0.0, 1.5E+39)	
Others	16	12 (75.0)	4 (25.0)	0.52 (0.15, 1.82)	
Address					
Rural	127	109 (85.8)	18 (14.2)	1	
Urban	88	75 (85.2)	13 (14.8)	0.95 (0.44, 2.06)	
Marital status					0.7
Married	135	117 (86.7)	13	1	
Widowed	13	11 (84.6)	2 (15.4)	0.85 (0.17, 4.13)	
Divorced	22	18 (81.8)	4 (18.2)	0.69 (0.21, 2.28)	
Single	39	32 (82.1)	7 (17.9)	0.70 (0.27, 1.83)	
Underage	6	6(100.0)	0(0.0)	206.42 (0.0, 1.1E+15)	

HP+= Helicobacter pylori positive ; *HP- = Helicobacter pylori* negative.

the aim of this study was to determine the prevalence of *H pylori* among dyspeptic patients and to assess the relationship between *H pylori* infection, blood group, HIV infection and life style of patients attending Gondar University Hospital in Northwest Ethiopia.

MATERIALS AND METHODS

The study was conducted at the Hospital of Gondar University, Northwest Ethiopia, from February 2003 to June 2003. Patients who presented with complaints of dyspepsia were included in the study. Institutional ethical clearance was obtained from the Research and Publication Committee of Gondar University.

Socio-demographic and relevant clinical information was collected by a questionnaire. A senior internist examined the patients and filled the questionnaires. The variables included sex, age, ethnicity, smoking, stress, nutrition, alcohol consumption, exercise and socio-economic factors. After informed consent was obtained from each patient and appropriate pre-test counseling was provided about 5mL of venous blood was collected in vacutainer tubes and sera were separated by centrifugation immediately after clotting. Serological status of *H pylori* infection was tested with commercial *H pylori* serology kits (HEXAGON H.PYLORI, Germany) following instructions of the manufacturer. The kit was used for the detection of *H pylori* IgG antibodies in serum. It could detect anti-*H pylori* antibodies with a highsensitivity (97%) and specificity (95%) when compared with ELISA (HEXAGON H.PYLORI, Germany). Blood grouping was performed by slide agglutination test using monoclonal anti-A, anti-B, anti-AB and anti-D (Rho) antibodies. Testing for the presence of HIV antibodies was determined by ELISA following the manufacturer's instructions (Vironostica HIV Uni-Form II plus O, Organon Teknika, Boxtel, The Netherlands). HIV serology test was carried out anonymously with all clinical and laboratory data identified by a code number. Data entry and analysis were performed using SPSS version 10 statistical package. χ^2 test was used to compare the categorical data and logistics regression was used to avoid the confounder effect and to calculate the risk ratio. Odds ratio (OR) and 95% confidence interval (CI) were used to measure the strength of association. $P<0.05$ was considered statistically significant.

RESULTS

A total of 215 dyspeptic patients were included in the study. Table 1 shows the socio-demographic characteristics of the study population in relation with *H pylori* serostatus. The mean age of the subjects was 35 years (range 14-74 years). A substantial majority (55.8%) of the patients were young adults aged 20-40 years. Females constituted 54% of the patients. Fifty three percent of the patients were farmers followed by students (13.5%). Regarding the marital status, 62.8% were married, 18.1% were singles and 10.2% were divorced. Fifty nine point one percent of the

Table 2 *H pylori* and HIV co-infection in dyspeptic patients attending Gondar University Hospital (Northwest Ethiopia, 2003)

	H pylori		Total	Statistics
	Seropositive *n* (%)	Seronegative *n* (%)	*n* (%)	
HIV seropositive	36 (19.6)	8 (25.8)	44 (20.5)	$P=0.4$
HIV seronegative	148 (80.4)	23 (74.2)	171 (79.5)	$\chi^2=0.63$

Table 3 Demographic factors for HIV infection in dyspeptic patients attending Gondar University Hospital (Northwest Ethiopia, 2003)

Characteristics	HIV serostatus		*P*
	Seropositive *n* (%)	Seronegative *n* (%)	
Male	19 (19.2)	80 (80.8)	$P=0.6$
Sex			OR= 0.87 (95% CI 0.42-1.77)
Female	25 (21.6)	91 (78.4)	$\chi^2=0.18$
Age group (yr)			
10-29	21 (25.3)	62 (74.7)	$P=0.3$
30-49	17 (18.7)	74 (81.3)	$\chi^2=2.23$
⩾ 50	6 (14.6)	35 (85.4)	$\gamma=2$
Residence			$P<0.01$
Rural	19 (15.0)	108 (85.0)	OR=0.45 (95% CI 0.21-0.92)
Urban	25 (28.4)	63 (71.6)	$\chi^2=5.78$
Occupation			
Farmer	18 (15.8)	96 (84.2)	$P<0.001$
Housewife	3 (21.4)	11 (78.6)	$\chi^2=24.78$
Gov't employee	2 (10.0)	18 (90.0)	$\gamma=7$
Students	4 (13.8)	25 (86.2)	
Unemployed	8 (57.1)	6 (42.9)	
Merchant	4 (66.7)	2 (33.3)	
Soldier	0	2 (100)	
Others	5 (31.3)	11 (68.7)	

patients came from Gondar town and the remaining 48.1% came from rural areas.

Among the patients, 184 (85.6%) were found to be positive for anti-*H pylori* antibodies. The sex specific prevalence in males was 88.9% which was not significantly different from that of females (82.8%). The prevalence of *H pylori* was significantly higher in patients aged 50 years and above (100%) when compared to patients aged < 50 years (82.2%) ($\chi^2=8.54$, $\gamma=1$, $P<0.05$). *H pylori* seroprevalence was not associated with residence, marital status, occupation and ethnic group of the patients ($P>0.05$) (Table 1).

The prevalence of HIV among the patients was about 20.5% (44/215). The HIV seropositivity was not associated with *H pylori* seropositivity as only 19.6% (36/184) of the patients who were positive for *H pylori* were also positive for HIV while 25.8% (8/31) patients who were negative for *H pylori* were positive for HIV (Table 2). Analysis of demographic factors for HIV infection showed a significant association by residence ($\chi^2=5.78$, $P<0.01$) and occupation ($\chi^2=24.78$, $\gamma=7$, $P<0.001$) of the dyspeptic patients (Table 3)

Analysis of blood group showed that 201 (93.5%) of the total subjects were rhesus positive (Rh+) and 14 (6.5%) were rhesus negative (Rh-). Among the total subjects blood group O was the most common blood group (43.3%) in the patients followed by blood groups B (28.4%), A (22.3%) and AB (6.0%), respectively. However, no statistically significant association was seen between *H pylori* infection and blood group of the patients (Table 4).

Table 4 Relationship between ABO blood groups and seropositivity of antibodies against *Helicobacter pylori* infection

Blood group	Serum anti HP+ [*n* (%)]	Serum anti HP- [*n* (%)]
O	76 (84.4)	14 (15.6)
B	59 (88.1)	8 (11.9)
A	41 (85.4)	7 (14.6)
AB	8 (80)	2 (20)
Total	184 (85.6)	31 (14.4)

Table 5 Relationship between *Helicobacter pylori* infection and life style in dyspeptic patients attending Gondar University Hospital (Northwest Ethiopia, 2003)

		Serum anti-HP+ *n* (%)	Serum anti-HP- *n* (%)	*P* value
Alcohol intake	Yes	128 (90.1)	14 (9.9)	<0.01
	No	56 (76.7)	17 (23.3)	$\chi^2=7.05$
Diets	Coffee	24 (82.8)	5 (17.2)	$\gamma=1$
	Spicy food	108 (89.3)	13 (10.7)	=0.2
	Others	52 (80)	13 (20)	$\chi^2=3.15$
				$\gamma=2$

History of diet, alcohol consumption and socio-economic status were obtained from the patients. It was observed that seropositivity for anti-*H pylori* antibodies was significantly associated with history of alcohol consumption (OR=2.78, 95% CI: 1.19-6.5, $P<0.01$) (Table 5). Although there was a higher prevalence of *H pylori* infection in those with low socioeconomic status than in those having average income, the association was not statistically significant. Likewise, no statistically significant interaction was observed in the prevalence of *H pylori* infection and consumption of coffee and spicy foods (Table 5).

DISCUSSION

The prevalence of *H pylori* IgG antibody among dyspeptic patients was 85.6%. This finding is higher than an earlier report from 136 patients with non ulcer dyspepsia from Addis Ababa, showing a prevalence of 65% for *H pylori*[9]. However it is comparable with a recent report from blood donors in Addis Ababa, where a seroprevalence of 89% has been observed[10]. When compared to studies from other countries like England, France, Scandinavia, Italy, Belgium and USA, the seroprevalence found in this study is much higher[11]. This may be explained by the association between *H pylori* and low socioeconomic status as evidenced by unsafe drinking water[12] and other factors like low educational level which would have an impact on personal hygiene and environmental sanitation[13].

Prevalence of *H pylori* increases with age. It is interesting to note that in patients aged 50 years and above its prevalence is 100%. Similar results have been reported in

other studies indicating the high frequency of infection in the elderly[13]. This tendency is believed to be attributable to the environmental factors specific to these age group rather than aging[13]. The lack of significant association between sex, residence of patients and *H pylori* is in line with previous reports from elsewhere[14,15].

In the present study, more than one fifth of the patients (20.5%) were found to be seropositive for HIV and the seroprevalence of HIV in *H pylori*-infected patients was also very high (19.6%). This high prevalence of HIV in dyspeptic patients reflects the severe magnitude of HIV infection in the general population in Northwest Ethiopia. Recent studies showed that 51.4% tuberculosis patients[16] and 5% cataract patients[17] were seropositive for HIV in the region substantiating our present observation. Co-infection with HIV can debilitate the defense mechanism of patients and increase morbidity and mortality.

Blood group O is associated with duodenal ulcer disease, while gastric ulcer and gastric carcinoma are associated with blood group A[18]. Since the identification of *H pylori*, no pathogenic mechanism has been identified to support this earlier finding. Lewis B blood group antigen has recently been shown to function as a receptor for *H pylori* adhesins, mediating bacterial adherence to the gastric epithelial surface, which is essential for bacterial colonization[19]. Furthermore, substitution of the Lewis B antigen with blood group A and B determinants results in failure of *H pylori* binding[19]. Reduced exposure of the Lewis B epitope in persons of blood groups A and B could result in lower *H pylori* infection rates and a predominance of *H pylori* infection in persons of blood group O[20]. This is consistent with the reported association of blood group O with duodenal ulcer disease, but at variance with the association of blood group A with gastric ulcer and carcinoma, which is also related with *H pylori* infection. The present study did not demonstrate any significant difference in *H pylori* serological status of dyspeptic patients with varying blood groups, which is consistent with similar studies from other countries[18,20-22].

Histories of alcohol (local alcoholic drinks like "Tella", "Teji" "Araki" and beer) consumption appears to be a risk factor for *H pylori* infection, which is in line with study from Finland[23]. However, different results have been reported in other countries[24]. The reason for this contradictory result might be due to the difference in the type of alcoholic beverages consumed and the life time history of alcohol consumption. Socioeconomic status does not appear to be a risk factor for *H pylori* infection, although a higher proportion of patients with low income are found to be positive for *H pylori* antibody. This result is in line with reports form Zambia and United Kingdom[25,26]. Spicy foods and coffee are considered to be risk factors for *H pylori* infection. However, no statistically significant interaction was observed in the present study, but it has been reported in Japan[27].

In conclusion, the prevalence of *H pylori* infection is very high and associated with history of alcohol intake and older age. Different diet, alcohol and socioeconomic status as risk factors for *H pylori* infection need further study.

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RAPID COMMUNICATION

Effect of electroacupunture on gastric mucosal intestinal trefoil factor gene expression of stress-induced gastric mucosal injury in rats

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Abstract

AIM: To investigate electroacupunture(EA) at the acupoints of Stomach Meridian of Foot-Yangming(SMFY), Gallbladder Meridian of Foot-Yangming(SMFY) on gastric mucosal intestinal trefoil factor (ITF) gene expression detection in stress-induced rats with gastric mucosal lesion, and to explore the regulatory mechanism and significance of EA-related gastric mucosal protective effect.

METHODS: Forty rats were randomly divided into 4 groups: Blank group, Model group, Model group+EA at acupoints of SMFY group("SMFY group"), and Model group+EA at acupoints of GMFY group(GMFY group). All rats (except blank group) were made model by water immersion and restraint stress (WRS). Then the gastric mucosa tissue in each rat was taken off after assessment of gastric mucosal lesion index(GUI), and the expression of ITF mRNA of the tissues was detected by reverse transcription-polymerase chain reaction(RT-PCR) method.

RESULTS: Compared with Model group(54.3 ± 1.34), the GUI value in SMFY group (31 ± 2.21) decreased significantly($P < 0.01$), so did that in GMFY group (39.8 ± 1.62, $P < 0.05$), meanwhile GUI value in SMFY group was significantly lower than in GMFY group($P < 0.01$). Compared with Model group (0.65 ± 0.01), EA had a tendency to improve the expression of gastric mucosal ITFmRNA gene: such tendency existed in GMFY group (0.66 ± 0.01) but with no signficant difference($P > 0.05$), in SMFY group(0.76 ± 0.01) with an extremely obvious difference ($P < 0.01$), furthermore the expression in SMFY group was significantly higher than in GMFY group ($P < 0.01$).

CONCLUSION: The gastric mucosal protective effect by EA at the acupoints of SMFY and GMFY was related to the expression variance of ITF, indicating certain meridian specificity exists. It could be one proof for the TCM theory "Relative particularity between SMFY and stomach".

Key words: EA; Relative particularity between Stomach Meridian of Foot-Yangming; Gastric mucosal damage; Stress; Intestinal trefoil factor; Gene expression

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INTRODUCTION

Acupuncture is one of the traditional Chinese medicine(TCM) therapeutic techniques that can be traced back at least 2500 years, gaining popularity in the West as an alternative and complementary therapeutic intervention[1-4]; while "Relative particularity between Foot Yangming meridian and stomach", is one of important TCM theories. Such theory was proved by our previous research results[5-7]: acupuncture at acupoints of Sibai (ST 2), Liangmen (ST 21), and Zusanli (ST36), could regulate gastric motion and gastric secretion. Recently, a group of new peptides have been discovered, called trefoil factor family (TFF) or trefoil peptides because of their uniquely distinctive cysteine-rich "three-leaf" secondary structure[8], which probably protects these peptides from the degradation by luminal acid and proteases within the gastrointestinal tract[9]. Intestinal trefoil factor (ITF) belongs to the growing family of trefoil peptides[10]. Most of the researches to date have revealed important roles for ITF in protection and repair against injury to the gastrointestinal mucosa[11-14].

To our knowledge, there existed still no evidence of ITF gene expression in stress-induced gastric mucosal lesion in rats by the treatment of acupuncture. In our present study, ITFmRNA was detected to probe gastric

mucosal protective mechanism of the factor, and to prove the classical TCM theory "Relative particularity between Stomach Meridian of Foot-Yangming(SMFY) and stomach".

MATERIALS AND METHODS

Reagents

Trizol reagent was obtained from Invitrogen Co. (USA). One tube RT-PCR kit was from Promega Co. (USA). Primers for rat ITF and GAPDH were designed by ourselves in accordance with gene sequence in GenBank, synthesized and purified by Gibco BRL Biological Engineering Co. All other reagents were analytically pure.

Animals

Forty spraque-dawley rats weighing 180-250 g, male and female mixture, were used. They were housed three to four per cage at temperature 22 ℃ ± 2 ℃ with a 12/12 h light/dark (0:00 a.m. to 20:00 p.m.) cycle under controlled environment. Rats were fed standard laboratory chow, and water was given *ad libitum*. The rats were kept for 7 d in laboratory for habituation. This study was carried out according to the protocol approved by the Ethical Committee of Hunan University of Traditional Chinese Medicine, Changsha, China.

Animal groups and model

All rats were randomly divided into 4 groups(ten rats in each). The design of the experimental animal groups is shown in Table 1.

Table 1 Design of experimental animal groups

Group/code	Treatment
Group A: Blank group	Untreated rats as normal control group
Group B: Model group	having no EA or other treatment for 7 d, then WRS rat model was established.
Group C: SMFY group	After EA at points of SMFY for 7 d, WRS rat model was established
Group D: GMFY group	After EA at points of GMFY for 7 d, WRS rat model was established

Acupuncture method

Acupoints location was defined by reference of rat-acupoint-atlas[15] and analogy to human body. According to the induction stated above, three pairs of acupoints consisting of Sibai (ST 2), Liangmen (ST 21), Zusanli (ST36), in the Foot Yangming Meridian, were designed, which represent acupoints of different level(head, trunk, limb); thus 3 pairs of acupoints of the Foot Shaoyang Meridian in the same horizontal level were selected: Yangbai(GB 14), Riyue(GB 24), Yanglingquan(GB 34). Acupoint location: Sibai acupoint, at the depression of the infraorbital foramen; Liangmen acupoint, at intersection of the midline between anterior midline and midclavicular line and the middle horizontal line of omphalos and xiphoid.

Pairs of stainless-steel needles of 0.25 mm in diameter were inserted into the acupoints stated above of experimental rabbits(Groups C and D). The needles were connected to the output of an electronic pulse generator, a medical EA stimulator (Model G6805-1, made by Shanghai Medical Electro-apparatus Factory, China), which achieves intermittent-and-irregular wave(intermittent wave:4 Hz, irregular wave:20 Hz, intensity of 6-15 V, the depth of acupuncture of 0.5 cm, constant time of 20 min), while there was a light vibration in the rats' lower limbs.

Induction of gastric mucosal lesion index

Seven days after corresponding treatment, each rat was immobilized in a restraint cage and immersed for 10 h to the height of the xiphoid in a water bath kept at 20 ℃ ± 1 ℃, and then sacrificed under anesthesia with 10% urethane intraperitoneally (1 ml/100 g). The abdomen was opened, and the stomach was removed, opened along the great curvature and the gastric mucosa was carefully examined under a dissection microscope to determine gastric mucosal lesion index(GUI) by the score systems suggested by Guth[16]. Briefly, the stomach mucosa was examined with magnifier for the presence of erosions and scored as follows: (1) for small, round hemorrhagic erosion; (2) hemorrhagic erosions less than 1 mm in length; (3) hemorrhagic erosions between 1-2 mm in length; (4) hemorrhagic erosions between 2-3 mm in length; (5) hemorrhagic erosions longer than 4 mm in length. The score value was multiplied by 2 when the width of erosion was larger than 1 mm. The extent of the lesion (lesion index) is expressed as the sum of the length of these breaks per stomach.

RNA extraction

After the treatment stated above, each rat's gastric mucosal tissue was collected and put in freezing-and-storing tubes and kept in the nitrogen tank quickly. Five samples of each group were selected randomly for experiment. Expression of EGFR mRNA was evaluated with RT-PCR. Total RNA was isolated from gastric mucosal samples using a guanidinium isothiocyanate/phenol chloroform single step extraction kit from Stratagene(Gibco BRL, USA), precipitated in ethanol and resuspended in sterile RNAase-free water for storage at -80 ℃ until use. Total RNA was quantified spectrometrically at 260 nm, and the quality of isolated RNA was analysed on agarose gels under standard conditions.

Reverse transcription reaction

Total RNA (10 μL, about 0.5 μg/sample) was reverse transcribed (RT) using oligo(dT)18 primers 1 μL (30 pmol/L), 5×RT buffer 4 μL(Promega Co.), dNTPs(10 mmol/L) 1 μL, RNasin(20 MU/μL, Promega Co. Madison, America) 0.5 μL, M-MULV reverse transcriptase (200 MU/μL, Promega Co., Madison, America) 1 μL, and DEPC water 2.5 μL in a 20 μL reverse transcription reaction system, and such system was performed at 42 ℃ for 30~60 min, then cooled and centrifuged for several seconds so that target mRNA of total RNA sample was transcribed into target cDNA.

Polymerase chain reaction (PCR)

An aliquot of the same RT product from each sample (1/20

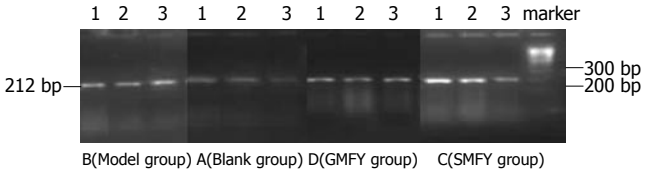

Figure 1 Electrophoresis of ITFmRNA RT-PCR product in gastric mucosal tissue (1, 2 and 3 are randomly selected from each group).

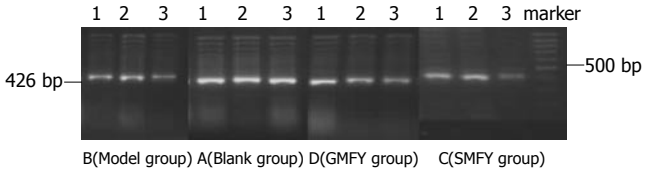

Figure 2 Electrophoresis of GAPDH mRNA RT-PCR product in gastric mucosal tissue (1, 2, and 3 are randomly selected from each group).

of the total volume) was used in the PCR amplification reactions for ITF and GAPDH. The PCR reaction system compound contained 4 μL cDNA, 10×PCR buffer(Promega Co., Madison, America)5 μL, dNTPS (10 mmol/L) 1 μL, oligonucleotide primers sense/ antisense (10 mmol/L) 1 μL (the related primer sequence as stated below), Taqase(5 MU/μL Promega Co.) 1 μL, ddH_2O 32 μL in a total volume of 50 μL. Reaction mixtures were incubated for predenaturation at 94 ℃ for 2 min, followed by 35 cycles for ITF (denaturation at 94 ℃ for 30 s, annealing at 58 ℃ for 1 min, and extension at 72 ℃ for 1 min) and 35 cycles for GAPDH (denaturation at 94 ℃ for 30 s, annealing at 58 ℃ for 30 s, and extension at 72 ℃ for 30 s), and a final extension at 72 ℃ for 5 min.

PCR primer design

By use of the relatively quantitative method to measure ITF-mRNA, rat GAPDH was selected as internal control substance. The primer sequences and sizes of amplification products are as follows: ITF sense, 5' -ACAACCCTGCTGCTGGTCCT -3'; antisense, 5' -TCTGTCTCTTGCAGAGGTTTG-3' (212 base pairs); GAPDH sense, 5'-TGCTGAGTATGTCGTGGAGTC-3'; antisense: 5'- AAGGCCATGCCAGTGAGCTTC-3' (426 base pairs).

RT-PCR product electrophoresis

Five microliter PCR products were analyzed on 10 g/L agarose gel containing ethidiumbromide with TBE buffer at 80 V for 40 min and photographed under UV illumination. The band intensities were quantified by densitometry. ITF and GAPDH PCR products were respectively 212 base pairs(Figure 1), and 426 base pairs(Figure 2). ITF and GAPDH were determined by computer-assisted densitometric scanning. Signals were quantified by density analysis of the digital images using Eagle Eye Ⅱ image software (Stratagene Co.) and ITF/ GAPDH quotient indicated the relative expression of ITF.

Table 2 Effect of EA on GUI and ITF-mRNA expression in gastric mucosal tissue (mean ± SD)

G	GUI score (n = 10)	ITF mRNA/GAPDH mRNA (n = 5)
A(Blank group)	0.3±0.48	0.45±0.01
B(Model group)	54.3±1.34[b]	0.65±0.01[b]
C(SMFY group)	31±2.21[bd]	0.76±0.01[bd]
D(GMFY group)	39.8±1.62[bcf]	0.66±0.01[bf]

[b]$P<0.01$ *vs* blank group, [c]$P<0.05$ *vs* Group B, [d]$P<0.01$ *vs* Group B, [f]$P<0.01$ *vs* Group C.

Experiments were performed in triplicate.

Statistical analysis

The data were expressed as mean±SD of 10 rats per group. Comparison between groups was assessed using one-way analysis of variance (ANOVA) on ranks. Differences were considered statistically significant if the P value was less than 0.05. Software SPSS 10.0 was used in all statistical tests.

RESULTS

Gastric mucosal injury condition and GUI

There were some dots and strips injury detected by the magnifier(10×). The GUI in Model group was highest, while in Blank group lowest, with significant difference between them ($P<0.01$). It demonstrated the ulcer model was successful. Compared with Model group, GUI in SMFY group and GMFY group reduced significantly ($P<0.05$ or $P<0.01$), and the GUI in SMFY group was lower than that in GMFY group($P<0.01$, Table 2).

Effect of ITF-mRNA expression on gastric mucosal tissue

The expression of ITF mRNA using RT-PCR was detected in the intact gastric mucosa of Blank group as a weak signal but it was well-defined among other groups: Model, SMFY, and GMFY groups($P<0.01$). Compared with Model group, EA at acupoints of SMFY group could upregulate significantly the expression of ITF-mRNA expression in gastric mucosal tissue($P<0.01$), while there did not exist difference of expression between GMFY group and model group($P>0.05$), but obvious difference between SMFY group and GMFY group was found ($P<0.01$, Table 2).

DISCUSSION

According to classical TCM theory, SMFY, running from head, via chest and abdomen, along anterior lateral lower limb to foot, is a crucial meridian for its good bidirectional modulation of digestive diseases. Previously, we studied the effect of single acupoint of SMFY on gastric function as well as the whole SMFY's functional mechanism. Acupuncture at acupoints of head and face, trunk, or lower limb (such as acupoint "Sibai", "Tianshu", "Liangmen", "Zusanli", "Shangjuxu"), could produce certain ameliorative effect through the following mechanisms: augmentation of gastric antrum anrea, reinforcement of

pressure power of gastric pyloric sphincter, promotion or inhibition of related gastrointestinal peptide secretion[17-19]. All of these have provided experimental evidence for the theory "Relative particularity between SMFY and stomach".

The cytoprotective functions in protecting gastrointestinal tract against ongoing damage may be accomplished in several ways, and evidences for participation in both the early phase of epithelial repair known as restitution (marked by increased cell migration but no proliferation), and in the subsequent, protracted phase of glandular renewal (marked by proliferation, differentiation and migration) have been published[20-22]. ITF is one of the most recently described members of the trefoil peptide family, a peptide of 59 amino acids, expressed normally by mucus secretory cells of the small and large intestine[9,23]. It is secreted onto the luminal surface of the gastrointestinal tract, and may act in conjunction with the mucin glycoprotein products of goblet cells to promote reestablishment of mucosal integrity after injury through mechanisms distinct from those that may act at the basolateral pole of the epithelium[11,24].

This study assessed for the first time ITF expression by RT-PCR analyses in rat gastric mucosa after exposure to water immersion and restrained stress. It showed that expression of ITF in gastric mucosa was enhanced shortly after the stress, leading us to hypothesize that this process might be mediated by ITF. Meanwhile, it was proved that EA had a tendency to improve the expression of gastric mucosal ITFmRNA gene, and such expression of SMFY group was strongly higher than model group and GMFY group, indicating that the expression discrepancy of ITFmRNA may be the underlying mechanism of different effect of EA at acupoints of SMFY and that of GMFY, thus it could be one proof for the TCM theory "Relative particularity between SMFY and stomach".

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CASE REPORT

Large Brunner's gland adenoma: Case report and literature review

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Abstract

Brunner's gland adenoma (BGA) is a very rare benign tumour of the duodenum, which is usually asymptomatic and discovered incidentally at endoscopy. Occasionally, this lesion may be large, causing upper gastrointestinal haemorrhage or intestinal obstruction. The case had a large Brunner's gland adenoma, presenting melena that was managed by endoscopic excision.

Key words: Brunner's gland adenoma; Endoscopic resection; Giant brunneroma

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INTRODUCTION

Brunner's glands, described by the anatomist Brunner in 1688, are submucosal mucin-secreting glands. They are predominantly localized in the duodenal bulb and proximal duodenum and progressively decrease in size and number in the distal portions. Brunner's glands exert a physiological "anti-acid" function by secreting alkaline fluid composed of mucin which protects the duodenal epithelium from the acid chime of the stomach[1]. Furthermore, they produce and secrete "enterogastrone", an enteric hormone inhibiting gastric acid secretion.

Brunner's gland adenoma (BGA), firstly described by Curveilheir in 1835, is a benign tumour arising from the Brunner's glands that exceptionally may evolve towards a malignant transformation[2-4]. BGA is an extremely rare tumour with an estimate incidence of 0.008% reported in a single series of 215 000 autopsies[1]. At present, < 200 cases have been reported in the world medical English literature.

Here, one case of large BGA managed by endoscopic resection is reported together with a review of the pertinent literature.

CASE REPORT

A 58-year old female was referred to our Gastroenterology Unit due to episodes of epigastric discomfort over the past few months. Past medical history included appendicectomy performed during childhood, cholecystectomy for gallstones performed 4 years previously and a self-limiting single episode of melena occurred two months previously. The patient denied weight loss or use of non steroidal anti-inflammatory drugs (NSAIDs).

Clinical examination and routine blood tests were normal. Esophago-gastro-duodenoscopy (EGDS) demonstrated normal esophagus and stomach. A large pedunculated polyp, 3 cm × 4 cm in size, completely occupying the duodenal bulb was found. Healing microerosions were present on the mucosa. Histological examination of the multiple biopsy specimens obtained during EGDS, suggested "Brunner's gland hyperplasia" in a context of mild chronic gastritis without evidence of *Helicobacter pylori* (*H pylori*) infection.

Abdominal CT-scan confirmed the presence of a polypoid mass originating in the mucosa of the duodenal bulb and extending to the second portion of the duodenum for about 4 cm, without evidence of duodenal wall infiltration (Figure 1). Endoscopic resection was carried out to remove the duodenal mass. Histological examination revealed closely packed Brunner's glands and ducts embedded in a fibrous stroma with a moderate degree of lymphocyte and monocyte infiltration (Figures 2A and 2B). A diagnosis of Brunner's adenoma was made. During the 6-month follow-up, the patient remained symptom-free and no further episodes of melena occurred. EGDS did not find any residual lesion.

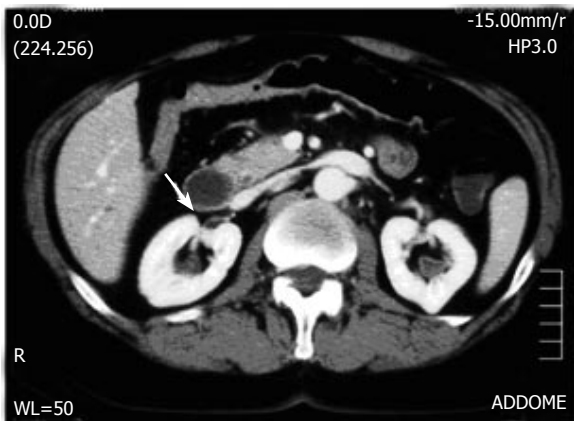

Figure 1 Evidence of polypoid mass in duodenal bulb extending to second duodenal portion (arrow).

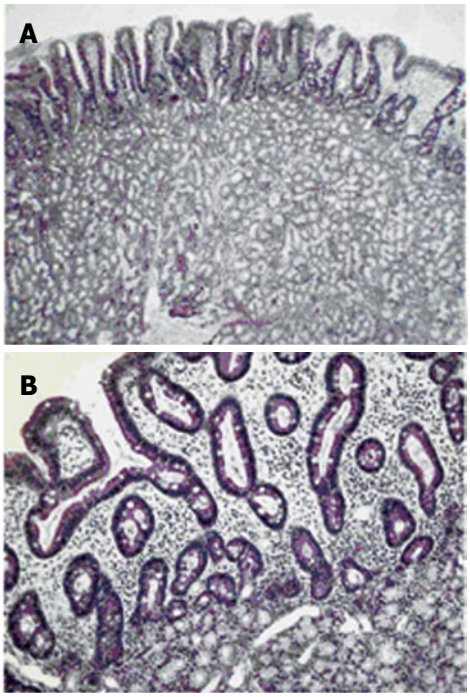

Figure 2 Gland hyperproliferation extending beyond muscolaris mucosae reaching lower portion of duodenal villi with irregular and squat profile of these structures (**A**) (H&E, original magnification X4) and detail of interface between Brunner's gland proliferation and duodenal mucosa with absence of muscularis mucosae and irregular profile of villi (**B**) (E&E, original magnification X20).

DISCUSSION

BGA has a tendency to be predominant in the fifth or sixth decade of life with equal gender distribution [5]. Clinical presentation is variable. However, the majority of cases are asymptomatic or present with non specific, vague symptoms such as abdominal pain or discomfort, nausea or bloating. BGA is usually an incidental finding during imaging studies or EGDS[5].

In symptomatic patients, the most common clinical presentations are gastrointestinal bleeding (37%) and obstructive symptoms (37%)[5]. Gastrointestinal bleeding manifests in the majority of cases as chronic loss of blood with iron deficiency and anaemia[6]. Less frequently, when erosion or ulceration of the tumour occurs, patients can present with melena or haematemesis. These findings are usually described in BGA occurring beyond the first portion of the duodenum, probably because these lesions are subjected to more stress and vascular damage from gastrointestinal motility[6]. In our case, the patient referred to our unit for a self-limiting episode of melena and showed a large polyp at EGDS with a short peduncle localised in the duodenal bulb with an ulcer scar on the surface.

Aetiology and pathogenesis of BGA still remain to be elucidated. Due to the "anti-acid" function of Brunner's glands, it has been postulated that an increased acid secretion could stimulate these structures to undergo hyperplasia[7]. Franzin *et al* [8] have reported an association between BGA and hyperchlorhydria in patients with chronic gastric erosions and duodenal ulcers, but Spellberg *et al*[9] have not found regression of the lesion with acid secretion inhibitors[9]. A second hypothesis suggests that this lesion is of inflammatory origin due to the presence of a dense inflammatory cell infiltration[10]. Since lymphocytes are usually present in the normal submucosa of the intestinal tract, the presence of inflammatory foci in the BGA is not sufficient to sustain the "inflammatory hypothesis". Finally, it has been suggested that *H pylori* infection may play a role in the pathogenesis of BGA. In a recent study involving 19 100 subjects, *H pylori* infection was found in five out of seven (71%) BGA cases[11]. In our patient, *H pylori* infection was not found. The extreme rarity of BGA and the high prevalence of *H pylori* infection in general population do not allow us to draw a clear pathogenetic link. At present, the most accredited pathogenetic hypothesis remains that BGA is a duodenal dysembryoplastic lesion or hamartoma[12].

The duodenal bulb is the most frequent localization of BGA (57%) [13]. In the majority of cases, these lesions develop into a polypoid mass, usually pedunculated (88%), being 1 to 2 cm in size[14] while few cases reaching several centimetres as the "giant BGA" have been reported[6,15-18]. On the other hand, lesions < 1 cm are referred to as Brunner's gland hyperplasia[19].

Diagnosis of BGA is not always easy at present. Radiological findings (X-ray and computed tomography) are often non specific[12]. Indeed, the duodenal filling defect can mimic several other lesions, such as leiomyoma, lipoma, lymphoma, aberrant pancreatic tissue or carcinoid tumours[20]. Computed tomography is useful only to confirm the absence of extra-luminal extension of BGA[20]. Diagnosis can be obtained by histological examination of the excised mass. Traditional endoscopy of pinching biopsies is usually negative since the biopsy forceps are unable to reach the tumoral tissue localized completely in the submucosa layer [21]. In our case, diagnosis was made on endoscopic biopsy samples of duodenal mass, because Brunner's gland hyperproliferation was extended beyond muscularis mucosae reaching lower portion of duodenal villi (Figure 2).

Endoscopic or surgical removal of BGA has been suggested to prevent the development of complications (haemorrhage, severe anaemia, obstruction or intussusception). Endoscopic polypectomy represents the ideal approach, which is more cost-effective and less invasive of the abdominal surgery [22, 23]. However, the success depends on site and size of the BGA and presence of a peduncle. Several cases of successful endoscopic resections have been reported [12, 24-26]. In our case, even though the lesion was large (3 cm × 4 cm) with a short peduncle occupying entirely the duodenal bulb (Figure 1), endoscopic polypec-

tomy was effective for the treatment of BGA, without complication and recurrence of the lesion during the 6-month follow-up.

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CASE REPORT

Metastasis of hepatocellular carcinoma to the small bowel manifested by intussusception

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Abstract

Hepatocellular carcinoma (HCC) is a so highly invasive tumor that metastasizes hematogenously and lymphogenously to distant site. Frequent sites are lung, regional lymph node, bone, and adrenal gland. But metastasis to the gastrointestinal (GI) tract is rare, and most common site is stomach. Metastasis to the small intestine is extremely rare. Moreover, metastatic HCC of the small bowel causing intussusception has not been reported until now. Here, we report a case of metastasis of HCC to the small bowel manifested by intussusception.

Key words: Hepatocelluar carcinoma; Small bowel metastasis; Intussusception

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a highly malignant tumor with frequent metastasis. Extrahepatic metastases have been reported in 25 to 65 percent of autopsy series, with lung, bones, and regional lymph nodes as the most commonly involved sites[1-5]. Metastasis of HCC to the gastrointestinal (GI) tract is rare and most common site of metastasis is reported stomach by direct invasion of tumor[4,5]. The distant hematogenous metastasis of HCC to the small intestine is extremely rare[6].

The majority of adult intussusceptions arising in the small bowel are due to benign lesions, and metastatic melanoma is the most frequent malignancy causing small bowel intussusception[7]. Intussusception caused by the metastatic HCC to the small bowel has not been reported until now. We report a case of metastasis of HCC to the small bowel manifested by intussusception.

CASE REPORT

A 65-year-old man was referred to emergency room with complaint of sudden onset, cramping periumbilical pain for 2 d. The pain was continuous with frequent exacerbation, and was associated with nausea and vomiting. The patient had been assigned a diagnosis of hepatitis B virus associated cirrhosis 3 years previously. Two months before, he was diagnosed as HCC with multiple bones and regional lymph nodes metastases according to the large enhanced hepatic mass on computed tomography scan with elevated level of serum alpha-fetoprotein (629 μg/L). On physical examination, the abdomen was distended, with provocable pain in the periumbilical area and increased bowel sound. Complete blood cell count revealed anemia (Hb 72 g/L) and leukocytosis (15.1×10^9/μL). Liver function tests and renal function test were not remarkable. Plain radiography of abdomen demonstrated presence of marked distension of bowel loops with air fluid level and absence of colonic gas, which were known as a characteristic finding of small bowel obstruction (Figure 1). Computed tomography scan showed ill defined hepatic mass with regional lymph node enlargement and a "target mass" lesion in the right lower abdomen (Figure 2). Abdominal ultrasound showed 10 cm long intussusception in the mid small bowel level (Figure 3A) and a small mass which was suspected of leading point of intussusception was also observed at the head of intussusception (Figure 3B).

After five days of conservative management, segmental small bowel resection and end-to-end anastomosis was performed. At surgery, intussusceptional segment was located at the level of jejunum 110 cm distal from the Treitz

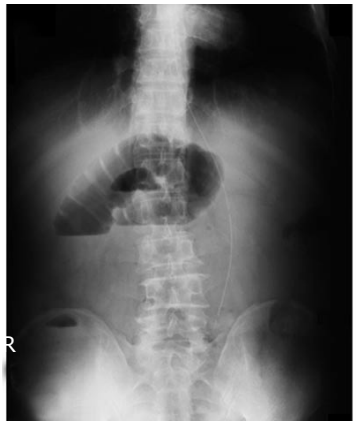

Figure 1 Plain radiography of abdomen showed stepladder appearance.

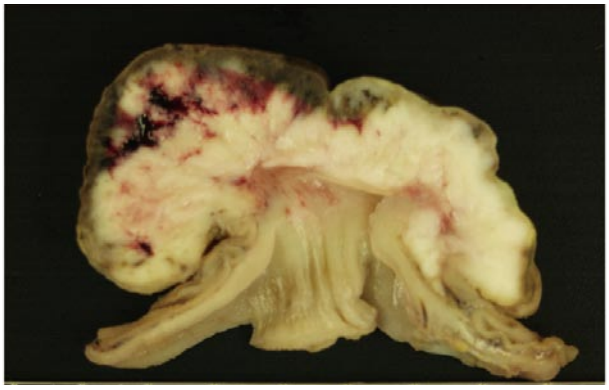

Figure 4 Surgical specimen showed that mass arising from submucosa grown into lumen and had dimpling portion on the top of tumor.

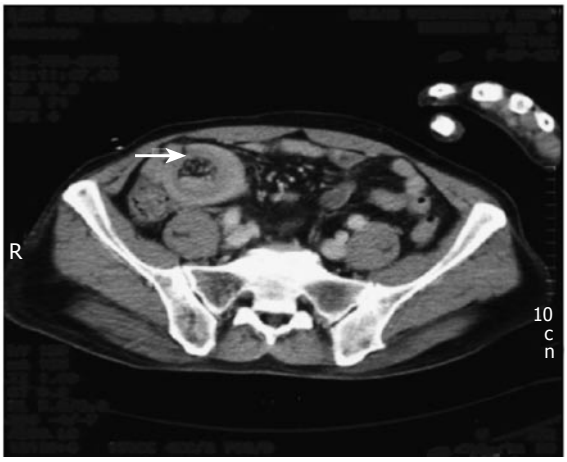

Figure 2 Computed tomography scan showed a "target mass" lesion (arrow) in the right lower abdomen, representing the intussuscepted mesenteric fat and vessel.

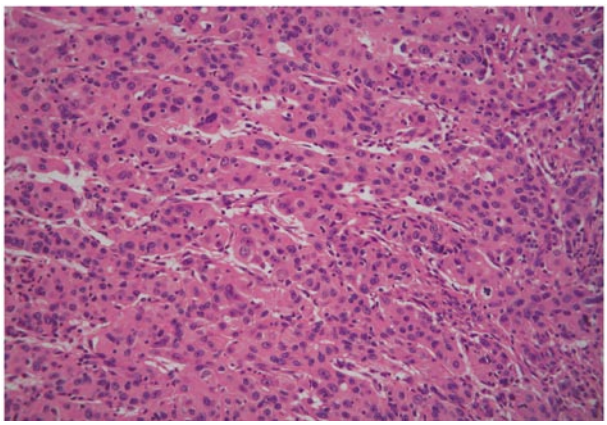

Figure 5 Histological examination demonstrated a poorly differentiated adenocarcinoma with hepatoid feature of trabecular pattern (HE X 100).

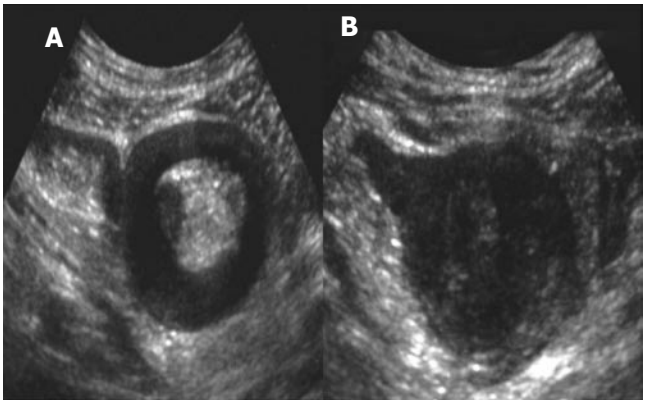

Figure 3 Abdominal ultrasound showed, **A:** "pseudo kidney sign" at the body of intussusception, **B:** Lobulated mass lesion at the head of intussusception, which was suspected of leading point of intussusception.

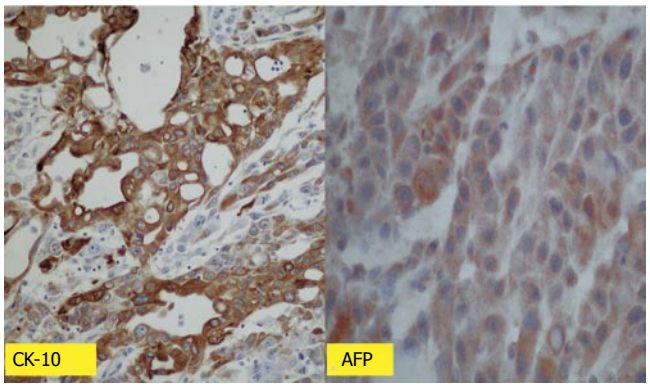

Figure 6 Strongly positive staining for alpha-fetoprotein and cytokeratin 18 (Immunoperoxidase × 100).

ligament and 8 cm sized protruding mass was grown into the lumen of small intestine within intussusceptional segment. Other three masses which measured 0.5 cm, 0.8 cm, and 3 cm respectively were also grown into the lumen at the level of jejunum 170 cm distal from the Treitz ligament.

Macroscopic examination of the surgical specimen showed multiple luminal protruding submucosal mass with extension to the subserosa (Figure 4). The largest mass measured 3.0 cm × 2.0 cm × 0.8 cm. In microscopic examination, the atypical epithelial cells were poorly differentiated carcinoma with hepatoid feature of the trabecular pattern (Figure 5). There tumor cells were positive for alpha-fetoprotein and cytokeratin 18 on the immunohistochemical staining (Figure 6). These evidences supported HCC metastasis to small bowel.

DISCUSSION

HCC is so highly invasive that extrahepatic metastasis is frequently observed [2,5]. The most common site of metastasis is lung, followed by regional lymph node, adrenal gland and bone. HCC metastasis to gastrointestinal tract is a rare condition, being found only in 4-12% of cases in autopsy series[5]. These metastatic lesions are usually asymptomatic, and most are discovered on postmortem examination[8]. Reports of premoterm diagnosis of GI tract involvement in HCC are extremely rare and are associated with poor prognosis[8,9]. Chen *et al* reported that median survival following the diagnosis of GI tract involvement in HCC was only one month[8].

The modes of metastasis of HCC are known as hematogenous, lymphatic spread and local infiltration[8]. The presumed mode of metastasis of HCC to GI tract is mostly direct invasion to adjacent GI tract via adhesion to the serosal side by bulky mass. Lin *et al.* reported that nine of eleven patients with HCC represented direct invasion of HCC to adjacent GI tract[9]. In Park's series, the mode of spread was direct invasion from a contiguous HCC (66.7 %), hematogenous metastasis (17%), peritoneal seeding (6%)[10]. Characteristic features of HCC with direct involvement of GI tract were large, located at the subcapsular area, and had exophyting growth pattern[9,10]. HCC has a high incidence of venous invasion and thrombosis formation. Hematogenous spread of HCC to the GI tract may be caused by tumor thrombi via the portal system and disseminated by hepatofugal portal spread to the GI tract. In previous reports, most of patients with hematogenous metastasis of HCC to the GI tract showed main portal vein thrombosis[8,9]. But in some cases, vascular involvement was not observed. In our case, the presumed mode of metastasis to small intestine was hematogenous spread. However despite of bulky mass with multiple bone metastases, main portal vein thrombosis was not observed.

The most commonly involved site of the GI tract in HCC was reported to be the stomach[9]. The small intestine can often be involved in tumors such as melanoma, carcinoma of the lung, cervix of the uterus, the breast, and colon[11,12]. HCC metastasis to small intestine was rarely reported. Few cases with the involvement of the duodenum have been reported, but in those cases, the duodenum was directly invaded by tumor in the liver[6, 8-10]. In our case, HCC metastasized to jejunum by hematogenous spread and formed multiple polypoid masses.

Metastatic tumors in the small intestine predominantly involve the bowel wall, the subserosa, the serosa or the mesentery. But submucosal involvement is rare. According to Farmer and Hawk, metastatic masses to small bowel are divided into three types: (1) spherical mesenteric masses encroaching on or extension into the intestine, (2) intramural masses, often with ulceration; and (3) bulky polypoid masses extending into the bowel lumen [11]. Polypoid lesions are resulted from mucosal or submucosal involvement rather than intramural involvement. In the present case, histological examination showed that polypoid mass sprouted from submucosa and extended to serosa. This finding suggested that the main metastatic site of HCC was the submucosa of the small intestine.

Intussusception, although common in children, is rare in adults and represents 1% of cause of small bowel obstruction in adult [13]. Adult intussusception presents with a variety of acute, intermittent, and chronic symptoms mimicking partial small bowel obstruction[14]. Thus, the diagnosis of intussusception is difficult to make before surgery and computed tomography scan is the most useful diagnostic tool. In distinction to intussusception in children, intussusceptions in adults are mainly resulted from organic lesion. The most common cause of benign lesion was postoperative band and malignant lesions were mainly consisted of metastatic tumor[13]. In previous report, melanoma was the most common tumor of small intestine and lymphoma, followed by sarcoma, squamous cell carcinoma[13]. In our case, cause of small bowel intussusception was diagnosed as metastatic HCC by pathologic examination.

We reported an extremely rare case of metastatic HCC to small intestine, which caused small bowel intussuception in adult. Up to our knowledge, this is the first case report of small bowel intussuception resulting from HCC metastasis.

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CASE REPORT

Patient with hepatocellular carcinoma related to prior acute arsenic intoxication and occult HBV: Epidemiological, clinical and therapeutic results after 14 years of follow-up

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Abstract

Little is known about the long-term survivors of acute arsenic intoxication. We present here a clinical case report of a man with chronic hepatitis B virus (HBV) infection who developed hepatocellular carcinoma four years after acute arsenic poisoning. HBsAg was detected in serum in 1990 when he voluntarily donated blood. In 1991, the patient suffered from severe psychological depression that led him to attempt suicide by massive ingestion of an arsenic-containing rodenticide. He survived with polyneuropathy and paralysis of the lower limbs, and has been wheelchair-bound since then. During participation in a follow-up study conducted among HBV carriers, abdominal ultrasound detected a two-centimeter liver mass consistent with hepatocellular carcinoma. The tumor was confirmed by computed tomography (CT) and magnetic resonance image (MRI). Because of his significant comorbidity, the patient received palliative treatment with transarterial lipiodol chemoembolization (TACE) on three occasions (1996, 1997 and 1999). At his most recent visit in May 2005, the patient was asymptomatic, liver enzymes were normal and the tumor was in remission on ultrasound.

Key words: HBV carriers; Occult HBV; HCC therapy; Arsenic intoxication

Casanovas-Taltavull T, Ribes J, Berrozpe A, Jordan S, Casanova A, Sancho C, Valls C, Bosch FX. Patient with hepatocellular carcinoma related to prior acute arsenic intoxication and occult HBV: Epidemiological, clinical and therapeutic results after 14 years of follow-up. *World J Gastroenterol* 2006; 12(12): 1972-1974

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INTRODUCTION

Arsenic is a recognized human carcinogen established by the International Agency for Research on Cancer[1]. Chronic arsenic intoxication has been associated with skin lesions (such as hyperpigmentation, hyperkeratosis and carcinoma) and liver disease (non-cirrhotic portal hypertension, hepatocellular carcinoma and angiosarcoma). Moreover, chronic arsenic exposure is associated with a higher frequency of diabetes mellitus, ischemic heart disease and hypertension[2-4]. Epidemiological studies mainly involving cohorts of chronic arsenic-exposed subjects suggest that chronic arsenic intoxication increases the risk of developing liver cancer as compared with non-exposed populations[5,6].

Patients who survive the initial effects of acute arsenic exposure may develop peripheral neuropathy and encephalopathy[7]. Hepatic injury is uncommon but is associated with severe liver disease and high mortality[8]. Little is known about the long-term survivors of acute arsenic intoxication.

We present here a clinical case report of a Caucasian man with chronic hepatitis B virus (HBV) infection who developed hepatocellular carcinoma four years after acute arsenic poisoning.

CASE REPORT

The man born in 1947 was obese with no HBV carriers

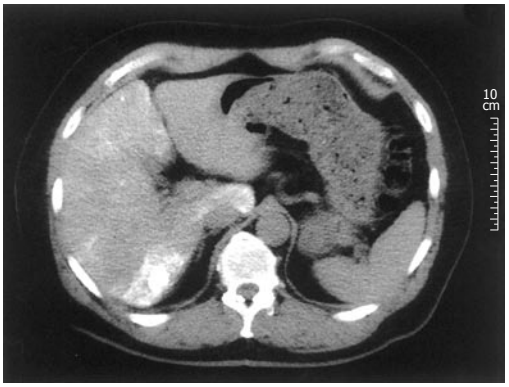

Figure 1 Non-contrast CT one month after arterial chemoembolization with iodized oil (Lipiodol) and doxorubicine emulsion. CT shows a 2-cm nodule with dense Lipiodol enhancement in segment VI (arrow), consistent with hepatocellular carcinoma.

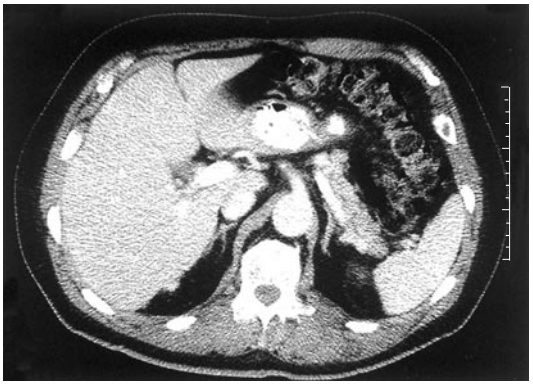

Figure 2 Follow-up contrast-enhanced CT five years later from 1995 shows a small hypervascular lesion (arrow) in segment VI consistent with residual tumor. Tumoral Lipiodol retention was absent and the size of the lesion was significantly decreased.

in his family and no previous hepatitis or parenteral drug use. He was a heavy smoker and alcohol consumer before 1987. HBsAg was detected in serum in 1990 when he voluntarily donated blood.

In 1978 he had self-limited cardiac arrhythmia because of stress and in 1991 arrhythmia due to atrial fibrillation that reverted with cardioversion. In 1991, the patient suffered from severe psychological depression that led him to attempt suicide by massive ingestion of an arsenic-containing rodenticide. He developed acute renal failure and generalized paralysis and was hospitalized for 11 days in the intensive care unit, requiring assisted ventilation and chelation therapy with dimercaprol. He survived with polyneuropathy and paralysis of the lower limbs, and has been wheelchair-bound since then. In 1994 and 1995 he suffered from several transient ischemic attacks that were managed with chronic decoagulation therapy. In 2000 diabetes was diagnosed. He also required long-term treatment for sleep apnea-hypoapnea syndrome with automatic continuous positive airway pressure.

In November 1995 he was invited to participate in a follow-up study conducted in HBV carriers[9]. The viral serology results were negative for HBsAg, anti-HBs, HBeAg, IgM-anti-HBc, and positive for anti-HBe, IgG-anti-HBc. Serum HBV-DNA was negative by PCR technique, while anti-HDV, anti-HCV and anti-HIV were all negative. ALT was 1.36 μKat/mL (normal <0.5 μKat/mL), AST and alpha-fetoprotein were normal. Abdominal ultrasound detected a pattern suggestive of chronic liver disease and a liver mass two centimeters in diameter consistent with hepatocellular carcinoma. The tumor was confirmed by computed tomography (CT) scanning and magnetic resonance imaging. Because of his comorbidity, the patient was not considered eligible for liver transplant or hepatectomy. Palliative treatment with transarterial lipiodol chemoembolization (TACE) was performed on three occasions (1996, 1997 and 1999) (Figure 1) with clinical, analytical and imaging follow-up studies (Figure 2). At his last visit in May 2005, he was asymptomatic, liver enzymes were normal and the tumor was in remission on ultrasound imaging.

DISCUSSION

To the best of our knowledge, this patient is the first known case of liver tumor developed after acute arsenic exposure. Standard chelation treatment for acute intoxication could not remove arsenic from intracellular sites because of its lipophobic nature, suggesting that this treatment cannot protect cells from long-term clinical consequences[10]. As has been described, the presence of intracellular arsenic may be related to the development of diabetes, arterial hypertension, ischemic arterial disease and liver cancer in such patients. It should also be taken into consideration that intraindividual variability in arsenic methylation can increase susceptibility to arsenic-induced cancer[11].

Given the fact that the patient was obese and had occult HBV infection (his anti-HBc and anti-HBe positive status could indicate integration of the virus in the host genome) prior to arsenic intake, we are not sure that some hepatic injury is not due to these risk factors[12,13]. The presence of these risk factors may enhance the hepatic lesion and the development of liver cancer.

Angiosarcoma is the type of liver tumor most often related with chronic arsenic exposure[5]. In our case, histological confirmation of the liver tumor could not be performed due to the anticoagulant treatment (Sintrom®). However, the features of the tumor on CT and TACE are consistent with hepatocellular carcinoma, particularly when the tumor characteristics are considered after lipiodol uptake during and after TACE[14]. Even though the most effective therapy for hepatocellular carcinoma is liver transplantation or partial hepatectomy[15], our patient remains asymptomatic after nine years of TACE treatment. This fact is consistent with the increasing survival rates of patients receiving this palliative treatment[16].

In conclusion, this is the first patient with occult HBV infection who developed hepatocellular carcinoma after acute arsenic exposure, and his tumor remitted after palliative treatment with TACE. The clinical implication of our observation is that periodical liver studies should be undertaken in patients who survive after acute arsenic exposure.

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CASE REPORT

Mucinous cystadenoma of the appendix associated with adenocarcinoma of the sigmoid colon and hepatocellular carcinoma of the liver: Report of a case

Srdjan P Djuranovic, Milan M Spuran, Nada V Kovacevic, Milenko B Ugljesic, Dragutin M Kecmanovic, Marjan T Micev

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Abstract

Mucinous cystadenoma of the appendix is a rare condition and represents one of the three entities with the common name mucocele of the appendix. It is characterized by a cystic dilatation of the lumen with stasis of mucus inside it. Histopathologically mucocele is divided into three groups: focal or diffuse mucosal hyperplasia, mucinous cystadenoma and mucinous cystadenocarcinoma. This condition is often associated with other neoplasia, especially adenocarcinoma of the colon and ovaries. We here describe a 57 year old male patient who presented with abdominal discomfort, constipation, fresh blood in stool and frequent urination. He had a big cystadenoma of the appendix associated with adenocarcinoma of the colon and hepatocellular carcinoma of the liver. The patient underwent right haemicolectomy, sigmoid colon resection and segmental resection of the liver. Now 3 years later he has no evidence of disease relapse. According to this, we stress the need of accurate preoperative diagnosis and intraoperative exploration of the whole abdomen in these patients.

Key words: Mucocele; Appendiceal cystadenoma; Colon carcinoma; Hepatocellular carcinoma

Djuranovic SP, Spuran MM, Kovacevic NV, Ugljesic MB, Kecmanovic DM, Micev MT. Mucinous cystadenoma of the appendix associated with adenocarcinoma of the sigmoid colon and hepatocellular carcinoma of the liver: Report of a case. *World J Gastroenterol* 2006; 12(12): 1975-1977

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INTRODUCTION

Mucocele of the appendix is a common name for three different entities with similar clinical presentations. Its main characteristic is cystic dilatation of the appendiceal lumen with mucus inside it. Focal or diffuse mucosal hyperplasia and mucinous cystadenoma are of benign nature, but could lead to complications due to rupture, invasion to adjacent organs or recurrence. Mucinous cystadenocarcinoma is a malignant disease and pseudomyxoma peritonei is its worst complication. On the other hand, this condition is often associated with other intra-abdominal neoplasia. According to this, it is necessary to apply strict oncologic principles for resection in order to minimize the possible complications. A correct preoperative diagnosis may help to avoid iatrogenic rupture during surgery and missing the possible associated intra-abdominal tumors. We describe here a case of correct preoperative diagnosis of big appendiceal mucinous cystadenoma associated with adenocarcinoma of the sigmoid colon and hepatocellular carcinoma of the liver.

CASE REPORT

The patient was male, 57 years old with pain in ileo-cecal region for 6 mo prior to administration. He had abdominal discomfort, constipation, fresh blood in stool and frequent urination. On physical examination he had palpable tumor mass in the lower right quadrant of abdomen, enlarged liver and subicterus of sclera. Laboratory findings showed inflammatory syndrome with sideropenic normocytic anemia, elevated alkaline phosphatase, carcinoembryonic antigen, carboanchidratic 19-9 antigen and alpha-feto protein. He had negative markers for hepatotropic viruses (B and C). Transabdominal sonography showed the presence of a large bilocular cystic tumor in the right lower quadrant of abdomen with outlined capsule and maximal dimensions of 106 mm × 74 mm, slightly enlarged liver with focal hyperechogenous tumor in the 6th and 7th liver segments (maximal diameter of 67 mm) and "pseudokidney" sign in the left lower quadrant of abdomen. CT scan displayed tumor of the right liver lobe (Figure 1). Barium enema showed extra luminal compression and medial displacement of cecum and terminal ileum with appendix not filled with the contrast and 4-cm long tubular stenosis of the proximal part of

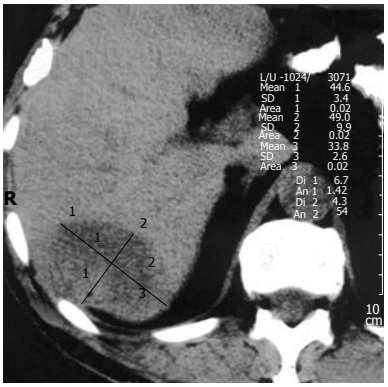

Figure 1 Abdominal CT showing presence of the right lobe tumor of the liver.

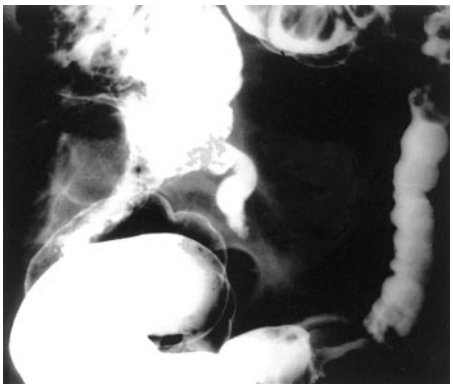

Figure 2 Barium enema displaying extraluminal compression and medial dislocation of the cecum due to cystadenoma of the appendix and tubular stenosis of the sigmoid colon due to the adenocarcinoma.

sigmoid colon (Figure 2). According to that we suspected that it was the mucocele of the appendix with neoplasia of the sigmoid colon and hepatic tumor. The patient underwent right haemicolectomy with ileo-transverso termino-lateral anastomosis and sigmoid colon resection with colo-recto termino-terminal anastomosis as well as paraaortal and paracaval lymphadenectomy. Segmetal resection of the liver (the 6th and 7th segments) was done and the whole tumor was resected. Histology showed big mucocele of the appendix caused by mucinous cystadenoma with severe displasia (Figure 3 and Figure 4), adenocarcinoma of the sigmoid colon (T3,N1,M0,L1,V0; Dukes C; Astler-Coller C-2) and carcinoma hepatis hepatocellulare (well-differentiated alveolar type). Adjuvant therapy with 5-fluorouracile and Leucovorine was administrated in five cycles. Now, three years later, the patient is doing well and has gained 17 kilograms with no evidence of disease relapse and his laboratory findings including tumor markers are within the normal range.

DISCUSSION

Mucocele of the appendix is a descriptive term that implies a dilated appendiceal lumen caused by abnormal accumulation of mucus. Mucocele is found in 0.2-0.3% of all appendectomy specimens. The male:female ratio is 1:4 and the mean age of patients is around 55 years[1,2]. Post-imflammatory obstruction was initially thought to be the main reason for the majority of mucoceles, but recent evidence suggests that neoplasia is the basis of most of these cases. The understanding of the nature of appendiceal mucocele and its terminology has been

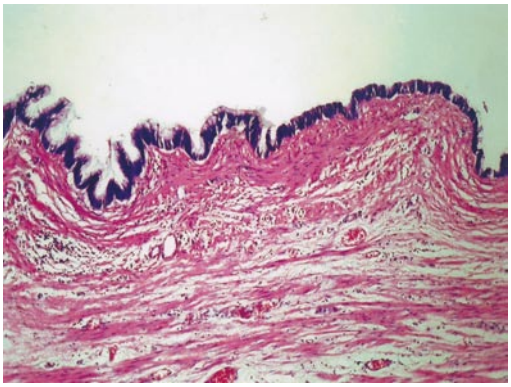

Figure 3 Cystadenoma mucinosum appendicis with obvious dysplastic epithelial lining and focally evident mucinous cytoplasmatic production (H&E, 64x).

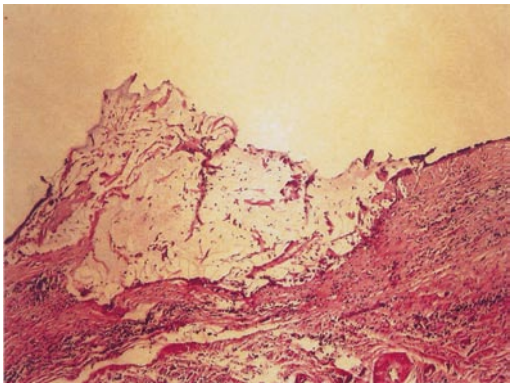

Figure 4 Cystadenoma mucinosum appendices with small extracellular mucinous deposit just beneath focally eroded adenomatous epithelium (H&E, 13x).

greatly changed since the initial description of Rokitansky in 1842[3] and Higa *et al*[4] in 1973. Appendiceal mucocele can be histologically divided into three groups: focal or diffuse mucosal hyperplasia without epithelial atypia, mucinous cystadenoma with some degree of epithelial atypia and mucinous cystadenocarcinoma. A large series of hyperplasia were found in 24.7% cystadenoma, 63% cystadenocarcinoma and 12.3 % of all cases[1,2,4]. Our case could be regarded as mucinous cystoadenoma with severe dysplasia.

Peritoneal (and/or retroperitoneal and pleural) pseudomyxoma is regarded as a worst complication of this disease which could have metastatic characteristics in case of mucinous adenocarcinoma. It is well known that this entity is often associated with colorectal carcinoma (11-20%), ovarian mucinous cystadenoma, uterine myoma, carcinoma of the pancreas and carcinoid tumor of the appendix[4]. Our patient is interesting because of the two associated tumors while one of them (hepatocellular carcinoma) has not so far been mentioned in literature in association with mucinous cystadenoma.

Clinical symptomatology of these patients is not specific. Abdominal pain is present in 64% of the patients and palpable ileo-cecal mass in 50% of them. Disease course is asymptomatic in 25% of the patients even when they have large tumors. Urinary infection and haematuria

are often associated (20%)[5]. Intestinal obstruction caused by intussusception and intestinal bleeding are rare complications[6]. Our patient had a long history of abdominal pain, blaunting, subicterus and urinary discomfort, constipation and rectal bleeding. Some of these symptoms are due to associated tumors.

Laboratory findings are usually non specific, but the elevation of tumor marker level often indicates neoplastic origin and/or associated tumors, which was seen in our patient.

Correct and complete preoperative diagnosis was rare in the past, but modern techniques of imaging allow us today to recognise most of the complications and associated conditions[7]. Sonography usually shows a cystic encapsulated lesion with liquid content adjacent to cecum. CT/NMR scan shows a low density, encapsulated, thin-walled mass that does not contain contrast medium and communicates directly with the cecum. Other solid or cystic abdominal and peritoneal tumors could also be visualised by these methods. Barium enema could point out a failure of the appendix to fill with contrast medium and signs of the extra luminal compression in the ileocecal region[8,9]. The "sign of volcano" is a pathognomonic colonoscopy finding[8,9]. We could not perform total colonoscopy due to neoplastic stenosis of the sigmoid colon.

Fine needle aspiration biopsy is not recomended because of great risk of pseudomyxoma dissemination.

Classical surgical approach is the best therapy option with intraoperative abdominal cavity exploration because of the already stressed associated tumors. Appendectomy is advised for focal or diffuse mucosal hyperplasia and cystadenoma when the appendiceal base is intact. Cecal resection is performed for cystadenoma with a large base and right colectomy is recommended for cystadenocarcinoma. Other surgical procedures depend upon the existence of associated tumors. In cases of disseminated pseudomyxoma peritonei, ultrasonic surgical aspirator can be used[10, 11]. Laparoscopic approach to cystadenoma of the appendix is safe if surgery can be performed without grasping the lesion and if the specimen is removed through the abdominal wall using a bag[12].

The five-year survival rate is 100% in cases of benign mucocele and about 45% in malignant cases. Radiotherapy, chemotherapy and new therapeutic modalities (radioimmunotherapy and matrix metalloproteinase inhibitors) have still to be proven by prospective analysis[13].

In conclusion, accurate preoperative diagnosis and intraoperative exploration of the whole abdomen can improve the prognosis of patients with appendiceal mucocele.

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LETTERS TO THE EDITOR

Cytomegalovirus gastritis after rituximab treatment in a non-Hodgkın's lymphoma patient

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TO THE EDITOR

Rituximab is a chimeric monoclonal antibody against CD20 antigen expressed on most B cells and is used for the treatment of malignant lymphomas expressing the CD20 antigen[1]. It temporarily eliminates normal B-lymphocytes without a substantial decrease in serum immunoglobulin levels[2]. Recently, several serious viral infections have been reported in association with rituximab use. Cytomegalovirus (CMV) is one of the agents that may be a cause of morbidity and mortality in immunocompromised individuals[2, 3]. In the face of exogenous or endogenous causes of immunosuppression, cytomegalovirus can result in retinitis, colitis, pneumonitis, or encephalitis. Presented here is a case of a patient with non-Hodgkin's lymphoma (NHL) who developed gastritis and enterocolitis due to CMV infection after treatment with rituximab.

A 65-year old female patient was admitted with complaints of epigastric pain and diarrhea. Prior to her admission she was treated for diffuse large cell type NHL, stage IIIB bulky disease with six courses of a regimen consisting of cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) and rituximab, and a good partial response was achieved. Due to the presence of bulky disease at presentation, she also received radiotherapy (RT) to the parailiac and inguinal regions at a dose of 3000 centigray (cGy), one month after chemotherapy. Two weeks after radiotherapy, she presented with epigastric pain and diarrhea without blood and mucous. Microscopic examination of stool specimens was unremarkable. Her blood and stool cultures were negative. Her chest X-ray was also normal. Initial investigations revealed normal hemoglobin concentration and platelet count as well as mild leucopenia. The number of white blood cells was 3.6×10^9/L (3.0×10^9/L neutrophils). Serum levels of glucose, amylase, liver transaminases, urea and electrolytes were normal. Based on the preliminary diagnosis of radiation enteritis, antidiarrheal therapy was initiated. The patient did not benefit from this, and her epigastric pain aggravated. A computerized tomography scan of the abdomen revealed gastric wall thickening as well as findings consistent with colitis of the ascending and transverse colon. Subsequent upper gastrointestinal endoscopic evaluation demonstrated multiple linear exudative gastric ulcers, the largest being 4 cm in diameter, from which multiple biopsy specimens were obtained. Histological examination revealed hyperchromatic epithelial cells with nuclear viral inclusions (owl's eye). These cells were positive for monoclonal antibodies against CMV. However, peripheral blood CMV viral load was negative. She failed to respond to acid suppression therapy and her clinical condition deteriorated further. Repeat upper gastrointestinal endoscopy and histological examination of biopsy specimens were consistent with the previous results. Consequently, treatment with intravenous gancyclovir (2×5 mg/kg) was initiated with a diagnosis of CMV gastritis and enterocolitis. Dramatic clinical improvement was achieved within one week and treatment was continued for 3 weeks. Her symptoms resolved completely and a subsequent endoscopy only showed signs of healing ulcers.

Pelvic or abdominal RT is a known cause of acute enteritis characterized by abdominal cramping and diarrhea in approximately 50% of treated patients, and the incidence is higher with concomitant chemotherapy[4]. Acute intestinal side effects of RT occur at doses of approximately 1000 cGy. Since the curative doses for most abdominal or pelvic tumors range between 5000 and 7000 cGy, enteritis is more often than not likely to occur[5]. Our patient received 3000cGy of RT and probably developed RT-induced enteritis. However, antidiarrheal therapy with oral opiates failed to alleviate the patient's complaints, which only resolved after anti-CMV therapy.

Cytomegalovirus enterocolitis should be considered in the differential diagnosis in patients receiving rituximab

whose symptoms fail to resolve after conventional anti-diarrheal therapy, irrespective of peripheral blood CMV viral load.

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Meetings

MAJOR MEETINGS COMING UP

Digestive Disease Week
107th Annual of AGA, The American Gastroenterology Association
20-25 May 2006
Loas Angeles Converntion Center, California

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
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Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
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Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
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ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
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Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
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6th Annual Gastroenterology And Hepatology
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Rio Grande
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World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
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International Conference on Surgical Infections, ICSI2006
6-8 September 2006
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European Society of Clinical Microbiology and Infectious Diseases
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Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
Seatlle - Washington
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EVENTS AND MEETINGS IN 2006

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29	6900	6 900	
30	1000 rpm	1 000 r/min	
31	sec	s	After Arabic numerals
32	1 pg $\cdot L^{-1}$	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.
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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
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45	**F*=9.87, $^§F$=25.9, $^\#F$=67.4	^1F=9.87,^2F=25.9, ^3F=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	$10\times10\times10cm^3$	10 cm×10 cm×10 cm	
66	N·km	KN·m	moment
67	$x\pm s$	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 *et al.*	
72	EcoRI	*Eco*RI	*Eco* in italic and RI in positive. Restriction endonuclease has its prescript form of writing.
73	Ecoli	*E.coli*	Bacteria and other biologic terms have their specific expression.
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75	Iga	*Iga*	writing form of genes
76	*igA*	IgA	writing form of proteins
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EDITORIAL

Co-localization hypothesis: A mechanism for the intrapancreatic activation of digestive enzymes during the early phases of acute pancreatitis

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Abstract

Acute pancreatitis is generally believed to be a disease in which the pancreas is injured by digestive enzymes that it normally produces. Most of the potentially harmful digestive enzymes produced by pancreatic acinar cells are synthesized and secreted as inactive zymogens which are normally activated only upon entry into the duodenum but, during the early stages of acute pancreatitis, those zymogens become prematurely activated within the pancreas and, presumably, that activation occurs within pancreatic acinar cells. The mechanisms responsible for intracellular activation of digestive enzyme zymogens have not been elucidated with certainty but, according to one widely recognized theory (the "co-localization hypothesis"), digestive enzyme zymogens are activated by lysosomal hydrolases when the two types of enzymes become co-localized within the same intracellular compartment. This review focuses on the evidence supporting the validity of the co-localization hypothesis as an explanation for digestive enzyme activation during the early stages of pancreatitis. The findings, summarized in this review, support the conclusion that co-localization of lysosomal hydrolases with digestive enzyme zymogens plays a critical role in permitting the intracellular activation of digestive enzymes that leads to acinar cell injury and pancreatitis.

Key words: Acute pancreatitis; Digestive enzymes; Pancreatic acinar cells

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INTRODUCTION

Acute pancreatitis is an inflammatory disease of the pancreas which, in its severe forms, leads to varying degrees of pancreatic necrosis. Passage of a biliary tract stone into or through the terminal biliopancreatic ductal system is the most frequent inciting cause for clinical acute pancreatitis and studies performed using an opossum model of biliary acute pancreatitis have suggested that the offending stone triggers pancreatitis by obstructing drainage from the pancreatic duct[1]. Other studies performed using that same model have indicated that the earliest changes of pancreatitis occur within the acinar cells of the pancreas and that periductal as well as perilobular changes, including cell injury and inflammation, occur at later times[2].

The morphological changes of acute pancreatitis suggest that an autodigestive process has occurred and, indeed, activated digestive enzymes can be detected within the pancreas during clinical acute pancreatitis. Activated digestive enzymes can also be detected within the pancreas during the very early stages of most experimental pancreatitis models. Since the pancreas normally synthesizes and secretes many potentially harmful digestive enzymes, it is generally believed that it is the premature, intrapancreatic activation of those pancreas-derived enzymes which, ultimately, leads to cell injury and acute pancreatitis. However, for the most part, the potentially harmful digestive enzymes that are synthesized and secreted by the pancreas are present, within the gland, as inactive pro-enzymes or zymogens and activation of those zymogens normally occurs only when they reach the duodenum. How, then, might these zymogens be prematurely activated within the pancreas during the early stages of acute pancreatitis and how might that activation lead to acinar cell injury/necrosis?

Not surprisingly, these questions have prompted the performance of many studies, using various experimental models of acute pancreatitis, over the past several decades and several hypotheses have been advanced which address these questions. One of those hypotheses is the so called "co-localization hypothesis" which suggests that, during the early stages of acute pancreatitis, pancreas derived

digestive zymogens become co-localized with lysosomal hydrolases in acinar cell cytoplasmic vacuoles and that, as a result of this co-localization, lysosomal hydrolases such as cathepsin B, activate trypsinogen. According to this hypothesis, trypsin then activates the other digestive enzyme zymogens and the activated digestive enzymes gain access to the cell interior leading to the cell injury/necrosis which typify acute pancreatitis[3].

This review will summarize current evidence that supports the co-localization hypothesis. To place this hypothesis in its proper context, we will begin by briefly summarizing current concepts regarding the physiological synthesis, intracellular trafficking, and secretion of certain types of proteins by the exocrine pancreas. This will be a highly selective review which will primarily draw upon work performed by many collaborators in the authors' laboratory over the past 25 years and the contributions of those individuals are gratefully acknowledged.

PANCREATIC ACINAR CELL BIOLOGY

Protein assembly

On a "gram of protein-per-cell" basis, pancreatic acinar cells synthesize more protein than any other cell in the body. Digestive enzymes, which are secreted from the pancreas and designed to act within the gastrointestinal tract, account for most of the protein mass synthesized by acinar cells but acinar cells also synthesize many other types of proteins, including the hydrolytic enzymes that are designed to digest un-needed material within intracellular lysosomes. Both types of enzymes, secretory digestive enzymes and lysosomal hydrolases, are assembled on ribosomes attached to the rough endoplasmic reticulum (ER). As they are assembled, they elongate within the cisternae of the rough ER until a signal peptide is added and, at that point, they are cleaved freeing them to assume their tertiary structure within the ER. Many secretory digestive enzymes are synthesized as pro-enzymes or zymogens which are inactive until they are proteolytically processed following secretion. Many of the lysosomal hydrolases are also synthesized as inactive pro-enzymes but their post-translational processing and activation is accomplished within the cell.

Intracellular trafficking of secretory proteins[4]

Following their assembly within the ER, the enzymes destined for secretion are carried within small transport vesicles to the Golgi stacks where they are sorted and some are post-translationally modified. Those that are destined to be secreted in a constitutive manner are then transported to the cell surface where they exit the cell. Most of the secreted digestive enzymes and their zymogens, however, are subject to regulated discharge - i.e. they are stored within the cell until their secretion is accelerated in response to extracellular stimuli. Following their assembly and transport to the Golgi, these proteins traverse the Golgi stacks and they are then packaged within condensing vacuoles which evolve into zymogen granules as they mature and migrate towards the luminal surface of the cell. There, by mechanisms which are regulated by neurohormonal secretagogue stimuli, fusion/fission of the zymogen granule membrane with the luminal plasmalemma occurs and this leads to the formation of a fusion pore which permits the granule contents to be discharged into the extracellular (i.e. ductal) space. In an overall sense, this regulated pathway allows for the massive and rapid discharge of intracellularly stored proteins which, in the case of pancreatic acinar cells, consists primarily of digestive enzymes[5].

Intracellular trafficking of lysosomal hydrolases[6]

The lysosomal hydrolases are a group of more than 50 dissimilar acid hydrolases which function to degrade un-needed cellular material as well as material taken up by cells via either endocytosis or phagocytosis. As noted for other proteins, lysosomal hydrolases are assembled within the cisternae of the rough ER and, in common with other newly synthesized proteins, the nascent lysosomal hydrolases are transported to the Golgi complex which they traverse from its *cis* to its *trans* surface, passing through the *medial* and *trans*-Golgi subcompartments. In contrast to proteins destined for either secretion or transport to other intracellular sites, the lysosomal hydrolases are sorted from other newly synthesized proteins as they traverse the Golgi stacks by a complex mechanism in which *N*-acetylglucosamine phosphates are added to mannose residues and then the *N*-acetylglocosamine groups are removed, leaving the remaining mannose residues phosphorylated at the 6-position. Those mannose-6-phosphorylated lysosomal hydrolases are then bound to receptors in the *trans*-Golgi that specifically recognize mannose-6-phosphate residues. The mannose-6-phosphate receptors along with their associated lysosomal hydrolases are then shuttled to the pre-lysosomal compartment where, as a result of the acidic pH of this compartment, dissociation of the hydrolase/receptor complexes occurs, thereby liberating the hydrolases within the pre-lysosomal compartment and allowing the now unoccupied receptors to return to the Golgi where they are free to bind and transport additional mannose-6-phosphorylated lysosomal hydrolases. Of potential significance however, is the fact that, even under physiological conditions, sorting of lysosomal hydrolases from secretory proteins in the Golgi is incomplete and, as a result, a fraction of the newly synthesized lysosomal hydrolases enters the secretory pathway[7-9]. This fraction of the lysosomal hydrolases is subject to regulated secretion from the cell along with other secretory proteins[10]. Another fraction of lysosomal hydrolases may also be secreted from the cell by being enclosed within so-called secretory lysosomes[11]. Lysosomal hydrolases may also be constitutively secreted from the cell without ever being packaged within lysosomes.

Many of the lysosomal hydrolases are initially synthesized as inactive or only partially activated pro-enzymes. Their complete activation is achieved through post-translational processing of the pro-peptide as it undergoes intracellular transport to pre-lysosomes[12]. Together, pre-lysosomes, lysosomes, endosomes, phagosomes, and autophagosomes function as an

interconnected network of organelles which contain a wide variety of acid hydrolases capable of degrading nucleic acids, proteins, carbohydrates, and lipids.

Activation of digestive enzyme zymogens

Some of the digestive enzymes (e.g. amylase, lipase, DNAase, RNAase) are synthesized and secreted from pancreatic acinar cells as active enzymes but others, including most of the potentially harmful digestive enzymes (e.g. trypsin, chymotrypsin, phospholipase, elastase, carboxypeptidase) are synthesized as inactive pro-enzymes or zymogens. Under physiological conditions, activation of these zymogens does not occur until they reach the duodenum where the brush border enzyme enterokinase (enteropeptidase) catalytically activates trypsinogen and trypsin then catalyzes the activation of the other zymogens. In most instances, activation involves cleavage of the zymogen and release of an "activating peptide" which, prior to its release, had maintained the zymogen in its inactive state. Thus, quantitation of free activating peptide levels may provide information regarding the extent of zymogen activation prior to that measurement[13].

Protective mechanisms

In a general sense, the acinar cells of the pancreas are protected from the injury which might be inflicted by premature, intracellular activation of trypsinogen and other digestive enzyme zymogens by virtue of 3 features. The first, as noted above, is the fact that most of the potentially harmful digestive enzymes are normally present within acinar cells as inactive zymogens. The second is the fact that potent inhibitors of trypsin are synthesized and co-transported through the cell along with trypsinogen and those inhibitors are available to dampen any trypsin activity that might arise as a result of intracellular trypsinogen activation. The third protective feature of pancreatic acinar cells is the fact that, throughout their intracellular trafficking within the cell, digestive enzymes and their zymogens are sequestered from the remainder of the cell by being enclosed within membrane bounded organelles. Very recently, two reports have appeared that suggest that acinar cells may have yet another protective feature. Pancreatic acinar cells express proteinase-activated receptor-2 (PAR2) which is a tethered ligand receptor that is activated by trypsin and, as shown by Namkung *et al*[14] and Sharma *et al*[15], activation of pancreatic PAR2 triggers events that protect the pancreas from pancreatitis.

THE CO-LOCALIZATION HYPOTHESIS

History

The so-called "co-localization hypothesis" grew out of studies, performed more than two decades ago, that employed the diet-induced and the secretagogue-induced models of acute experimental pancreatitis[16-20]. Those studies had shown that digestive enzyme synthesis and intracellular transport continue during the early stages of pancreatitis, but secretion of newly synthesized digestive enzymes from acinar cells is blocked. Those studies had also shown that, prior to the appearance of evidence reflecting acinar cell injury, the normal segregation of digestive enzyme zymogens from lysosomal hydrolases is perturbed and, as a result, both types of enzymes become co-localized within intra-acinar cell cytoplasmic vacuoles. That co-localization phenomenon could be demonstrated using techniques of immunolocalization as well as using techniques of subcellular fractionation. Finally, those studies had shown that digestive enzyme zymogens, including trypsinogen, are activated within acinar cells at very early stages during the evolution of experimental pancreatitis-i.e. prior to the appearance of cell injury. Since the lysosomal hydrolase cathepsin B is known to be capable of activating the digestive zymogen trypsinogen[21,22] and, since trypsin can activate the other digestive zymogens, these various observations suggested the following 3-tiered hypothesis: (a) that perturbation of normal intracellular trafficking of digestive zymogens and lysosomal hydrolases, is a very early event during the evolution of pancreatitis and that, as a result of this perturbation, digestive enzyme zymogens become co-localized with lysosomal hydrolases within acinar cell cytoplasmic vacuoles; (b) that, as a result of this co-localization phenomenon, the lysosomal hydrolase cathepsin B (and, most likely, other lysosomal hydrolases) activates trypsinogen and trypsin activates the other zymogens; and (c) that the organelles containing intracellulary activated digestive zymogens become fragile and they release their content of activated enzymes within the cell interior where those enzymes trigger changes leading to cell injury/death[23].

Challenges

Subsequent to its initial proposal, the co-localization hypothesis was challenged on two grounds. The first was the fact that, while most lysosomal hydrolases have a pH optimum of roughly 5.0, the site of co-localization during the early stages of pancreatitis appears to have a neutral pH. Subsequent studies characterizing the pH optimum for cathepsin B, however, indicated that considerable (although perhaps not optimal) activity was also present at pH 7.0[24] and, thus, this concern regarding the co-localization hypothesis appears to be unwarranted. The second challenge to the hypothesis, however, proved to be more substantial because it focused on the issue of causality. Its advocates suggested that the co-localization phenomenon, although real, might be a response to the early injury of pancreatitis. They argued that the co-localization of digestive enzymes with lysosomal hydrolases might simply be a reaction to injury, rather than causing this injury[25]. Some even suggested that cell injury-induced co-localization might be a protective response which could allow lysosomal hydrolases to inactivate and degrade those prematurely activated digestive zymogens[26].

THE EVIDENCE THAT CO-LOCALIZATION LEADS TO ZYMOGEN ACTIVATION IN PANCREATITIS

The remainder of this review will be devoted to an

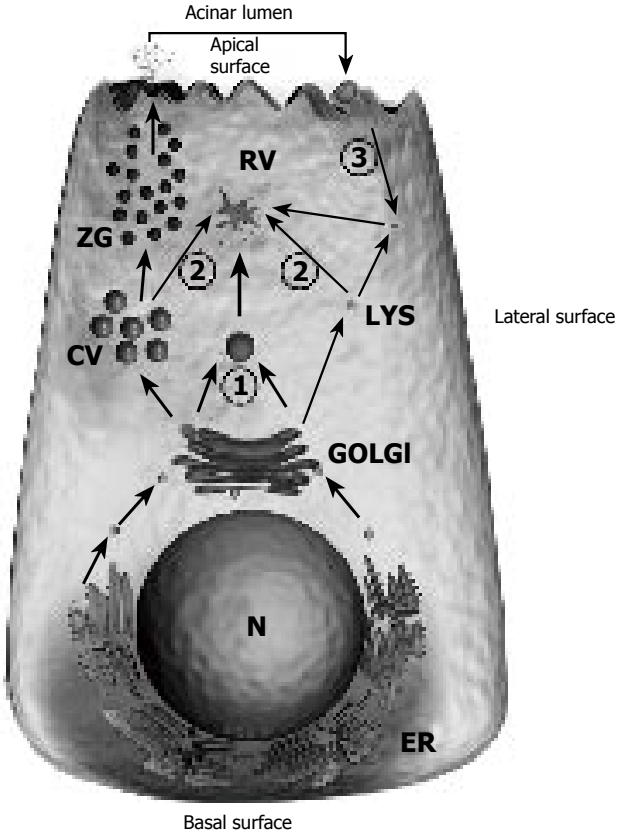

Figure 1 Acinar cell demonstrating mechanisms of co-localization. Arrows represent physiological and pathological pathways. Co-localization can occur as a result of (1) missorting of lysosomal hydrolases from digestive zymogens in the Golgi, (2) fusion of zymogen granules with lysosomes and (3) reuptake into the lysosomal compartment of secreted digestive zymogens. Note the ruptured co-localization vacuole (RV) and release of activated digestive enzymes inside the cell. (ER = endoplasmic reticulum, N = nucleus, LYS = lysosomes, CV = condensing vacuoles and ZG = zymogen granules).

examination of the evidence which suggests that the co-localization phenomenon is not simply a reaction to injury or a protective response but that it is, in fact, an early and critical event which leads to zymogen activation and acinar cell injury in pancreatitis. Ideally, studies addressing these issues would be performed using clinically derived material and patients with acute pancreatitis but, unfortunately, those studies are not possible since patients with acute pancreatitis are only identified after the very early phases of the disease have been completed and access to the pancreas of patients with early acute pancreatitis is generally not possible. Thus, by necessity, studies testing the co-localization hypothesis have all been performed using various models of acute pancreatitis in experimental animals.

Validation of the co-localization hypothesis would require fulfillment of the following 6 postulates: (1) The co-localization phenomenon should be observed in all experimental models of acute pancreatitis. (2) Lysosomal hydrolases should be capable of activating digestive enzyme zymogens such as trypsinogen. (3) During the evolution of pancreatitis, the co-localization phenomenon should occur prior to zymogen activation and cell injury. (4) The co-localization phenomenon and digestive zymogen activation should occur within the same intracellular compartment. (5) Digestive zymogen activation should be dependent upon the presence of lysosomal hydrolase activity. (6) Prevention of co-localization should interfere with zymogen activation but prevention of zymogen activation should not interfere with co-localization.

The evidence supporting fulfillment of these 6 postulates will now be summarized.

The co-localization phenomenon occurs in all models of experimental pancreatitis

As noted above, early studies performed using the diet-induced and the secretagogue (caerulein)- induced models of experimental pancreatitis and employing the techniques of both subcellular fractionation and immunolocalization, indicate that the co-localization phenomenon occurs in both of those models[16-20,9]. Subsequent studies using the rabbit model of duct obstruction[27], the closed duodenal loop model[28], and the taurocholate model of retrograde duct injection[29], indicate that the co-localization phenomenon occurs in each of those models as well. In each of these models, co-localization is an early event which can be demonstrated to occur prior to the appearance of cell injury/necrosis.

The mechanism(s) responsible for the co-localization phenomenon in each of these models appears to be different (Figure 1). In the diet-induced model, it reflects fusion of zymogen granules with lysosomes (i.e. crinophagy) while, in the secretagogue-model, it reflects both crinophagy and defective sorting of lysosomal hydrolases away from secretory proteins because of a defect in processing lysosomal hydrolases prior to their passage through the medial Golgi compartment. In the opossum model, co-localization occurs because previously secreted digestive zymogens are taken up by acinar cells via endocytosis and then transported to the endosome/lysosome compartment.

Lysosomal hydrolases such as cathepsin B can activate trypsinogen

Studies independently performed and reported by at least 3 groups of investigators have shown that cathepsin B can activate trypsinogen under in-vitro conditions[21,22,24]. The most recent of those studies evaluated the pH profile of this activation process and found that a considerable degree of activation could occur at pH 7.0[24].

The co-localization phenomenon occurs prior to acinar cell injury/necrosis

If the co-localization phenomenon is a reaction to cell injury, it should occur after changes indicative of that injury are already demonstrable while, if it is the cause of cell injury, it should occur prior to the appearance of that injury. Time-dependence studies using the secretagogue-induced model have been performed to evaluate this issue[30]. In these studies, pancreatic tissue was homogenized and subcellular fractionated to obtain zymogen granule-enriched and lysosome-enriched fractions at selected times after the start of caerulein administration. Redistribution of cathepsin B from the lysosome-enriched to the zymogen granule-enriched fraction was noted within

15 minutes after the start of caerulein administration and, at roughly this same time, evidence of trypsinogen activation (i.e. increased trypsin activity and increased pancreatic levels of trypsinogen activation peptide) was also first detected. In contrast, hyperamylasemia, pancreatic edema and acinar cell injury/necrosis were only detected at later times. Thus, these studies indicate that the co-localization phenomenon occurs prior to, and not after, cell injury in pancreatitis.

Co-localization and zymogen activation occur within the same intracellular compartment during pancreatitis

Studies addressing this issue have been performed using antibodies directed against the lysosomal hydrolase cathepsin B and antibodies directed against the peptide released during the process of trypsinogen activation (i.e. trypsinogen activation peptide, TAP). During the early stages of secretagogue-induced pancreatitis, both types of antibodies were found localized in the same cytoplasmic vacuoles[31,9,20]. Similar observations have been made in studies using antibodies directed against lysosomal membrane proteins[32]. In both cases, the site of co-localization appeared to be in the area of the *trans*-Golgi network. Taken together, these immunolocalization studies support the conclusion that co-localization and activation occur within the same intracellular compartment.

Zymogen activation and pancreatitis severity are dependent upon cathepsin B activity

This issue has been addressed in two ways. In one, cathepsin B inhibition was achieved using the cell permeant and highly specific cathepsin B inhibitor CA-074me. The effect of this inhibitor in two dissimilar models of pancreatitis (secretagogue-induced and taurocholate-induced) was evaluated[33] and, in both models, inhibition of cathepsin B was found to prevent trypsinogen activation. That inhibition was also found to reduce the severity of pancreatitis. The second approach to this issue involved the use of genetically modified mice that did not express cathepsin B and, in those mice, both trypsinogen activation and the severity of pancreatitis were found to be reduced[34]. These observations support the conclusion that cathepsin B (either alone or, perhaps with other lysosomal hydrolases) plays a critical role in trypsinogen activation during pancreatitis and that, in pancreatitis, trypsinogen activation is closely related to the severity of the disease.

Prevention of the co-localization phenomenon interferes with zymogen activation but inhibition of zymogen activation does not prevent co-localization

The co-localization of digestive zymogens with lysosomal hydrolases in both the secretagogue-induced and the taurocholate-induced models of pancreatitis is dependent upon the activity of phosphoinositide-3-kinase (PI3K) and co-localization can be prevented in those models by administration of the PI3K inhibitors wortmannin and LY49002. In a recently reported study, administration of those PI3K inhibitors was found to reduce trypsinogen activation[35]. These findings indicate that prevention of co-localization, by administration of PI3K inhibitors, can reduce zymogen activation in these models of pancreatitis.

As noted above, CA-074me is a potent in-vivo cathepsin B inhibitor and administration of CA-074me to animals can prevent trypsinogen activation during pancreatitis. To further evaluate the possible causative role of co-localization in zymogen activation, mice subjected to secretagogue-induced pancreatitis were given CA-074me and the effect of inhibiting trypsinogen activation (via inhibition of cathepsin B) on the co-localization phenomenon was evaluated (Van Acker *et al*, submitted for publication). Under these conditions, co-localization of lysosomal hydrolases with digestive enzymes was still observed even though zymogen activation had been prevented. Taken together, these findings are consistent with the conclusion that zymogen activation is dependent upon the co-localization phenomenon and they are inconsistent with the theory that zymogen activation leads to the co-localization phenomenon.

SUMMARY

Acute pancreatitis is generally believed to be a disease in which the pancreas is injured by digestive enzymes that it normally produces. Most of the potentially harmful digestive enzymes produced by pancreatic acinar cells are synthesized and secreted as inactive zymogens which are normally activated only upon entry into the duodenum but, during the early stages of acute pancreatitis, those zymogens become prematurely activated within the pancreas and, presumably, that activation occurs within pancreatic acinar cells. The mechanisms responsible for intracellular activation of digestive enzyme zymogens have not been elucidated with certainty but, according to one widely recognized theory (the "co-localization hypothesis"), digestive enzyme zymogens are activated by lysosomal hydrolases when the two types of enzymes become co-localized within the same intracellular compartment.

This review has been focused on the evidence supporting the validity of the co-localization hypothesis as an explanation for digestive enzyme activation during the early stages of pancreatitis. Indeed, there is considerable evidence that, under appropriate conditions lysosomal hydrolases such as cathepsin B are capable of activating digestive enzyme zymogens such as trypsinogen. Normally, that activation does not occur because, under physiological conditions, newly synthesized lysosomal hydrolases are segregated from digestive enzyme zymogens as the two types of enzymes traffic through acinar cells. However, during pancreatitis, that segregation is perturbed and lysosomal hydrolases become co-localized with digestive enzyme zymogens within cytoplasmic vacuoles. Evidence supporting the co-localization hypothesis as an explanation for intracellular zymogen activation include the following: (a) the co-localization phenomenon is observed in virtually all of the experimental models of pancreatitis that have been studied; (b) lysosomal enzymes such as cathepsin B can activate digestive zymogens such as trypsinogen and trypsin can activate the other zymogens; (c) the co-localization phenomenon occurs prior to the appearance of cell injury/necrosis during pancreatitis; (d) co-localization and zymogen activation occur within the

same intracellular compartment; (e) zymogen activation is dependent upon the presence of lysosomal enzyme (i.e. cathepsin B) activity; and finally, (f) preventing the co-localization phenomenon prevents zymogen activation during pancreatitis but preventing zymogen activation does not, necessarily, prevent the co-localization phenomenon. Taken together, these findings support the conclusion that co-localization of lysosomal hydrolases with digestive enzyme zymogens plays a critical role in permitting the intracellular activation of digestive enzymes that leads to acinar cell injury and pancreatitis.

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REVIEW

Novel susceptibility genes in inflammatory bowel disease

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Abstract

The inflammatory bowel disease, Crohn's disease and ulcerative colitis, are polygenic disorders with important environmental interactions. To date, the most widely adopted approach to identifying susceptibility genes in complex diseases has involved genome wide linkage studies followed by studies of positional candidate genes in loci of interest. This review encompasses data from studies into novel candidate genes implicated in the pathogenesis of inflammatory bowel disease. Novel techniques to identify candidate genes-genome wide association studies, yeast-two hybrid screening, microarray gene expression studies and proteomic profiling, are also reviewed and their potential role in unravelling the pathogenesis of inflammatory bowel disease are discussed.

Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Yeast- two hybrid; Genomics

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INTRODUCTION

Following the identification of the NOD2/CARD15 gene in 2001[1,2], the impetus has grown to identify novel genetic determinants of susceptibility and phenotype in the inflammatory bowel diseases, Crohn's disease and ulcerative colitis. The inflammatory bowel disease are now considered to be non-Mendelian polygenic disorders with important environmental interactions[3], and as such the accepted approach to identifying candidate genes in complex disease genetics has been adopted, namely genome wide scanning followed by studies of positional candidate genes in subchromosomal regions of association (loci).

A number of candidate genes and susceptibility loci have been discussed elsewhere in this issue, so in this review we first summarise recent data with regards to genome wide scanning and candidate gene studies, which may lead to gene identification. Novel techniques to identify candidate genes - genome wide association studies, yeast- two hybrid screening, microarray gene expression studies and proteomic profiling, are subsequently discussed.

GENE FINDING APPROACHES

Genome wide scan approach

Genome-wide linkage analysis using highly polymorphic microsatellite markers identified during the course of the Human Genome Project has led to success in identifying genetic determinants both in single gene disorders and in complex genetic diseases. To date, successful genome scans have typically involved several hundred microsatellite markers and a large number of multiply affected inflammatory bowel disease families (typically sibling pairs), with the aim of examining whether the degree of sharing variant alleles between affected individuals exceeds that as expected by chance alone[4].

To date, fourteen genome wide scans have been carried out in patients with inflammatory bowel disease. The most widely accepted guidelines for assessing the results are those defined in 1995 by Lander and Kruglyak who proposed criteria for reporting areas of linkage[5], with areas of 'suggestive linkage' having LOD scores of 2.2 and above and P values of less than 7×10^{-4}, areas of 'significant linkage' having LOD scores above 3.6 and P values of less than 2×10^{-5}, and areas of 'highly significant linkage' having LOD scores of 5.4 and above and P values of less than 3×10^{-7}. Areas of' confirmed linkage defined as areas of significant linkage have been replicated in an independent cohort, with a nominal P value of less than 0.01. Using these criteria, loci with confirmed linkage have been identified on chromosomes 1[6], 3 (IBD9)[7,8], 5 (IBD5)[9-11], 6 (IBD3; HLA)[10,12], 12 (IBD2)[13], 14 (IBD4)[9,14], 16 (IBD1)[13,15] and 19 (IBD6)[10] (Figure 1).

These confirmed regions of linkage need to be further narrowed by fine mapping of these areas, as each spans a large genomic region. The classical positional cloning approach is used by Hugot and colleagues to identify NOD2/CARD15 as the gene confers the critical mutations in IBD1 on chromosome 16[1]. Alternative strategies for fine mapping these regions or providing a short-cut to the critical genes are discussed in the following.

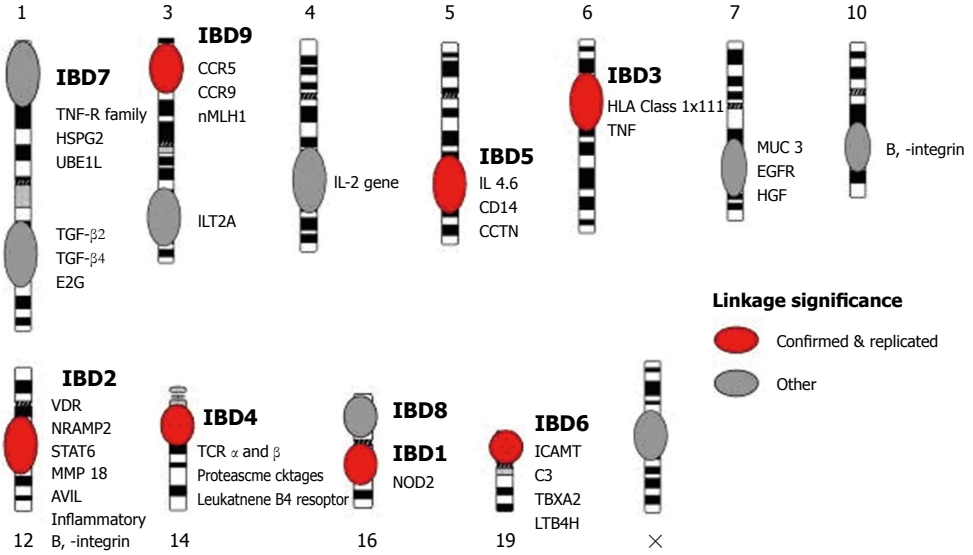

Figure 1 Confirmed and suggested linkage intervals from genome wide scans in inflammatory bowel diseases with the potential candidate genes coloured in red.

Significance levels defined by Lander and Kruglyak [95].

Positional candidate gene approach

The positional candidate gene approach to identifying association involves the use of data concerning the chromosomal location of the candidate gene as well as functional or expression data. In inflammatory bowel disease genes involved in the regulation of the innate immune system, mucosal integrity and cell-cell interactions are all clearly plausible candidate genes. By comparing the allelic frequencies of variants in these genes between patients with IBD and matched controls (case control analysis), or by investigating intra-familial association/ linkage, genes containing critical disease causing mutations may be identified.

Using this approach, Ogura and colleagues[2] identified the frameshift mutation in the NOD2/CARD15 gene (Leu1007fsinsC). Stoll and colleagues[16] also identified the DLG5 (Drosophila Discs Large Homolog 5) gene located on chromosome 10q23 by candidate gene approach allied to its position as being associated with inflammatory bowel disease in the German population[16].

However, whilst these approaches have had notable success, especially with the identification of the NOD2/CARD15 gene within the IBD1 locus, there have been difficulties in identifying candidate genes within other loci, notably the IBD5 locus as discussed elsewhere. Due to the tight linkage disequilibrium across the IBD5 locus and the wealth of candidate genes in the region[17], it has been impossible to identify with certainty the causative mutations in this region. Although Peltekova and colleagues[18] have suggested that the OCTN1/2 genes contain the causative mutations, considerably larger cohorts of inflammatory bowel disease patients (around 3000 patients) are needed to confirm these findings using genetic studies alone and functional and expression data can help delineate this region[19].

NOVEL GENES AND LOCI CURRENTLY UNDER INVESTIGATION

Genes involved in initiation and regulation of the innate immune system

The function of the immune system is to be able to recognise the vast number of antigens that are present in our environment, to mount an appropriate response to invasive pathogens and also to limit damage during inflammation and immune response to these pathogens. Dysregulation of the immune system can lead to immune-mediated diseases such as inflammatory bowel disease. Following the discovery of NOD2/CARD15, attention has focussed on the innate immune system[20]. From an evolutionary prospective, the innate immune response is more ancient than the adaptive immune response present in nearly all taxa[21]. As a further measure of the importance of the innate immune response in plants, genes involved in plant disease resistance occupy >1% of the genome of primitive species, such as *Rockcress*[22].

Toll like receptors (TLRs) are transmembrane proteins which play a pivotal role in mediating the innate immune response to viral, bacterial and fungal pathogens[23]. TLRs were initially identified in *Drosophila* and they are widely conserved across animal species[24]. In mammals TLRs are integral membrane glycoproteins which recognise conserved products unique to microbial metabolism and signal via a number of downstream signalling molecules (MyD88, Il-1R-associated kinases, TGF-β, and TNF- receptor associated factor 6)[25]. To date 11 members of the TLR family have been identified in mammals[24].

TLR4

TLR4 functions as a sensor for lipopolysaccharide (LPS), part of the cell membrane of Gram negative bacteria[26], and as such has been considered a strong functional candidate gene in the pathogenesis of inflammatory bowel disease as well as in other immunoinflammatory diseases. Variant alleles of the TLR4 Asp299Gly polymorphism have been associated with decreased bronchial responsiveness to LPS in humans and reduced activation in transfection experiments[27]. The Asp299Gly polymorphism has been associated with Crohn's disease and ulcerative colitis both in a cohort of Belgian patients[28] and in cohorts from Greece[29] (Table 1).

An association was observed between ulcerative colitis

Table 1 Number of patients studied and Toll-like receptor 4 Asp299Gly allele frequency (as a percentage of each group) in healthy controls, ulcerative colitis and Crohn's disease patients

	Healthy controls (%)	Ulcerative colitis (%)	Crohn's disease (%)	*P* value for controls versus Crohn's disease (NS: not significant)
Franchimont *et al*, Belgium (28)	139 (5.0)	163 (10.0)	334 (11.0)	0.007
Arnott *et al*, Scotland (32)	189 (8.8)	246 (6.8)	234 (10.3)	NS
Torok *et al*, Germany (30)	145 (4.0)	98 (9.0)	102 (7.0)	NS
Gazouli *et al*, Greece (29)	100 (3)	85 (3.5)	120 (7.9)	0.026
Brand *et al*, Germany (31)	199 (7.5)		204 (14.2)	0.038
Oostenbrug *et al*, Netherlands (33)	296 (4.6)	179 (5.9)	393 (6.7)	NS

These data from 6 European case control studies looking at the allelic frequencies of the Asp299Gly polymorphism of the TLR4 gene illustrate the contrasting results from these studies, and furthermore suggest a degree of genetic heterogeneity between the different populations.

and the TLR4 Thr399Ile polymorphism but not the Asp299Gly variant in one German cohort[30]. However, in a further cohort of German patients Asp299Gly variants were associated with Crohn's disease[31]. No association between inflammatory bowel disease and the TLR4 Asp299Gly polymorphism has been observed in cohorts of patients from Scotland[32]. In the light of these discordant data-sets, it is difficult to ascertain whether these two polymorphisms represent the critical disease causing mutations in the TLR4 gene[33]. Recent data from two independent mouse models have shown that treatment with TLR4 antagonists may prevent the development of colitis, further suggesting that this innate immune receptor may be central to the pathogenesis of inflammatory bowel disease[34].

TLR5

TLR5 functions by recognising flagellin, a common bacterial antigen that is present in enteric bacteria[35, 36]. Strong serological response to flagellin in multiple animal models of colitis has been observed by Lodes and colleagues[37], who demonstrated that colitis can be induced by transferring flagellin-specific T cells to immunodeficient animals. Further evidence implicating TLR5 polymorphisms in the pathogenesis of inflammatory bowel disease suggests that carriage of a dominant negative TLR5 polymorphism (TLR5-stop) appears to protect against Crohn's disease and results in a significant reduction in flagellin-specific circulating concentrations of IgA and IgG[38]. These data linking a genetic defect in TLR5 to an alteration in the acquired immune response are intriguing and of great pertinence as recent studies report synergism between NOD2/CARD15 and TLR5 signalling[39].

NOD-LLR protein family

NOD (nucleotide- binding oligomerization domain) - LLR (leucine rich region) proteins are part of the CATERPILLER (CARD, transcription enhancer, R [purine]- binding, pyrin, lots of purine repeats) family, and these proteins are key regulators of innate immunity and apoptosis in mammals and plants[40]. NOD- LLR proteins play a central role in recognising pathogens on the cell surface and in the cytosol, and a family of greater than twenty human proteins possessing a NOD domain has been identified[41]. NOD-LLR proteins are comprised of three distinct functional domains: the amino terminal effector domain involved in signalling, the centrally located NOD domain and the LLR ligand-sensing domain[42]. Three of the human NOD-LLR proteins have effector domains, namely caspase-recruitment domains (CARD): NOD1/CARD4, NOD2/CARD15 and Apaf-1.[40]

With the established role of NOD2/CARD15 in the pathogenesis of Crohn's disease, attention has focused on NOD1/CARD4 identified by two groups in 1999[43,44]. NOD1/CARD4 has a similar structure to NOD2/CARD15 with it having only one CARD domain, a central NOD domain and a LLR[45]. NOD1 detects intracellular diaminopimelic acid, a tripeptide motif found in many Gram negative bacteria and unique to Gram negative proteoglycans[46,47]. NOD1/CARD4 plays a role in colonic epithelial defence against the intracellular pathogens *E. coli* and *Shigella flexeneri*[48,49]. Its effector domain is associated with RipK2(a CARD-containing interleukin-1 beta-converting enzyme-associated kinase), thus mediating NFκB activation[43,44,50].

Linkage to the area that encompasses the NOD1/CARD4 gene, chromosome 7p14, was first found on a genome wide scan of the UK population in 1996[13]. This initial finding is replicated in two further genome wide scans involving patients in North America (USA and Canada)[6,10]. In a pan-European study involving 381 inflammatory bowel disease families from France, Sweden, Belgium, Spain, Denmark, Italy and Ireland (235 CD and 58 UC), no association between susceptibility to IBD and the E266K variant which lies within the CARD domain of NOD1/CARD4 was observed[51].

However, a further recent UK study of 556 IBD trios (294 CD and 252 UC) a significant association was observed between a complex insertion deletion allele (CCCCCCAC/ CCCCCCCCC) of NOD1/CARD4 at nucleotides 30, 258, 950 (Ensembl build 35) which is partially identified by rs6958571 and is located within intron 9, and predisposition to inflammatory bowel disease ($P = 0.002$)

and, also to ulcerative colitis ($P=0.01$)[52]. By creating a sliding two marker haplotype using this insertion deletion allele and the rs2907748 (T→ C) variant at nucleotides 30,246,263, the association was strengthened with *P* values of 3×10^{-6} for inflammatory bowel disease, 7×10^{-4} for Crohn's disease and 3×10^{-5} for ulcerative colitis. The deletion allele was significantly associated with an early age of onset of IBD (<25 years) and there was no evidence of an epistatic effect when NOD1/CARD4, NOD2/CARD15 and IBD5 are investigated.

Interestingly allelic variation of the same insertion deletion polymorphism (CCCCCCAC/ CCCCCCCCC) at nucleotides 30, 258, 950, which appear to alter the protein binding of NOD1/CARD4, is also associated with susceptibility to childhood onset of asthma and elevated serum IgE levels[53]. Further genetic and functional data with regards to NOD1/CARD4 are required to discover whether these variants play a role in the pathogenesis of inflammatory bowel disease in racially diverse populations.

CCL20

CC chemokine ligand (CCL) 20 is responsible for the chemoattraction of immature dendritic cells (DC) expressing the CCR6 receptor in the epithelium and Peyer's patches in the bowel mucosa[54]. The ligand- receptor pair of CCL20 and CCR6 also play a role in the chemoattraction of effector/memory T-cells and B-cells under homeostatic and inflammatory conditions, in diseases including cancer and rheumatoid arthritis[55]. Microarray and real-time PCR analysis of endoscopic colonic biopsies of patients with IBD have revealed increased levels of CCL20 mRNA in histologically inflamed biopsies when compared to non-inflamed IBD biopsies and inflamed non-IBD colonic biopsies[56,57].

Using microarray analysis of peripheral blood mononuclear cells in 8 patients with ulcerative colitis and 8 healthy controls, Choi and colleagues showed that the CCL20 gene is upregulated in ulcerative colitis samples[58]. Five SNPs in the promoter region of CCL20 are identified and analysed in a case control study of 118 ulcerative colitis patients and 300 healthy controls in the Korean population. Three of the CCL20 variants are significantly associated with ulcerative colitis ($P<0.0038$) and further replication data in ethnically diverse cohorts of patients with IBD are required.

Other loci

The identification of other genetic variants in loci identified by genome wide scan continues to yield other potential determinants of inflammatory bowel disease. These genes may play a role that is limited to specific populations and disease phenotypes. However, their identification can unravel the pathogenesis of inflammatory bowel disease and may reveal new therapeutic targets.

Other loci of current interest include the IBD2 locus on chromosome 12 first described in the UK[13] and subsequent analysis suggests that this locus is most strongly associated with colonic disease[59]. In the Flemish population the IBD4 region on chromosome 14 appears to be an important determinant of Crohn's disease[60] and this association has been replicated in the USA[14] and by the International IBD genetics consortium[61]. Fine mapping of the IBD2 and the IBD4 loci are required to establish which genes in these regions contain critical disease causing mutations.

NOVEL TECHNIQUES TO IDENTIFY CANDIDATE GENES

Genome wide association studies

With the advent of the HapMap project[62] and plans to include information on around 300 million genotypes, genome-wide association studies have been proposed as a powerful tool in identifying common variants that contribute to complex genetic traits[63]. Genome wide association studies compare the frequency of alleles and genotypes between cases and controls on a genome-wide scale, thus creating a comprehensive unbiased method to identify candidate genes. Advances in technology lowering the price of genotyping on a large scale and genome wide linkage disequilibrium mapping using data from the HapMap project have made this method available. However, significant concerns remain about the power of these studies and it has been proposed that levels of significance of $P<5\times10^{-8}$ are required to constitute significance in genome wide association studies[64].

Using this approach Tamiya and colleagues[65] placed 27 039 microsatellite markers across the human genome at 100kb intervals in 470 patients with rheumatoid arthritis and 470 controls. Forty seven candidate regions were identified and the previously implicated major histocompatibility complex gene HLA- DRB1 in the chromosomal region 6p21.3 was shown to be associated with rheumatoid arthritis. In the UK, the Wellcome Trust has provided initial funding for a phase I study including 1 000 patients with Crohn's disease and 3 000 controls, 675 000 SNPs will be genotyped to cover the entire genome and results are expected in 12 months.

Yeast two- hybrid screening

The yeast two-hybrid assay is an elegant means of investigating protein-protein interactions, which have become increasingly important in our understanding of biological systems and pathways. The yeast two- hybrid model can also be used to characterize interactions already known to occur, thus helping delineate the protein domains responsible for interaction and the environmental conditions involved[66].

The yeast two-hybrid assay is performed in the budding yeast, *S cerevisiae* using two fusion proteins: the target protein of interest, known as 'bait' is fused to a DNA binding domain attached to its N-terminus. The second protein, the 'prey' is fused to an activation domain. If the bait protein interacts with the prey, these bring the binding domain and the activation domain of transcriptional activator together, which in turn switches on the expression of the reporter genes. The reporter genes are constructed to allow growth of the yeast in a selective medium when the interaction occurs. When investigating 'protein- protein' interactions, a single bait protein is used to search for interaction with a library of proteins fused to the activation domain. The choice of library is determined

by the tissue of interest, e.g. intestinal cell library for inflammatory bowel disease[67].

Schizophrenia is a disease of polygenic genetic susceptibility where the yeast two hybrid systems have been successfully used to identify candidate genes. The disrupted-in- schizophrenia 1 (DISC1) gene was identified in 2 000[68] and confirmed in other cohorts,[69,70] was used as bait. DISC1 encodes a novel protein of unknown function and full-length human DISC1 protein was used to screen human adult and foetal brain libraries for interacting proteins, using the yeast two-hybrid system[71]. Twenty-one proteins from a variety of locations have been identified implicating DISC1 in several aspects of central nervous system signalling and confirming data from other yeast two- hybrid scans using DISC1 as bait[72,73]. From these data the authors are able to identify a number of potential DISC1 interactions and to speculate that DISC1 may be at the centre of an extensive protein interaction network.

Yeast two- hybrid as an investigative tool in inflammatory bowel disease

Barnich and colleagues[74] used NOD2/CARD15 gene as 'bait' to screen a bone marrow library and identified GRIM19, a protein with homology to the NADPH dehydrogenase which interacts with endogenous NOD2[74]. GRIM19 is required for NFκB activation following NOD2-mediated recognition of bacterial muramyl dipeptide and the authors hypothesised that GRIM19 is a key component of the CARD15/NOD2 signalling pathway which currently remains under detailed investigation[75-77]. In our own protein yeast two- hybrid studies a series of 12 candidate genes which interact with CARD15/NOD2 (*Nimmo, Satsangi,* unpublished data) have been identified.

GENOMICS

Microarray expression studies

Gene expression technology using microarray allows a comprehensive picture of gene expression at the tissue and cellular level, thus helping understand the underlying physiological and pathological processes. Microarray technology has developed from spotted nylon array technology used to identify genomic inserts in bacterial colonies by hybridisation with preidentified cDNAs[78]. In the seminal microarray experiment in 1995, a two-colour fluorescent pattern of differential gene expression is generated when comparing 48 genes in the root and the shoot of *Arabidopsis*[79].

Since 1995 there has been a rapid increase in the number of papers published using microarray technology from 7 in 1995-1996 to 139 in 1999 and to 3 000 in 2003[80]. The initial optimism set out by Mark Schena, one of the authors of the *Arabidopsis* microarray experiment suggesting that all human diseases can be studied by microarray technology, With the ultimate goal of this work to develop effective treatments and cures for every human disease by 2 050 has not yet fully been born out by the published data. However, a number of exciting and novel observations have been generated.

Microarray design

RNA isolated from the tissue sample or cells can be used to generate cRNA or cDNA[78]. The probe set is then designed with transcripts targeting the genes of interest and care needs to be taken to prevent cross- hybridization. It is worth noting at this point that the probe refers to the reporter sequence placed at a particular position on the array which interrogates the sample and not the other way around. The probes are hybridised on the chip with the target unknown cRNA or cDNA. Following this process which can take several hours, the unbound target is washed off and the arrays are fluorescently labelled, so that they can be analyzed by confocal laser scanning. Expression of the entire human genome can be analyzed on one chip and complex computational and statistical techniques have been developed to analyze expression data because of the large amount of data microarrays[81].

Clinical data generated by microarray

Probably the most provocative and clinically relevant data generated by microarray have been in the field of cancer research. An example of the potential of microarray has demonstrated by Alizadeh and colleagues[82]. By comparing expression analysis on a 'lymphochip' panel of 3186 genes between patients with diffuse large B- cell lymphoma (DLBCL) prior to treatment, the investigators are able to group these patients into two discrete groups: germinal centre DLBCL and activated DLBCL. When the clinical progress of these two groups of patients were examined, the germinal cell DLBCL had a higher five year survival rate than the activated DLBCL (75% $n=25$ versus 16% $n=37$ respectively, $P \leqslant 0.01$). Gene expression profiles have also been successfully used to predict prognosis in 295 patients with breast cancer[83]. However, a meta- analysis of 84 studies found that DNA microarrays have a variable performance in measuring prognosis in a number of different cancers[84].

Microarray experiments in subjects with inflammatory bowel disease

Microarray has been used to compare synovial tissue obtained from patients with severe rheumatoid arthritis and macroscopically affected bowel of patients undergoing surgery for Crohn's disease[85]. A number of inflammatory genes were commonly expressed in diseased tissues. In 2000 Dieckgraefe and colleagues[86] published a study using microarray technology to compare patients with ulcerative colitis undergoing colectomies for disease refractory to medical management and a control group. Six thousand five hundred genes were analysed and the results confirmed an increase in a number of genes previously implicated in the pathogenesis of ulcerative colitis (IL-1, IL-1 RA and IL8) and suggested that multiple members of the chemokine subfamily may play a role in disease pathogenesis.

In a further microarray experiment using surgically resected inflammatory bowel disease tissue, 7 070 genes were examined and 170 genes were differentially expressed in ulcerative colitis and Crohn's disease with almost an equal number up regulated and down regulated[87]. Twenty percent of the differentially regulated genes were common to both forms of inflammatory bowel disease and when the locations of these genes were mapped several were found to lie within IBD2 locus on chromosome 12.

More recently Langman and colleagues[88] used microarray to analyze biopsies taken from patients with Crohn's disease, ulcerative colitis and control patients, 22 283 genes were analysed. They found that genes involved in cellular detoxification and biotransformation (pregnane X receptor and MDR1) are significantly down regulated in the colon of patients with ulcerative colitis.

Endoscopic investigation of inflammatory bowel disease with the ability to take pinch mucosal biopsies has allowed investigators to take microarray tissue from a larger range of patients including those with less severe disease compared to patients requiring rescectional surgery. In a study of 24 patients with ulcerative colitis, Okahara and colleagues[89] investigated the difference in gene expression between endoscopic biopsies taken from inflamed and non inflamed areas using a 1300 gene microarray, and found that migration inhibitory factor- related protein 14 (MRP14), growth-related oncogene gamma (GROγ) and serum amyloid A1 (SAA1) were upregulated whereas TIMP1 and PDZ and LIM domain 1 (elfin) were down regulated in the inflamed biopsies when compared to the non- inflamed biopsies.

In a study involving endoscopic biopsies of patients with Crohn's disease, ulcerative colitis and controls, Costello and colleagues[90] found that 500 and 272 transcripts are differentially regulated in CD and UC, respectively. Candidate genes are confirmed by real-time PCR and immunohistochemistry, and a number of genes involved in immune regulation were identified.

Further microarray studies in inflammatory bowel disease

Microarray has been useful in studies thus far and continues to be a powerful tool in investigation of the pathogenesis of inflammatory bowel disease. Further studies need to be designed in order to allow the collection of accurate clinical data describing the phenotype and activity of inflammatory bowel disease at the time of sample collection as these data are critical in analysis and interpretation of the results. The confounding problems associated with the heterogeneous mixture of cells, which is inevitable in the analysis of entire biopsy samples, can be reduced by using laser capture micro-dissection, in order to analyze only the cells of interest. By obtaining painstakingly accurate clinical information at the time of biopsy collection and narrowing down cell heterogeneity, the amount of background 'noise' that has hampered previous microarray studies can be reduced[91]. Minimum standards of information reported on microarray data have been proposed with the aim of establishing a standard for recording micoarray-based gene expression data[92], and replication of results with real-time PCR helps to validate these studies.

PROTEOMICS

Proteomic analysis is another relatively new investigative tool that has not been used to any large extent in the field of inflammatory bowel disease. Proteomics refers to the study of the total protein content in cells and the products of genes, so as to identify differences between normal and diseased tissue[93]. This is achieved by combining the techniques of protein electrophoresis and mass spectrometry. Expression profiles of proteomes may be generated from samples of serum or secreted fluid, and may be able to differentiate disease progression, response to therapy and to identify novel therapeutic targets.

Preliminary data have been generated in our unit by comparing serum of patients with severe ulcerative colitis who responded to corticosteroid therapy and matched patients who were resistant to and failed corticosteroid therapy[94]. Proteomic profiles of corticosteroid resistant and responsive groups are significantly different at 19 protein biomarkers: 12 proteins were up-regulated and 7 proteins were down-regulated in the corticosteroid resistant group. These results suggest that protein profiling may be useful in predicting patient response to corticosteroid therapy and identification of these proteins is currently underway.

CONCLUSION

The application of novel technologies has catalyzed the search for novel determinants in inflammatory bowel disease. Proof of the principle for genome wide studies is provided by the NOD2/CARD15 discovery and investigation of genes at other loci is underway. Rigorous attention to statistical design and phenotypic classification of disease is of paramount importance in gene discovery.

With the advent of new powerful investigative tools such as microarray and proteomic analysis, large amounts of data can be generated from small studies in inflammatory bowel disease. Among the clinical challenges in harnessing the power of these new investigative tools will be to accurately define clinical phenotype in patients studied, given the heterogeneity inevitable in these studies.

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REVIEW

Chemotherapy as a component of multimodal therapy for gastric carcinoma

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Abstract

Prognosis of locally advanced gastric cancer remains poor, and several multimodality strategies involving surgery, chemotherapy, and radiation have been tested in clinical trials. Phase III trial testing the benefit of postoperative adjuvant chemotherapy over treatment with surgery alone have revealed little impact on survival, with the exception of some small trials in Western nations. A large trial from the United States exploring postoperative chemoradiation was the first major success in this category. Results from Japanese trials suggest that moderate chemotherapy with oral fluoropyrimidines may be effective against less-advanced (T2-stage) cancer, although another confirmative trial is needed to prove this point. Investigators have recently turned to neoadjuvant chemotherapy, and some promising results have been reported from phase II trials using active drug combinations. In 2005, a large phase III trial testing pre- and postoperative chemotherapy has proven its survival benefit for resectable gastric cancer. Since the rate of pathologic complete response is considered to affect treatment results of this strategy, neoadjuvant chemoradiation that further increases the incidence of pathologic complete response could be a breakthrough, and phase III studies testing this strategy may be warranted in the near future.

Key words: Surgery; Radiation; Anticancer drug; Adjuvant chemotherapy; Neoadjuvant chemotherapy

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INTRODUCTION

Despite its declining incidence in Western Europe[1] and the United States[2], gastric carcinoma remains the second most common cause of cancer death worldwide with over 600 000 deaths per year[3]. The curative treatment of gastric carcinoma remains primarily surgical. Although developments in surgery have been slowed in the West by the large percentage of patients presenting at advanced stages[4] radical gastrectomy with extended lymphadenectomy[5,6] has been performed in Japan and other East Asian countries[7] as well as at specialized centers in the West[8-12] and is now recognized as a reasonably safe procedure in experienced hands[13,14]. However, the survival benefit of extended lymphadenectomy is yet to be proven in a large-scale randomized trial[15,16], and the prognosis of patients with locally advanced gastric cancer remains dismal even after potentially curative resection. Consequently, multimodal treatment strategies involving surgery, chemotherapy, and radiation have been explored to improve on the survival of the patients with resectable advanced cancer. The current review focuses on the development and states of the art of chemotherapy given as a component of such multimodal treatments. It is not within the scope of this review to describe in detail the chemotherapeutic regimens given concurrently with radiation.

Postoperative adjuvant chemotherapy

One straightforward strategy against resectable advanced gastric cancer is to do the best that can be done by surgery and then supplement it with chemotherapy to eliminate micrometastases that may have developed before the surgery or viable cells that may have been disseminated during the surgical procedure. Based on the fractional cell kill hypothesis [17], it would be expected that the highest tolerable drug doses given at the shortest possible interval after surgery would maximize the rate of cell kill[18, 19]. The authors have shown through an in vivo model of peritoneal carcinomatosis that either oral S-1 (1M tegafur-0.4M gimestat-1M otastat potassium)[20] or intraperitoneal paclitaxel[21] can control viable intraperitoneal cancer cells at an early stage of the metastatic process, although the therapeutic effect proved limited once they have developed into gross metastases. This strategy has long been a standard of care in other cancer types including colorectal cancer, reflecting the results of several randomized phase III trials. In the case of gastric carcinoma, however, the results of phase III trials testing this strategy have been inconsistent. An early success was reported from Japan in the 1970s, where a treatment by twice weekly intravenous administrations of

0.08 mg/kg mitomycin for 5 weeks reportedly improved the 5-year survival of curatively resected Stages I~IV gastric carcinoma by 13.5%[22]. This was the result of a large-scale nationwide trial in which 714 patients had been enrolled. Survival analysis, however, showed that the number of patients receiving chemotherapy was 242, whereas the number treated by surgery alone was 283. Details of a large number of patients who had been excluded from the survival analysis have not been reported, and the reliability of the study is questionable from the current point of view. Nevertheless, the result was taken seriously at the time, and several trials in the decade to follow explored the benefits of new combinations, mostly with oral fluoropyrimidines, as well as of new routes of delivery versus intravenous mitomycin, the gold standard. It was only in late 1980s after a long dispute that the Gastric Cancer Surgery Study Group in the Japan Clinical Oncology Group (JCOG) declared that treatment with surgery alone should once again be considered as the standard of care for curatively resected gastric cancer. Since that time, several new phase III trials have been launched in Japan with surgery alone as a standard arm. However, these new generation studies have repeatedly produced negative results. The failure to prove a survival benefit may be attributed to inadequate eligibility criteria that allowed the inclusion of early-stage cancers[23], faulty study design[24], and selection of ineffective antineoplastic agents[25]. The first JCOG study to be reported was a phase III study testing the impact of intravenous mitomycin (1.4 mg/m^2) and fluorouracil (166.7 mg/m^2) twice weekly for 3 week followed by oral UFT for 18 months[23]. From subset analyses of this negative study that enrolled 579 patients, Nakajima suggested that T1 stage cancer should be excluded from future trials, whereas T2 stage cancer could be a promising target for postoperative adjuvant chemotherapy. However, eligibility criteria of the next JCOG trial testing intravenous mitomycin 1.33 mg/m^2, fluorouracil 166.7 mg/m^2, and cytosine arabinoside 13.3 mg/m^2 given twice weekly for 3 wk followed by oral fluorouracil 134 mg/m^2 daily for the next 18 mo were based on surgical rather than histopathologic findings, and this again resulted in the inclusion of several T1 stage cancers. Consequently, the excellent survival achieved by surgery alone left little room for improvement by adjuvant chemotherapy, and the study, designed unfortunately to detect a large difference of 15% in 5-year survival, wasdestined to be under-powered[24]. Another JCOG phase III trial testing the survival benefit of a combination chemotherapy with intraperitoneal CDDP (70 mg/m^2) on d1, intravenous CDDP at 70 mg/m^2 on d14, and continuous 5FU at 700 mg/m^2 on d14~16 followed by one year of oral UFT (267 mg/m^2) over surgery alone for T3~T4 stage cancer had been powered to detect a difference of 12% in 5-year survival[25]. Survival curves in this study that were found to be almost identical suggest that this trial failed because of an inadequate regimen rather than a flaw in the statistical considerations, since intraperitoneal CDDP had already proven ineffective as was reported in 1994 by a much smaller Austrian study[26].

After the aforementioned JCOG trials, a trial testing oral UFT (350 g/m^2) to be continued for 16 months was conducted for pathological T2/N1 and T2/N2 stage cancer (NSAS-GC). Although there were several new participants in the study in addition to the members of JCOG (33 institutions in all), the patient accrual was poor with only 199 patients participating in 4 years, whereas 488 were needed to detect an 8.8% difference in 5-year survival. The trial was eventually discontinued in order to carry out the next randomized trial testing a new and more promising drug, S-1[27], in the adjuvant setting (ACTS-GC). Nevertheless, a planned interim analysis at the median follow-up of 3.8 years revealed significant improvements in overall and relapse-free survival in the chemotherapy arm[28]. Patient characteristics had been well-balanced between the arms, and the study had been carefully conducted. However, this was considered too small a study to definitively prove the benefit of postoperative chemotherapy in gastric cancer. In the meantime, ACTS-GC, a nationwide trial comparing postoperative adjuvant chemotherapy by oral S-1 with surgery alone for Stages II and III gastric cancer, completed a planned enrollment of 1000 patients in the year 2005. It is hoped that the results of this trial will in the near future provide a decisive answer regarding the survival benefit of adjuvant chemotherapy with oral fluoropyrimidines following formal D2 dissection. Since the result of ACTS-GC could turn out to be negative after all, another confirmative phase III trial testing the benefit of UFT is currently planned by JCOG so that the enrollment may be completed before the interim analysis for the ACTS-GC study due to take place in 2008. Although the response rate of S-1 in cancer with measurable lesions was higher than that of UFT, there is currently no guarantee that S-1 is more effective than UFT in the adjuvant setting.

In the meantime, two randomized trials in the West have shown the advantage of adjuvant chemotherapy, one with mitomycin and tegafur[29], and the other with epidoxorubicin, 5FU and leucovorin[30]. However, prognosis of those patients treated with surgery alone in these trials had been so dismal that the patients enrolled for the trial might have been treated with or inadequately staged by suboptimal surgery. On the contrary, an adjuvant postoperative chemotherapy regimen consisting of EAP (etoposide, doxorubicin, cisplatin) followed by intravenous 5FU/LV had no survival benefit in a trial by the Italian Medical Oncology Group. Interestingly, the 5-year survival rate of the surgery alone group in that trial was relatively good at 44% [31]. Survival benefit was not proven in other modern adjuvant trials exploring the FAM (5-FU, doxorubicin, mitomycin) regimen[32] or 5FU/LV and cisplatin[33], though meta-analyses of the Western trials demonstrated some potential of this strategy[34-38]. A meta-analysis of the three Japanese randomized trials with serosa-negative cancer as a target[23, 24, 27] also suggested a survival advantage[39]. Thus, attempts to confirm a definitive survival advantage of postoperative adjuvant chemotherapy should be continued.

There is a significant difference between the Japanese and Western principles for the selection of chemotherapeutic regimens to be used in the postoperative adjuvant setting. In the West, any regimen found to be active in the treatment for unresectable/metastatic cancer could also be regarded as a candidate to be tested in the adjuvant setting. For instance, the EAP regimen tested in the Italian Trials in the Medical Oncology group study[33] had caused

concern due to its severe toxicity[40]. A Swiss study currently exploring the impact in both a neoadjuvant and adjuvant setting of a combination of 5-FU, cisplatin and docetaxel which has revealed a superior response rate compared with 5FU and cisplatin among advanced gastric cancers [41] but has also been recognized as highly toxic. The Gruppo Onocologico Italia Merideionale (GOIM) recently conducted a trial to explore the efficacy and tolerability of the addition of epirubicin to a combination of etoposide, leucovorin and 5FU (ELF), and found this regimen (ELFE) to be active for advanced gastric cancer[42]. Here again, the next step they took was to test this regimen in the adjuvant setting in a phase III study with surgery alone as a control arm[43]. Since most of the patients treated with potentially curative surgery still have dismal prognosis in the West, investigators there do not hesitate to introduce toxic regimens that were nevertheless found to be active against advanced cancer into trials for postoperative adjuvant chemotherapy.

The situation is different in Japan where regimens used in adjuvant settings tend to be relatively mild. This is because a larger proportion of patients to be treated with postoperative adjuvant chemotherapy will survive by surgery alone, so that treatment-related toxicities and impairment of patient's quality of life are less likely to be tolerated. For these reasons, adjuvant chemotherapies in Japan have centered around oral fluoropyrimidine-based regimens with mild toxicities. The aforementioned regimen of intravenous mitomycin, fluorouracil, and cytosine arabinoside followed by oral fluorouracil, for example, induced Grade III leucopenia in 1.6% and Grade III gastrointestinal symptoms in 0% of patients treated in the adjuvant setting[24]. A highly regarded combination of oral S-1 (80 mg/m^2 for 21 d with 14 d of rest) and cisplatin (60 mg/m^2 on d8) that achieved a response rate of >70%[44], for instance, had been frequently used in Japan for unresectable/metastatic cancer, but has not been considered as a candidate for postoperative adjuvant chemotherapy. Use of mild regimens demonstrating moderate reponse rates are regarded by some investigators as old-fashioned[45], but as described earlier, the possibility that these regimens may still prove useful in pT2 stage cancers treated with adequate surgery cannot be currently denied.

Finally, a positive result in a trial testing adjuvant chemoradiation needs to be mentioned. In the United States, an Intergroup Trial (SWOG-9008/INT0116) was launched in 1991 to test the effect of combined radiation and fluorinated pyrimidine in the adjuvant setting[46]. The treatment consisted of five consecutive days of bolus 5FU (425 mg/m^2) /LV (20 mg/m^2) before and after 45 Gy of radiation given for 5 weeks, with intravenous 5FU (400 mg/m^2) /LV (20 mg/m^2) on the first four and last three days of radiotherapy. The trial enrolled 556 patients, and the median overall survival in the surgery-only group was 27 mo as compared with 36 mo in the chemoradiotherapy group, showing a significant survival benefit for chemoradiation. Since radiation was delivered to the gastric bed and regional lymph nodes, the object of chemoradiation would seem to have been to combat any locoregional residual disease, and it was indeed for this pattern of recurrence that a significant decrease in the incidence was observed among the treatment group. The extent of lymph node dissection being D0 in 54% of the patients enrolled, some skepticism arose as to whether chemoradiotherapy might have effectively compensated for the suboptimal surgery in terms of local control[47, 48]. These observations point to the importance of quality control in surgery even when multimodality treatments are being discussed[26]. Although postoperative chemoradiation has not been seriously explored in Japan where investigators believe that local control can be achieved through extended lymphadenectomy, this may be a useful option in countries where systematic lymphadenectomy has not become a standard practice.

Future perspective regarding postoperative adjuvant chemotherapy

A breakthrough in clinical trials testing postoperative adjuvant chemotherapy may be achieved through a customized approach in which the candidates for adjuvant chemotherapy are more meticulously selected. Detection of minimal residual disease may be one of the options, since chemotherapy given to a patient with no residual disease would only be needlessly harmful. This detection can be done through immunostaining or polymerase chain reaction of protein, gene mutation, or mRNA expression that may be present in cancer cells while absent in non-cancer cells that may be included in the samples[49]. Although the prognostic value of micrometastasis detected in the lymph node through immunostaining remains controversial[50, 51], detection of free cancer cells in peritoneal washing samples by a conventional cytologic examination is a strong prognostic factor predicting the risk for peritoneal carcinomatosis[52]. Enhanced detection through reverse-transcriptase polymerase chain reaction was found to be even more potent as a prognostic factor[53-55].

Identification of patients at risk will have little value unless effective drugs are available. Adequate selection of anticancer drugs could be achieved through *in vitro* chemosensitivity testing in which the rates of growth in relation to a control of viable cancer cells from the surgical specimens are tested in culture media containing various antineoplastic drugs. A retrospective study has shown that patients treated with postoperative adjuvant chemotherapy using a certain drug had a better outcome when the result of *in vitro* chemosensitivity testing for that drug had been positive[56]. Prospective studies to confirm this phenomenon are currently underway by several study groups in Japan. However, a randomized trial comparing patients whose treatments are selected based on chemosensitivity testing and those who were treated with empirical treatments is still needed to definitively assess the benefit of this costly and time-consuming procedure[57,58].

Neoadjuvant chemotherapy for resectable gastric cancer

Due to the consistently poor outcome of patients treated with a strategy of surgery followed by chemotherapy, preoperative chemotherapy has for several years attracted the attention of investigators in the West[59]. This is considered effective for patients in advanced T and N stages, and may result in downstaging of the tumors and consequently improving the curative resection rate. Since the best response is likely to be achieved within a few months from

the initiation of therapy, 2 to 3 courses of preoperative chemotherapy are usually performed in most clinical trials. Naturally, a high response rate is required for the chemotherapeutic regimens to be used in this setting in order to achieve downstaging. Chemotherapeutic regimens with high response rates usually are associated with greater toxicity and may not be feasible in the postoperative adjuvant setting, since chemotherapy performed immediately after gastric surgery is often marred by surgery-related gastrointestinal symptoms. Preoperative delivery of chemotherapy would be much less problematic, and this underscores the major appeal of neoadjuvant therapy[60]. Another theoretical benefit of neoadjuvant chemotherapy concerns micrometastases that are undetected at the initiation of treatment. Patients with locally advanced cancer are more or less likely to harbor distant micrometastases which could remain untreated for several weeks when the surgery-first strategy is selected.

It appears so far that survival rates are excellent among patients who respond to neoadjuvant chemotherapy[60]. However, whether a group of patients as a whole benefits from this strategy needs to be carefully elucidated, and several phase III trials are ongoing after producing promising phase II results[61-63]. Of these, a MAGIC trial exploring pre- and postoperative ECF regimens (a combination of epirubicin, CDDP, and continuous infusion of 5FU) has enrolled patients as planned and has proved the significant benefit of chemotherapy (improvement of 13% in a 5-year survival rate)[64]. Although three cycles each of ECF were to be performed before and after surgery, compliance with chemotherapy was unexpectedly lower after surgery (only 42% of patients completed postoperative chemotherapy), so that the result of MAGIC trial is considered mainly to reflect the effect of preoperative ECF. Tumor diameter, depth of invasion, and nodal status of the surgical specimens were significantly decreased or improved in the chemotherapy group, indicating that downstaging actually took place in a significant proportion of patients. Despite the toxicity of neoadjuvant chemotherapy, the mortality and morbidity in surgery following chemotherapy seems to have been comparable to what it would have been without preoperative chemotherapy.

In Japan, two phase II studies await survival analysis after enrollment as planned: one is exploring a combination of CPT-11 and CDDP[65] performed against gastric cancer with bulky nodal metastasis, and the other is testing CDDP and oral S-1[44] against linitis plastica type (type 4) and large type 3 cancers. A phase III trial comparing neoadjuvant chemotherapy by CDDP and S-1 with surgery alone for types 3 and 4 cancers will soon be launched. The concept of chemotherapy in the neoadjuvant setting is similar between Japan and Western countries in that regimens with high response rates have usually been selected. It has been proven in other types of cancer that only neoadjuvant chemotherapy using a regimen with a high pCR (pathological complete response) rate can really affect survival[66]. A high incidence of PR (partial response) was reported in a phase II trial of chemotherapy with CDDP and S-1, but CR was seldom observed as a result of treatment with this combination[44]. Thus, there is a theoretical concern that the expected survival benefit may not be obtained by a neoadjuvant chemotherapy relying on this regimen, although the consequences of neoadjuvant chemotherapy followed by gastrectomy plus extended lymphadenectomy with sufficient nodal clearance remain of interest.

The pCR rate can be enhanced by the addition of radiation, which has been shown to have a beneficial impact on the surgical outcome in esophageal and rectal cancer. Anticancer drugs in this case are in part considered as radiosensitizers, and 5FU, cisplatin, and paclitaxel have been used alone and in combination[67-69]. A pCR was achieved in 20% of patients who received two cycles of continuous 5FU, paclitaxel, and cisplatin followed by 45 Gy radiotherapy with concurrent 5FU and paclitaxel[70]. Although chemotherapy performed concurrently with radiation may be somewhat inadequate against potential micrometastases that may exist outside the field of radiation, a three- step strategy with intense induction chemotherapy prior to the preoperative chemoradiation and surgery may overcome this weakness. The validity of this strategy needs to be proven by a well-designed phase III trial.

CONCLUSIONS

Since the prognosis of locally advanced gastric cancer remains poor, several multimodality strategies involving surgery, chemotherapy, and radiation have been tested in clinical trials. Postoperative adjuvant chemotherapy with highly active regimens generally had disappointingly little impact on survival for advanced cancer, although there is a hard evidence that locoregional control could be improved by postoperative chemoradiation where systemic removal of the regional lymph nodes has not been conducted.

Moderate chemotherapy with oral fluoropyrimidines may be effective against T2 N+ stage cancer, although another confirmative trial is needed to prove this point. For more advanced disease, investigators have turned to neoadjuvant chemotherapy in the hope that downstaging might facilitate an R0 resection and that distant micrometastasis could be eliminated. A large randomized study testing pre- and postoperative ECF has confirmed the survival benefit of this strategy, and several phase III trials exploring other promising regimens are ongoing. The outlook is even brighter with neoadjvant chemoradiation that further increases the incidence of pathologic CR, and phase III studies testing this strategy are warranted.

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ESOPHAGEAL CANCER

Expression of midkine and its clinical significance in esophageal squamous cell carcinoma

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Abstract

AIM: To investigate the expression of midkine in esophageal squamous cell carcinoma (ESCC) and analyze its relationship with clinicopathological features.

METHODS: RT-PCR and immunocytochemical staining were used to detect the expression of midkine mRNA and protein in EC109 cells, respectively. Then the expression of midkine in 66 cases of ESCC samples were detected by immunohistochemistry using monoclonal antibodies against human midkine.

RESULTS: Midkine was expressed in EC109 cell by RT-PCR and immunocytochemistry. The immunoreactivity was detected in 56.1% (37/66) of the ESCC samples. The expression of midkine was found in cytoplasm of tumor cells. Notably, the intensity of midkine was stronger at the area abundant in vessels and the invading border of the tumors. Midkine was more intensely expressed in well differentiated tumors (76.9%) than in moderately and poorly differentiated tumors (43.1% and 41.2%, respectively) ($P < 0.05$). There was no statistically significant correlation between midkine expression and gender, age, clinical stage, lymph node metastasis or survival in ESCC.

CONCLUSION: Midkine is overexpressed in ESCC. It may play a role in tumor angiogenesis and invasion. The expression of midkine is correlated with tumor cell differentiation in ESCC. The more poorly tumor cells differentiate, the weaker midkine expresses.

Key words: Midkine; Esophageal squamous cell carcinoma; Expression

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INTRODUCTION

Midkine is a novel heparin-binding growth factor, originally identified as the product of a retinoic acid-responsive gene. Midkine and pleiotrophin (PTN; also called HB-GAM) are the only members of a family distinct from other heparin-binding growth factor families[1,2]. In the last few years, midkine has been found to be overexpressed in various human malignant tumors, such as esophageal[3-5], gastric[4,5], colorectal[4-6], liver[4,7,8], lung[9], thyroid[10], urinary bladder[11] and prostate carcinomas[12], as well as neuroblastomas[13] and astrocytomas[14,15]. Especially in neuroblasmas[13] and bladder carcinomas[11], the level of midkine expression correlates negatively with the patients' prognosis. Furthermore, it has also been reported that midkine is extensively expressed in the early stages of carcinogenesis[6,12]. All of these studies suggested that midkine may play an important role in carcinogenesis, development and metastasis of tumors, and that it could serve as a novel tumor marker.

In the present study, we investigated midkine expression in esophageal squamous cell carcinoma (ESCC). First, we detected midkine mRNA and midkine protein expression in EC109 cells (a cell line of well differentiated human esophageal squamous cell carcinoma) by means of RT-PCR and immunochemical staining, respectively. Then we examined midkine expression in 66 cases of ESCC tissues by immunohistochemical staining using monoclonal antibody against human midkine, and analyzed the correlation between midkine expression and clinicopathological features.

MATERIALS AND METHODS

Cell lines

EC109 cells, a well differentiated human ESCC cell line, established by National Laboratory of Molecular Oncology, Cancer Institute and Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College

in 1976, and generously supplied by Department of Immunology, the School of Oncology, Peking University, were maintained in RPMI1640 with 100 mL/L heat-inactivated fetal bovine serum in humidified 50 mL/L CO_2 atmosphere at 37 ℃.

RT-PCR

Total RNA was extracted from EC109 cells with a single method using the Trizol reagent according to the manufacturer's protocol. Complementary DNA (cDNA) of EC109 cells was synthesized from 5 μg of total RNA using M-MLV reverse transcriptase. PCR was performed in a total of 20 μL reaction mixture, comprising Taq DNA polymerase, dNTPs, buffer, 10 μmol/L of each gene-specific forward and backward primers and 2 μL of cDNA. β-actin mRNA levels were used as internal controls. The sequences of PCR primers are as follows: for midkine, 5' AAAGAATTCGAGATGCAGCAC CGAGG 3' (forward) and 5' AAACTCGAGCCAG GCTTGGCGTCTAGTC3' (backward); for β-actin, 5' AACTGGGACGACATGGAGAA 3' (forward) and 5' GGTAGTCAGTCAGGTCCC3' (backward). Expected RT-PCR product sizes were 477 bp for midkine and 318 bp for β-actin. PCR conditions consisted of an initial denaturation step for 5 min at 94 ℃, followed by 35 cycles of amplification (denaturation for 30 s at 94 ℃, annealing for 30 s at 62.5 ℃, and extension for 30 s at 72 ℃) and a final extension for 10 min at 72 ℃. Then 7 μL of PCR product was electrophoresed on 15 g/L agarose gels to visualize cDNA products.

Immunocytochemical staining of EC109 cells

Dako EnVision system was used for imm-unocytochemical staining of midkine. EC109 cells were fixed with acetone/methanol (1∶1, V/V). Endogenous peroxidase activity was blocked by incubating the sections with 30 mL/L hydrogen peroxide for 10 min at room temperature. After washed with PBS, the slides were blocked with 50 mL/L horse serum for 1 h at 37 ℃, followed by incubation with mouse anti-human midkine antibody overnight at 4 ℃. After washed with PBS, the slides were incubated with EnVision for 1.5 h at 37 ℃ and then further washed for 3 times with PBS. After that the sections were reacted with DAB for 5 to 7 min to allow visualization. Finally, the slides were counterstained with hematoxylin, dehydrated, and evaluated under a light microscope.

Patients

Sixty-six specimens of ESCC were obtained from patients who underwent surgery at Beijing Cancer Hospital from Aug. 1998 to Dec. 2000. None of the patients received chemo- or radiotherapy prior to resection. These patients included 56 males and 10 females, aged from 38 to 76 years with a median age of 57.5 years. All the cases had been pathologically proven to be ESCC, including 26 well, 23 moderately and 17 poorly differentiated tumors; and 35 metastatic lymph node specimens.

Immunohistochemical staining of tumor tissues

Mouse monoclonal antibody against human midkine was prepared by our laboratory[16]. Secondary antibody of EnVision kit was purchased from Dako Inc. The sections were stained using standard EnVision, peroxidase method. Four-micrometer-thick tissue sections were dewaxed with xylene and rehydrated with graded ethanol, then briefly immersed in water. Endogenous peroxidase activity was blocked by incubating the sections with 30 mL/L hydrogen peroxide for 10 min at room temperature. Then heat-mediated antigen retrieval was performed by heating the sections (immersed in 0.01 mol/L citrate buffer, pH 6.0) in a microwave oven for 10 min. After washed with PBS, the slides were blocked with 50 mL/L milk to prevent nonspecific binding for 1 h at 37 ℃, followed by incubation with mouse anti-human midkine antibody overnight at 4 ℃. After washed with PBS, the slides were incubated with EnVision for 1.5 h at 37 ℃ and then further washed for 3 times with PBS. Finally, the sections were reacted with DAB for 5 to 7 min to allow visualization, and counterstained with hematoxylin, dehydrated, and evaluated under a light microscope. The sections known to be midkine positive were stained under the same conditions as above and served as positive controls. For negative controls, sections were processed as above but the primary antibody was replaced by PBS. Anti-midkine immunoreactivity was confined primarily to the cytoplasm. One hundred cells from 5 randomly selected representative fields (× 400) of each section were counted. It was consid-ered positive when all immunoreactivity levels were more than 10% with anti-midkine antibody.

Statistical analysis

The χ^2 test was used to examine the differences of midkine expression between groups, and Cox regression was employed to analyze the correlation between midkine expression and patients' post-operation survival time by SPSS 11.0 software. $P<0.05$ was considered as statistically significant.

RESULTS

Midkine expression in EC109 cells

Midkine mRNA transcription was detected in EC109 cells by means of RT-PCR (Figure 1). After amplification of RT-PCR, we observed that there were two strands located in the gaps from 400 bp to 500 bp and from 300 bp to 400 bp, respectively. The sizes of RT-PCR product indicated by strands were conformed with expected RT-PCR product sizes of 477 bp for midkine and 318 bp for β-actin. The positive signals of midkine protein immunocytochemical staining were located in cytoplasm. Expression of midkine protein was observed in EC109 cells (Figure 2).

Expression of midkine protein in ESCC tissues

Midkine expression was confined primarily to the cytoplasm, shown as brown granules (Figure 3A). Midkine was expressed in 37 of 66 ESCCs (56.1%), while it was present at a very low level in just a few normal esophageal epithelia adjacent to tumor tissues. Midkine positive tumor cells were distributed in foci, and the intensity of midkine was stronger at the area abundant in vessels and the invading border of tumors (Figure 3B). In addition, Midkine ex-

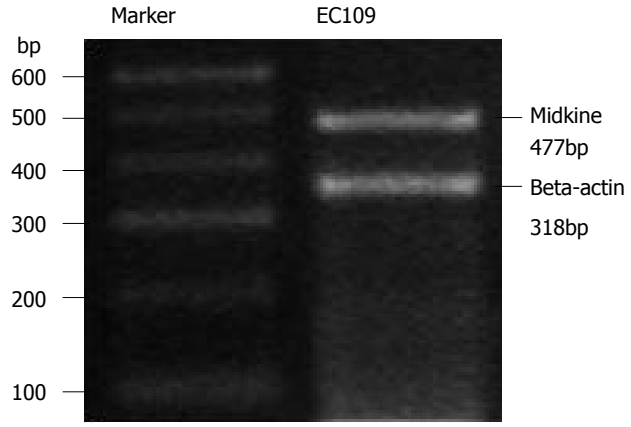

Figure 1 Amplification of midkine mRNA in EC109 cells by RT-PCR.

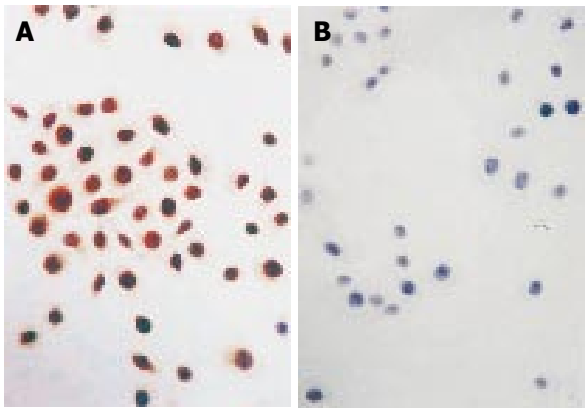

Figure 2 Midkine expression in EC109 cells (Immunoperoxidase method ×400). **A**: Positive in EC109 cells; **B**: Negative control.

pression was also found in epithelial cells and smooth muscle cells of vessels in tumor stromal elements.

Correlation between midkine protein expression and clinicopathological features

Midkine was expressed in 20 of 26 well differentiated ESCCs (76.9%), more than that in moderately (10/23, 43.1%) and poorly (7/17, 41.2%) differentiated tumors ($P<0.05$). There was no statistically significant correlation between midkine expression and gender, age, clinical stage, lymph node metastasis or post-operation survival time in ESCC ($P>0.05$, Table 1).

DISCUSSION

Many studies have shown that growth factors not only promote tissue proliferation but also induce malignant transformation, and they play important roles in the development of neoplasm[17]. Over-expression of growth factors has been found in many human tumors. Midkine, a novel heparin-binding growth factor, is strongly expressed in mid-gestational period. While in normal adult tissues, its expression is highly restricted. It is normally highly expressed in small bowel epithelium, moderately expressed in the thyroid and lowly expressed in lung, stomach, colon and kidney[18]. In the last few years, Midkine has been found

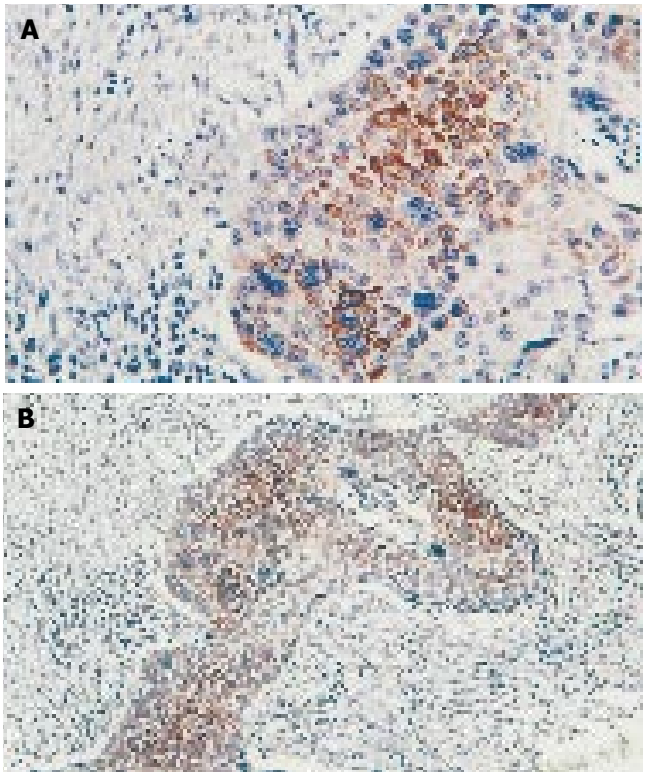

Figure 3 Midkine expression in ESCC (Immunoperoxidase method). **A**: In cytoplasm (×400); **B**: Stronger at the area abundant in vessels (×200).

to be over-expressed in various human malignant tumors, such as esophageal[3-5], gastric[4,5], colorectal[4-6], liver[4,7,8], lung[9], thyroid[10], urinary bladder[11] and prostate carcinomas[12], as well as neuroblastomas[13] and astro-cytomas[14,15]. Especially in neuroblasmas[13] and bladder carcinomas[11], the level of midkine expression correlates negatively with the patients' prognosis. It has also been reported that midkine is extensively expressed in the early stages of carcinogenesis[6,12]. Further studies showed that midkine had several cancer-related activities. Midkine could transform NIH3T3 cells[19] and enhanced the plasminogen activator/plasmin levels in bovine endothelial cells[20]. It also promotes cell growth[21,22], cell survival[23] and migration of various cells such as neutrophils and macrophages[24]. These biological activities of midkine supported the possible involvement of midkine in carcinogenesis and tumor advancement.

To further investigate the role of midkine in carcinogenesis and tumor advancement, we examined midkine expression in ESCC. First, we found that midkine was expressed in EC109 cell by means of RT-PCR and immunocytochemistry. Further, we examined midkine expression in 66 ESCC samples using immunohistochemistry. Our data showed that midkine was over-expressed in ESCC with the positive rate of 56.1% (37/66), while it was present at a very low level in just a few normal esophageal epithelium adjacent to tumor tissues. And the intensity of midkine was stronger at the area abundant in vessels and the invading border of tumors. Midkine expression was also found in epithelial cells and smooth muscle cells of vessels in tumor stromal tissues. These characteristics of distribution were confirmed in Kato's study[10], suggesting

Table 1 Intensity of midkine expression in relation to clinicopathological variables in esophageal squamous cell carcinoma

Variable		*n*	Positive *n*	Negative *n*	Positive rate (%)	χ^2 value	*P* value
Gender	Male	56	31	25	55.4	0.074	0.785
	Female	10	6	4	60.0		
Age (yr)	60	35	15	20	57.1	0.035	0.851
	⩾60	31	17	14	54.8		
Differentiation[a]	Well	26	20	6	76.9	7.906	0.019
	Modertate	23	10	13	43.5		
	Poor	17	7	10	41.2		
TNM Classification	I~II	15	16	7	69.6	2.669	0.102
	III~IV	13	21	22	48.8		
Lymph node metastasis	Positive	35	18	17	51.4	0.651	0.420
	Negative	31	19	12	61.3		

[a]$P<0.05$ as compared between well differentiated and moderately or poorly differentiated tumors.

that midkine may play a role in tumor angiogenesis and invasion. It has been demonstrated that midkine could promote the endothelial cells growth and it is a novel molecular mediator in tumor angiogenesis[25].

Our study also showed that the expression of midkine was correlated with tumor cell differentiation in ESCC. Midkine was more intensively expressed in well differentiated tumors (76.9%) than in moderately (43.1%) and poorly (41.2%) differentiated tumors ($P<0.05$). The phenomenon that the more poorly tumor cells differentiated, the weaker midkine was expressed was also observed in other studies on midkine expression in esophageal cancer and vulvar tumors[2,26].

In summary, our study showed that midkine was overexpressed in ESCC. It may play a role in tumor angiogenesis and invasion. The expression of midkine was correlated with tumor cell differentiation in ESCC: the more poorly tumor cells differentiated, the weaker midkine was expressed. Since midkine is a secretory protein, midkine level in blood can be detected when it over-expresses in tumors. It has been reported that an elevated serum midkine level can be detected in many malignant tumors, and serum midkine level decreases after the removal of tumors[27, 28]. In addition, the elevated midkine level even can be detected in urine[29]. These studies suggest that midkine could serve as a tumor marker which can be conveniently detected.

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Relationship between somatostatin receptor subtype expression and clinicopathology, Ki-67, Bcl-2 and p53 in colorectal cancer

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Abstract

AIM: To study the SSTR1, 2, 3, 4, 5 expression and their relationships with clinico-pathological factors, cell proliferation, Bcl-2 and p53 expression in colorectal cancer cells.

METHODS: Immunohistochemical staining of five SSTR subtypes, Ki-67, Bcl-2 and p53 was performed by the standard streptavidin-peroxidase (SP) technique for the paraffin sections of 127 colorectal cancers. and expression of five SSTR subtypes in 40 specimens of normal colorectal mucosae was detected with the same method.

RESULTS: Positive staining for five SSTR subtypes was observed in colorectal cancer cells and normal colorectal mucosae. SSTR1 was the most predominant subtype in both colorectal cancer and normal colorectal mucosa, and the second was SSTR5 or SSTR2. As compared with normal colorectal mucosa, SSTR4 was more frequently expressed in colorectal cancer cells (2.5% *vs* 18.9%, $P < 0.05$); the expression of SSTR2, 4, 5 in moderately to well differentiated colorectal adenocarcinoma was significantly higher than that in poorly differentiated ones ($P < 0.05$), the SSTR1 expression in colorectal cancer with positive lymph node metastasis was significantly higher than that with negative lymph node metastasis (72.2% and 54.5%, $P < 0.05$). In addition, in the ulcerative type of colorectal cancer, SSTR2 expression was obviously decreased ($P < 0.05$); the correlation did not reach a statistical significance between the five SSTR subtypes expression and Dukes'stages ($P > 0.05$), but the frequency of SSTR1 expression increased with Dukes' stage, while SSTR3 and SSTR5 expression decreased with Dukes' stage. Moreover, there was no correlation between expression of the five SSTR subtypes and other clinicopathological factors such as age, sex, tumor site, tumor depth, distant metastasis. The proliferative indexes in colorectal cancer cells with negative expression of SSTR2 and SSTR3 were significantly higher than that with positive expression ($P < 0.05$). The Bcl-2 expression in colorectal cancer cells with positive expression of SSTR1, 2, 3, 5 was significantly lower than that with negative expression ($P < 0.05$). There was no correlation between five SSTR subtypes and p53 expression.

CONCLUSION: The most predominant SSTR subtype is SSTR1, and the second is SSTR2 or SSTR5. Five SSTR subtypes play different roles in the development of colorectal cancer. SSTR2 and SSTR3 can inhibit the proliferation and promote apoptosis of tumor cells.

Key words: Somatostatin receptor subtype; Cell proliferation; Apoptosis; p53; Colorectal cancer; Immunohistochemistry

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INTRODUCTION

Somatostatin analogue (SSTA) may inhibit the growth of various tumor cells by direct interaction with specific somatostatin receptors (SSTR) on tumor cells. However, conflicting clinical results were obtained in patients with colorectal cancers treated with SSTA[1]. Many authors think the rationale for the clinical efficacy of SSTA in the management of colorectal cancer may be related to the direct inhibitory effect of SSTA, consequently, with the expression of SSTR subtypes on tumor cells[2,3]. To date, five SSTR subtypes, SSTR1-SSTR5, have been cloned in human tissues, however, few researches on their expres-

cance were reported, and the results were inconsistent[3-6]. In this study, we used an immunohistochemical method to detect the expression of five SSTR subtypes protein in colorectal cancer cells and to explore correlation between each subtype expression and clinicopathological factors, cell proliferation or apoptosis. It will provide the basis for the treatment of colorectal cancer with SSTA.

MATERIALS AND METHODS

Patients and samples

A total of 127 cases of colorectal adenocarcinoma were involved in this study. The patients with colorectal carcinomas, who underwent surgical resection at the Second Hospital Affiliated to Fujian Medical University (Quanzhou, China) from January 1, 2003 to June 30, 2004, had received neither chemotherapy nor radiation therapy before surgery. There were 73 men and 54 women, and their mean age was 58.5 (SD, 13.9; range, 24-84) years. specimens were obtained from right colon (31), left colon (27), rectum (69). According to the Chinese Dukes' Staging criteria, 15 cases were stage A, 31 cases were stage B, 46 cases were stage C and 14 cases were stage D. Among 127 patients, 102 were moderately to well differentiated and 25 poorly differentiated adenocarcinomas. Meantime, 40 specimens of morphologically normal colorectal mucosae were examined from the same patients at a minimal distance of 10 cm from the diseased area.

All specimens were routinely fixed in neutral-buffered formalin and embedded in paraffin. Serial 4 μm sections were cut from archived paraffin blocks and subjected to immunohistochemical study. In each case, standard hematoxylin-eosin staining was employed for routine histological examination.

Reagents

Goat anti-human SSTR2, SSTR3, SSTR4 and SSTR5 polyclonal antibody were purchased from Santa Cruz Biotechnology Co. (California, U.S.A.). Rabbit anti-human SSTR1 polyclonal antibody, mouse anti-human Ki-67 monoclonal antibody (MBI.1), mouse anti-human Bcl-2 monoclonal antibody (100/D5), mouse anti-human p53 monoclonal antibody (DO-7), SP staining kit and DAB kit were supplied by Maixin-Bio Co., Fuzhou, China.

Methods

The immunohistochemical staining for five SSTR subtypes, Ki-67, Bcl-2 and p53 expression in colorectal cancer cells and five SSTR subtypes expression in normal colorectal mucosae were carried out by the standard streptavidin-peroxidase (SP) technique. Briefly, after deparaffinization and rehydration, the antigen retrieval of the sections was achieved by incubation in 0.01mol/L citric acid buffer (pH 6.0) and boiled for 1 min in a pressure cooker, and then cooled and washed in tap water. The sections were incubated with hydrogen peroxide for 10 min and washed in PBS. Nonspecific reactions were blocked by incubating the sections in a solution containing normal serum. The sections were incubated with a primary antibody (anti-SSTR1, SSTR2, SSTR3, SSTR4, SSTR5, Ki-67, Bcl-2 or p53 antibodies) overnight at 4℃. The working dilution of anti-SSTR2, SSTR3, SSTR4, SSTR5 antibodies was 1:400. Rinsed with PBS, then the sections were incubated for 10 min at room temperature with biotinylated secondary antibody. After washing, streptavidin biotin complex conjugated to horse-radish peroxidase was applied for 10 min at room temperature. Again after three rinses with PBS, the sections were incubated with diaminobenzidine substrate, then rinsed with distilled water and counterstained with hematoxylin, finally dehydrated and cover slipped. The sections were prepared for microscopic observation. The known positive colorectal cancer tissues were used as positive control. As negative control, PBS was used to replace primary antibody.

Scoring criteria for SSTR subtypes expression

Intensity and percentage of positive cells were used to evaluate each tissue section. The mean percentage of positive tumor cells to normal epithelial cells in at least five areas at 400 magnification was determined and assigned to one of five categories: 1) 0, < 5%; 2) 1, 5% to 24%; 3) 2, 25% to 49%; 4) 3, 50% to 74%; and 5) 4, ≥ 75%. The intensity of SSTR subtype immunostaining was scored as 0 (achromatic), 1(light yellow), 2(yellow), and 3(brown). The percentage of positive cells and staining intensity were multiplied to produce a weighted score for each case. Cases with weighted scores less than 1 were defined as negative; all others were defined as positive.

Evaluation of staining for p53 and Bcl-2

For p53 or Bcl-2 expression, cases with ≤ 10% positively-stained tumor cells were defined as negative; otherwise, the definition was positive.

Determination of the Ki-67 proliferative index

At least 5 high-power fields were chosen randomly in each section, and 500 cells were counted for each field. The Ki-67 proliferative index was defined as the number of Ki-67-positive nuclei divided by the total number of colorectal cancer cells counted and was expressed as a percentage.

Statistical analysis

The statistical software package SPSS 11.5 was used. Clinicopathological variables associated with SSTR subtypes expression as well as the correlation between SSTR subtypes and p53 or Bcl-2 expression were analyzed by either the χ^2 test or Fisher's exact test. The correlation between SSTR subtypes and proliferative index was analyzed by t test. $P < 0.05$ was considered significant.

RESULTS

Expression of five SSTR subtypes

Positive-staining for all SSTR subtypes was identified in the cytoplasm and membrane of colorectal cancer cells and normal colorectal mucosa with brown-yellow granules (Figures 1-5). The positive rates of SSTR1, SSTR2, SSTR3, SSTR4 and SSTR5 expression were respectively 64.6% (52/127), 36.2% (46/127), 18.9% (24/127), 18.9% (24/127), 38.6% (49/127) in colorectal cancer and 52.5% (21/40), 40% (16/40), 30% (12/40), 2.5% (1/40), 32.5% (13/40) in normal colorectal mucosa (Table 1). SSTR1 was

Table 1 SSTR expression in colorectal cancer

Tissue	*n*	Expression *n* (%)				
		SSTR1	SSTR2	SSTR3	SSTR4	SSTR5
Normal mucosa	40	21(52.5)	16(40.0)	12(30.0)	1(2.5)	13(32.5)
Colorectal cancer	127	52(64.6)	46(36.2)	24(18.9)	24(18.9)[a]	49(38.6)

[a]$P<0.05$ *vs* normal mucosa.

the most predominant subtype in both colorectal cancer and normal colorectal mucosa, and the second was SSTR5 or SSTR2. As compared with normal colorectal mucosa, SSTR4 was more frequently expressed in colorectal cancer cells (2.5% *vs* 18.9%, $P < 0.05$), and the positive rates of other subtypes expression were not different between colorectal cancer and normal colorectal mucosa ($P > 0.05$).

Correlation between SSTR expression and clinicopathological factors

The expression of SSTR2, 4, 5 in moderately to well differentiated colorectal adenocarcinoma was significantly higher than that in poorly differentiated colorectal adenocarcinoma ($P < 0.05$)(Table 2). The SSTR1 expression in colorectal cancer with positive lymph node metastasis was significantly higher than that with negative lymph node metastasis (72.2% and 54.5%, $P < 0.05$). The correlation did not reach a statistical significance between the five SSTR subtypes expression and Dukes' stages ($P > 0.05$), but the frequency of SSTR1 expression increased with Dukes' stage, while SSTR3 and SSTR5 decreased with Dukes' stage. In addition, in the ulcerative type of colorectal cancer, SSTR2 expression was obviously decreased ($P < 0.05$); however, there was no correlation between expression of the five SSTR subtypes and other clinicopathological factors such as age, sex, tumor site, tumor depth, distant metastasis.

Correlation between SSTR expression and proliferative index and Bcl-2 or p53

The proliferative indexes in colorectal cancer cells with negative expression of SSTR2 and SSTR3 were significantly higher than that with positive expression ($P < 0.05$, Table 3). A positive cytoplasmic immunoreactivity for Bcl-2 was detected in 67 of 127 cases (52.8%). The Bcl-2 expression in colorectal cancer cells with positive expression of SSTR1, 2, 3 ,5 were significantly lower than that with negative expression ($P < 0.05$, Table 3). In contrast, nuclear accumulation of p53 was demonstrated in 81 of 127 cases (63.8%). There was no correlation between five SSTR subtypes and p53 expression.

DISCUSSION

Five SSTR subtypes belong to G proteins family, and different SSTR subtypes can be expressed in various patterns in different normal tissues or tumors. Evaluation of five SSTR subtypes expression in tumors may help understand carcinogenesis, and progression of tumors, as well as diagnosis and treatment. There are some controversies on the dominant SSTR subtype and the correlation between five SSTR subtypes expression and clinicopathological factors in colorectal cancer cells. Buscail *et al* found that SSTR2 expression was lost in colorectal cancer[3]. However, some subsequent studies demonstrated that there was no difference between SSTR subtypes expression between normal colorectal mucosae and colorectal cancer[5-7]. Also some results indicated the most frequent subtype was SSTR2 or SSTR5 in colorectal cancer[3,6,8]. The expression of SSTR2 or SSTR5 was significantly lower in Dukes' C and D stages of colorectal cancer than that in Dukes' A and B stages. The decrease of SSTR2 or SSTR5 expression may be related to tumor local invasion and metastasis, which may provide a growth advantage in colorectal cancer. It is possible that SSTR2 or SSTR5 acts as a tumour suppressor[3,6]. But recent research showed there was no correlation of SSTR mRNA expression with Dukes' stages[7].

In this study, only the frequency of SSTR4 expression in colorectal cancer cells was higher than that in the colorectal mucosae. SSTR1 was expressed predominantly in colorectal cancer cells, followed by SSTR5 and SSTR2. There was no correlation between five subtypes expression and Dukes' stages. The differences of our results from previous findings may be accounted for by different research methods. SSTR subtypes can express not only in colorectal cancer cells but also in other constituents of cancer tissue, such as vessels[9]. Thus, the PCR method tends to overestimate the real contribution of the various messengers for receptors owing to the outstandingly sensitive technique, and mRNA levels do not necessarily reflect the presence of the SSTR protein in certain cancers. To detect protein expression of SSTR by receptor autoradiography can not completely reflect protein levels of five SSTR subtypes, because there are major differences in binding affinity of SSTA to various SSTR subtypes[1]. In addition, the discrepancy may be relevant to different reagents and the small sample size of our study. Cascade reaction induced by activation of SSTR2 and SSTR5 can inhibit proliferation of tumor cells. In our study, the expression of SSTR2, SSTR4 and SSTR5 reduced in poorly differentiated colorectal cancer, and the SSTR3 and SSTR5 expression showed a tendency to reduction with Dukes' stages. The loss of these receptors can weaken inhibition on the proliferation of tumor cells, and may provide a growth advantage in colorectal cancer. It suggests SSTR2, 3, 4, 5 may be related to the development of colorectal cancer.

This study also demonstrated that the Ki-67 proliferative index in colorectal cancer cells with positive expression of SSTR2 or SSTR3 was significantly lower than that with negative expression of SSTR2 or SSTR3. This further proved that somatostatin *in vivo* can directly inhibit colorectal cancer cell proliferation by activating SSTR2 and SSTR3. The transplantable models of pancreatic primary tumor and hepatic metastases were established in hamsters which were xenografted with human pancreatic cancer cell line transferred with the SSTR2 gene, and the following effects were induced: growth of tumors with SSTR2 positive expression was delayed and the Ki-67 proliferative index was decreased significantly[10,11]. Activation of SSTR2 and

Table 2 Correlation between SSTR expression and clinicopathology in colorectal cancer *n* (%)

Clinico-pathological factor		*n*	SSTR1	SSTR2	SSTR3	SSTR4	SSTR5
Sex	Male	73	47(64.4)	26(35.6)	13(17.8)	13(17.8)	28(38.4)
	Female	54	35(64.8)	20(37.0)	11(20.4)	11(20.4)	21(38.9)
Age (yr)	< 60	66	45(68.2)	20(30.3)	12(18.2)	12(18.2)	24(36.4)
	⩾ 60	61	37(60.7)	26(42.6)	12(19.7)	12(19.7)	25(41.0)
Tumor site	Right colon	31	18(58.1)	11(35.5)	7(22.6)	6(19.4)	11(35.5)
	Left colon	27	17(63.0)	12(44.4)	5(18.5)	6(22.2)	13(48.1)
	Rectum	69	47(68.1)	23(33.3)	12(17.4)	12(17.4)	25(36.2)
Macroscopic type	Elevated	40	27(67.5)	17(42.5)	10(25.0)	9(22.5)	17(42.5)
	Ulcerative	59	37(62.7)	13(22.0)[a]	8(13.6)	8(13.6)	21(35.6)
	Infiltrative	28	18(64.3)	16(57.1)	6(21.4)	7(25.0)	11(39.3)
Differentiation	Moderate to well	102	70(68.6)	43(42.2)[a]	22(21.6)	24(23.5)[a]	46(45.1)[a]
	Poor	25	12(48.0)	3(12.0)	2(8.0)	0(0.0)	3(12.0)
Tumor depth	Muscularis	24	14(58.3)	11(45.8)	5(20.8)	3(12.5)	9(37.5)
	Serosa	103	68(66.0)	35(34.0)	19(18.4)	21(20.4)	40(38.8)
Lymph	(+)	72	52(72.2)[a]	24(33.3)	12(16.7)	15(20.8)	26(36.1)
node metastasis	(-)	55	30(54.5)	22(40.0)	12(21.8)	9(16.4)	23(41.8)
Distant metastasis	(+)	20	15(75.0)	6(30.0)	3(15.0)	2(10.0)	7(35.0)
	(-)	107	67(62.6)	40(37.4)	21(19.6)	22(20.6)	42(39.3)
Dukes′ stage	A	13	7(53.8)	5(38.5)	4(30.8)	2(15.4)	6(46.2)
	B	40	22(55.0)	16(40.0)	8(20.0)	7(17.5)	16(40.0)
	C	54	38(70.4)	19(35.2)	9(16.7)	13(24.1)	20(37.0)
	D	20	15(75.0)	6(30.0)	3(15.0)	2(10.0)	7(35.0)

[a]$P < 0.05$.

Table 3 Correlation between SSTR expression and proliferative index, Bcl-2 or p53 expression

		n	Ki67 proliferation index mean ± SD	Expression of Bcl-2 positive (%)	Expression of p53 positive (%)
SSTR1	(+)	82	34.22 ± 24.33	37(45.1)	53(64.6)
	(-)	45	40.21 ± 22.97	30(66.7)[a]	28(62.2)
SSTR2	(+)	46	28.65 ± 18.80	15(32.6)	33(71.7)
	(-)	81	40.71 ± 25.50[a]	52(64.2)[a]	48(51.3)
SSTR3	(+)	24	25.72 ± 17.88	5(20.8)	17(70.8)
	(-)	103	38.82 ± 24.55[a]	62(60.2)[a]	64(62.1)
SSTR4	(+)	24	28.02 ± 20.12	9(37.5)	16(66.7)
	(-)	103	38.29 ± 24.42	58(56.3)	65(63.1)
SSTR5	(+)	49	32.20 ± 21.92	17(34.7)	34(69.4)
	(-)	78	38.95 ± 24.91	50(64.1)[a]	47(60.3)

[a]$P < 0.05$.

SSTR3 can inhibit the proliferation of cancer cell through the following mechanisms: SSTR2 and SSTR3 can inhibit adenylate cyclase activity, which results in reduction of intracellular cAMP concentration; they also can stimulate tyrosine phosphatase activity, modulate the mitogen activated protein kinases (MAPK) and upregulate the expression of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ so that cell cycle arrest is induced; the effects on K^+ and Ca^{2+} channels lead to increased intracellular K^+ concentration and reduced intracellular Ca^{2+} concentration[12-15]. This study further verified that five SSTR subtypes play different roles in development of colorectal cancer. SSTR2 and SSTR3 can obviously inhibit the cell proliferation of colorectal cancer and have an important effect on the progression of colorectal cancer.

The activation of some SSTR subtypes not only directly inhibits the proliferation of tumor cells, but also is relevant to apoptosis. Bcl-2 is an apoptosis suppressor gene and important parameter of apoptosis. In this study, Bcl-2 expression in colorectal cancer cells with positive expression of SSTR1, SSTR2, SSTR3 or SSTR5 significantly decreased, indicating the four SSTR subtypes can promote the apoptosis of tumor cells and affect the progression of colorectal cancer by down-regulating the Bcl-2 expression or counteracting the anti-apoptosis effect of Bcl-2. They promote apoptosis via different mechanisms: Apoptosis is mediated by SSTR1 via a block in the G1/S progression in the cell cycle[16]. SSTR2 activation can promote apoptosis through the following pathways, it sensitizes cancer cells to apoptosis induced by tumor necrosis factor, induces caspase-8 activation cascade, stimulates mitochondrial cytochrome c released into the cytosol and downregulates the Bcl-2 expression[17]. In the mouse models of pancreatic primary tumor and hepatic metastases, which were xenografted with human pancreatic cancer cell line transferred with the SSTR2 gene, it has been demonstrated that activation of caspase-3 and apoptotic index increased, expression of anti-apoptosis protein Bcl-2 decreased in tumor cells expressing SSTR2[10,18]. Apoptosis can be signaled by SSTR3 which can activate phosphoprotein phosphates and lead to cellular acidification and activation of acidic endonuclease[19].

In conclusion, five SSTR subtypes play different roles in the development of colorectal cancer. SSTR2 and SSTR3 can inhibit the proliferation and promote apoptosis of tumor cells. The expression of SSTR2, SSTR4 and SSTR5 is correlated with malignant degree of colorectal cancer cells. Though SSTR1 is the predominant SSTR subtype, its ex-

pression is correlated with lymph node metastasis. SSTA, which has a high affinity to SSTR2, SSTR3 and/or SSTR5 should be a treatment choice for patients with colorectal carcinomas.

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BASIC RESEARCH

Adeno-associated virus-mediated heme oxygenase-1 gene transfer suppresses the progression of micronodular cirrhosis in rats

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Abstract

AIM: To test the hypothesis that enhancement of the activity of heme oxygenase can interfere with processes of fibrogenesis associated with recurrent liver injury, we investigated the therapeutic potential of over-expression of heme oxygense-1 in a CCl_4-induced micronodular cirrhosis model.

METHODS: Recombinant adeno-associated viruses carrying rat HO-1 or GFP gene were generated. 1×10^{12} vg of adeno-associated viruses were administered through portal injection at the time of the induction of liver fibrosis.

RESULTS: Conditioning the rat liver with over-expression of HO-1 by rAAV/HO-1 significantly increased the HO enzymatic activities in a stable manner. The development of micronodular cirrhosis was significantly inhibited in rAAV/HO-1-transduced animals as compared to controls. Portal hypertension was markedly diminished in rAAV/HO-1-transduced animals as compared to controls, whereas there are no significant changes in systolic blood pressure. This finding was accompanied with improved liver biochemistry, less infiltrating macrophages and less activated hepatic stellate cells (HSCs) in rAAV/HO-1-transduced livers.

CONCLUSIONS: Enhancement of HO activity in the livers suppresses the development of cirrhosis.

Key Words: Cirrhosis; Portal hypertension; Heme oxygenase; Gene therapy; Adeno-associated virus

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INTRODUCTION

Heme oxygenase (HO) is a rate-limiting enzyme that cleaves pro-oxidant heme into equimolar amounts of carbon monoxide (CO), biliverdin/bilirubin, and free ferrous iron [1,2]. Up to date, three isoforms of HO have been identified. Among of them, HO-1 and HO-2 are expressed in livers. HO-2 expresses constitutively in hepatocyte and functions as an important enzyme in catalyzing the endogenous and exogenous heme. In contrast, HO-1 is an inducible form of HO in livers [3]. Although expression of HO-1 in the liver is primarily restricted to a subpopulation of Kupffer cells [3,4], the protein is expressed in both parenchymal and particularly non-parenchymal liver cells, and therefore results in higher enzymatic activities of HO under stress conditions [4]. The cytoprotective effects of enhanced HO activities in liver might be through the mechanisms associated with its catalytic products. All of its three catalytic products can contribute the protective effects of HO [5-7].

HO-1 was induced in the livers with cirrhosis [8]. It was thought to be an adoptive response to oxidative stresses, inflammatory insults of persistent or recurrent liver injury as well as the responsiveness to the increase of intrasinusoidal resistance. HO-1 was mainly expressed in Kupffer cells, sinusoidal endothelial cells, stellate cells and in a part of hepatocyte in livers with cirrhosis [8,9]. HO-1 was also expressed in vascular system that might contribute to the development of hemodynamic changes in the rats with cirrhosis [9]. In addition, higher levels of CO production might be associated with the development of hepatopulmonary syndrome in patients with cirrhosis [10]. Thus, it becomes controversy whether the expression of HO-1 or enhancement of HO activities contribute to the pathophysiological changes in the development of cirrhosis, or whether HO-1 functions as homeostatic molecule in controlling of disease progression. To elucidate the role of

HO-1 in the context of recurrent hepatocellular injury and the development of cirrhosis, here we take the advantage of adeno-associated viral vector to increase the overall HO enzymatic activity of the liver in a stable manner and to investigate the effects of HO-1 in the disease progression in carbon tetrachloride (CCl_4)-induced cirrhosis model.

MATERIALS AND METHODS

Generation of recombinant adeno-associated viral vectors (rAAVs)

rAAVs were produced and purified as previously described [11]. In brief, a full-length HO-1 gene originally cloned from the spleen of a LEW rat or the reporter gene GFP was inserted into the vector plasmid to construct pSNAV1/GFP or pSNAV1/HO-1. rAAV/GFP or rAAV/HO-1 was generated in BHK-21 cells (American Type Culture Collection, Manassas, VA) by transfection of vector plasmid and subsequent rescue rAAVs (serotype 2) by co-infection with recombinant HSV1-rc/ΔUL2, which is essential for viron packaging. A large scale of rAAV was purified as described [12].

Animal model and gene delivery protocol

Inbred LEW rats (230-250 g) were purchased from the Animal Institute of Medical School Hannover, Germany and were maintained in the Laboratory Animal Unit of the University of Hong Kong. The study was approved by the Committee on the Use of Live Animals for Teaching and Research, the University of Hong Kong. Micronodular liver cirrhosis in LEW rats was induced using a protocol described previously [13]. In brief, phentobarbital sodium (35 mg/L) was given one week before the first dose of CCl_4 in order to increase the sensitivity of the rat liver to CCl_4 and was present in drinking water in the whole period of induction. A total of 9 doses of CCl_4 (0.2 mL/kg per wk) were given to the rats intragastrically. rAAVs (1×10^{12}v.g.) was given through the portal vein of rats at the time pentobarbital was given in drinking water and 7 d before the first dose of CCl_4.

Liver biochemistry, histology, immunohistochemistry and ELISA

Serum samples were collected at the end point of experiments. The activities of alanine aminotransferase (ALT) and total bilirubin in the plasma were measured in the Department of Clinical Biochemistry, the University of Hong Kong. Liver samples were snap frozen and stored at -75 ℃ until further applications. Five micrometers of frozen sections were used for hematoxylin and eosin staining, Masson's trichrome staining, and immunohistochemistry. The area of fibrotic tissues in the cross section of livers was measured by the computer software (MetaMorph imaging system, Universal Imaging Corporation, PA) after Masson's trichrome, collagen 1α and fibronectin staining. All measurements were done in a double-blind manner with at least 20 areas/liver (fibrotic area, original 200×) in the 5 livers/group. Mouse anti-rat ED1 (infiltrating macrophage), anti-ED2 (tissue residential macrophage), anti-HO-1 (OSA-111) monoclonal antibodies and polyclonal anti-TGF-β1, anti-desmin, anti-collagen 1α, and anti-fibronectin antibodies (Serotec Ltd., Oxford, UK; Stressgene, Victoria, British Columbia, Canada; Chemicon, Temecula, CA; Santa Cruz, CA) were applied in this study for immunohistochemistry using the standard horseradish peroxidase protocol. Serum macrophage migration inhibitory factor (MIF) level was detected by ELISA according to the instruction of manufacturer (Chemicon).

Measurement of portal and systolic blood pressure

Animals were anesthesized with ketamine (100 mg/kg, ip) and Rompun (0.2 mg/kg, ip). Portal pressure and systolic blood pressure were measured by directly introducing 24G Angiocath® (BD Biosciences, San Jose, CA) into the portal vein or abdominal aorta and connecting with a saline filled strain gauge transducer. The signals were monitored by Colin BP-408 Mark III (Japan) and were set to zero before the measurement.

HO enzymatic activity

The HO enzymatic activity was measured by the production of microsomal bilirubin in the liver. Frozen samples of livers were homogenized in ice-cold sucrose and Tri-HCl buffer. Microsomal pellet was obtained after centrifugation and was then re-suspended in $MgCl_2$-potassium phosphate buffer. Sample protein was further incubated with the reaction mixture containing rat liver cytosol, hemin, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and NADPH (Sigma-Aldrich, St. Louis, MO) for 60 minutes at 37 ℃. The generated bilirubin was measured using spectrophotometry. The level of bilirubin production was demonstrated by the ratio of sample/normal liver.

RNase protection assay

The mRNA level of target genes was determined by RNase protection assay according to the instruction of manufacturer (RiboQuant kit, BD Biosciences Pharmingen, San Diego, CA). In brief, total RNA of transduced and non-transduced liver samples were extracted and purified using Rneasy kit (Qiagen, Hilden, Germany). Three micrograms of RNA/samples were hybridized with complimentary [^{32}P]UTP labeled riboprobes overnight. The probes were digested with Rnase and were loaded on a denatured polyacrylamide gel. The radioactive signals were then detected by exposure to X-ray film (BioMax, Kodak, Rochester, NY) and quantified by phosphorimaging (Strom, Molecular Dynamics, Sunnyvale, CA).

Statistical analysis

Data were demonstrated as mean ± SEM. One-way ANOVA was used to compare the difference of means between the experimental groups with the Bonferroni's *t*-test. $P<0.05$ was considered statistically significant.

RESULTS

rAAV-mediated stable HO enzymatic activity in rat liver

To enhance HO activity in a stable manner, we administered the rAAV carrying rat HO-1 cDNA to the liver through portal injection. After the injection, a large num-

ber of HO-1 positive non-parenchymal cells were found in both rAAV/GFP and rAAV/HO-1-transduced livers on d 7 (17.7 ± 0.5 cells/mm^2 versus 10.8 ± 0.7 cells/mm^2, n = 3). However, the number of positive cells decreased dramatically to a level similar to normal livers in rAAV/GFP transduced liver on day 30, whereas there was a significantly larger number of HO-1-positive non-parenchymal cells in rAAV/HO-1-transduced livers (3.9 ± 0.8 cells/mm^2 *vs* 5.8 ± 1.0 cells/mm^2, n = 3, P < 0.05; Figures 1A and 1B). No HO-1 positive hepatocyte was found in both rAAV/GFP and rAAV/HO-1 treatment groups on d 7. The expression of HO-1 in hepatocyte was first detected sporadically on d 14 after portal injection of rAAV/HO-1, whereas there was no HO-1 positive hepatocyte in rAAV/GFP-transduced livers (data not shown). More HO-1 positive hepatocytes could be detected in rAAV/HO-1-transduced livers on d 30 (2.2 ± 0.3 cells/mm^2, n = 3, P < 0.05), whereas they remained undetectable in rAAV/GFP-transduced livers (Figures 1A and 1B). To evaluate the biological activity of transduced HO-1 in the livers, we determined the enzymatic activity of HO by measuring the generated bilirubin of microsomal protein isolated from the livers. There was a significant increase of HO activity of the rAAV-transduced livers on d 7 (the earliest time point that we detected). Both rAAV/GFP and rAAV/HO-1 transduced livers showed increased amount of generated bilirubin in isolated microsomal proteins. Impressively, the amount of generated bilirubin (0.9 fold over the basal level of normal rat liver) remained elevated in the rAAV/HO-1 treatment group and sustained for over 3 mo of observation time. In contrast, the amount of generated bilirubin decreased to the level of normal rats in the rAAV/GFP treatment group on d 14. (Figure 1C). This suggests the specificity of rAAV/HO-1 in the induction of stable HO activity in rat liver.

Enhancement of HO activity suppressed the development of cirrhosis in rats

Intragastrically administration of CCl_4 to adult LEW rats for 9 wk resulted in the formation of micronodular cirrhosis. To further evaluate the effects of elevated HO activity on the development of cirrhosis, we examined various parameters relating to cirrhosis after over-expression of HO-1 in rat liver by rAAV gene transfer (Figure 2A). Histological examination showed massive amount of fibrotic tissues accumulated in the portal tract areas of non-transduced or rAAV/GFP-transduced livers and led to the formation of micronodular cirrhosis accompanied with portal hypertension and lower systolic blood pressure in the majority of rats. rAAV/HO-1 gene transfer markedly diminished the amount of accumulated fibrotic tissues as shown in Masson's trichrome (MT) staining and immunohistochemistry. No micronodular cirrhosis was formed and only minimal fibrotic tissues could be found in the portal tract areas of rAAV/HO-1-transduced livers (Figure 2B). Analysis of fibrotic areas using the computer software showed that there was significantly less fibrotic element deposition in the rAAV/HO-1-transduced liver (7.9% ± 0.9% by MT staining, 3.1% ± 2.6% by collagen 1α

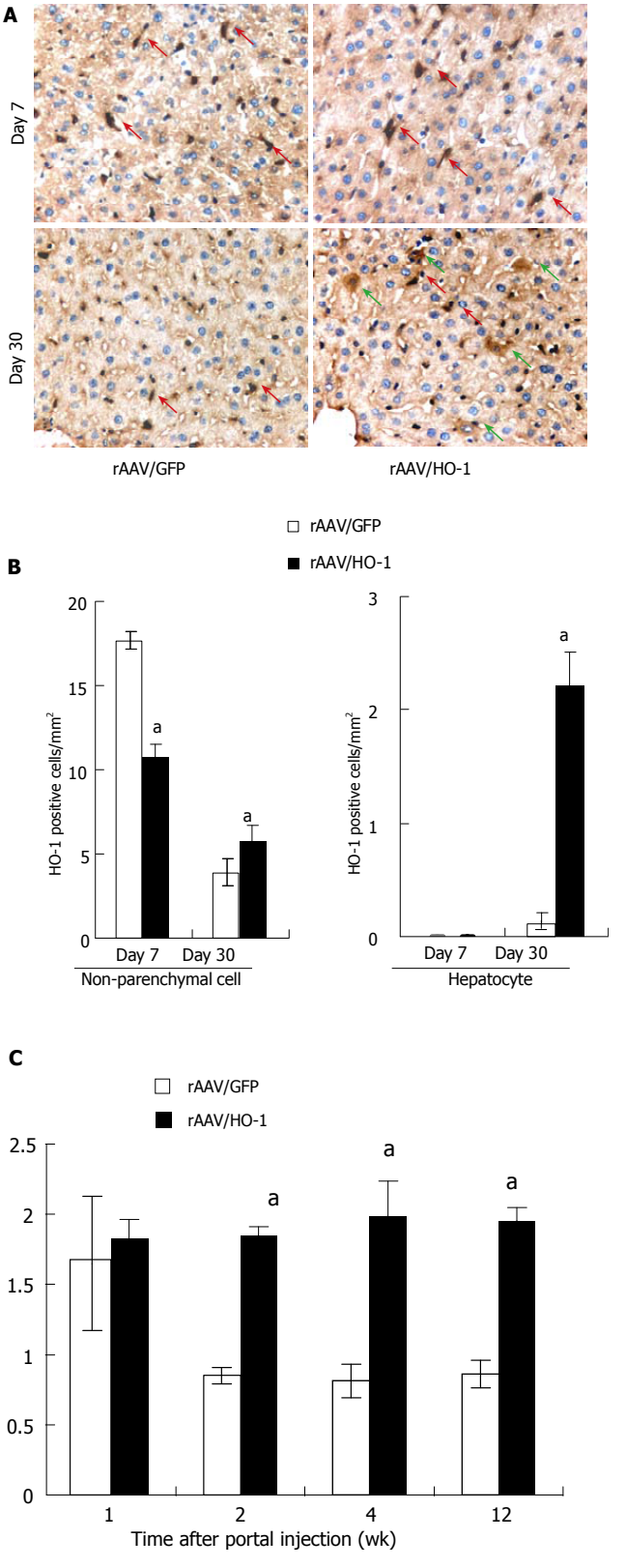


Figure 1 **A:** The expression patterns of HO-1 in the livers after portal injection of rAAV/HO-1 in LEW rats. Red arrow indicated the HO-1-positive non-parenchymal cells; green arrow indicated the hepatocyte; **B:** The quantification of the HO-1 positive cells in the liver after administration of rAAV/HO-1. Data were presented as number of HO-1 positive cells per mm^2; n = 3-5, mean ± SE, ^aP<0.05; **C:** The HO enzymatic activity of rAAV/GFP or rAAV/HO-1 transduced livers. The data were demonstrated as the amount of generated bilirubin of microsomal protein from liver tissues and were shown as the fold changes over the normal rat liver, mean ± SE, n = 3, ^aP < 0.05.

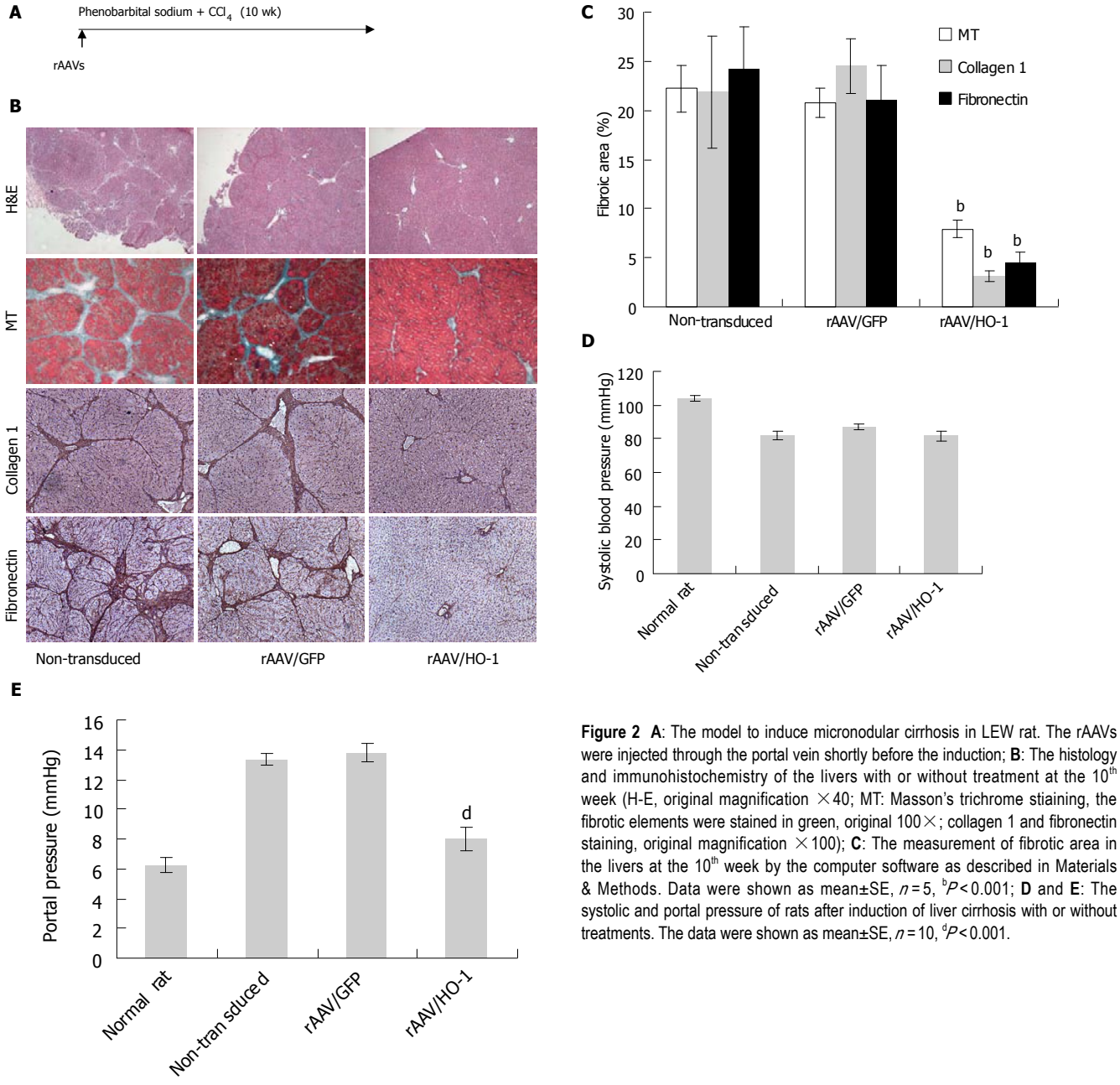
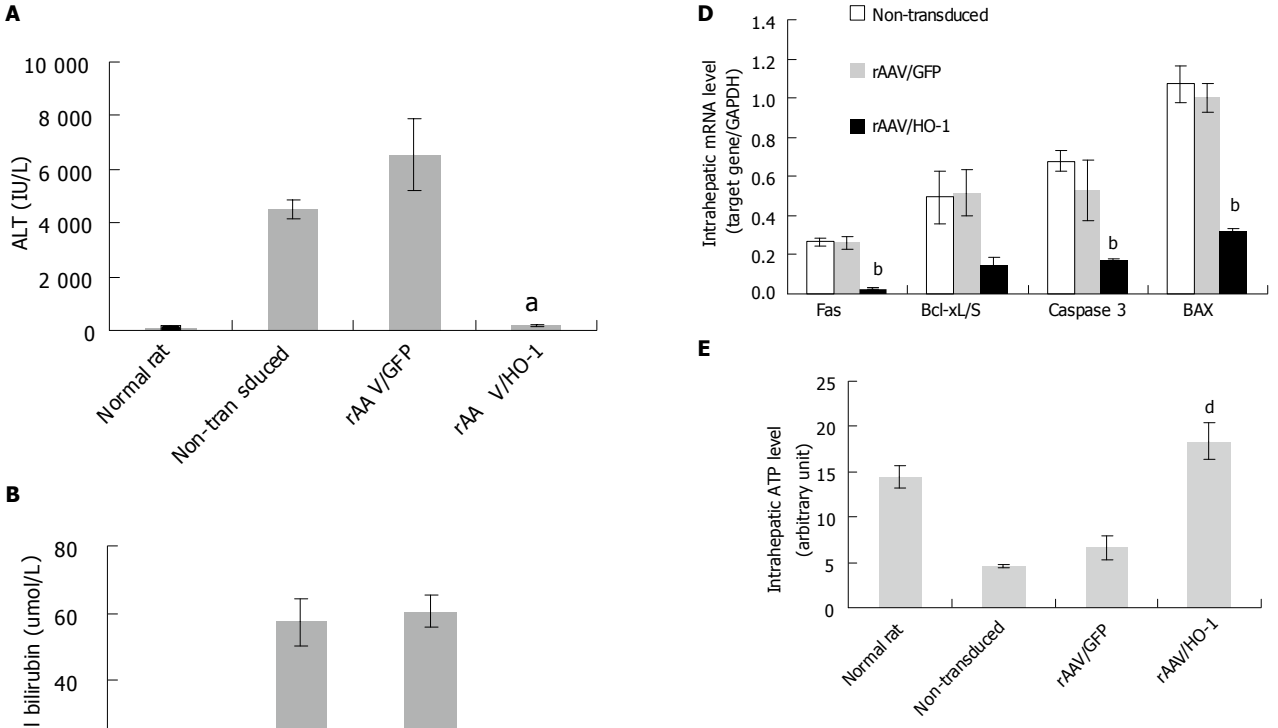


Figure 2 **A**: The model to induce micronodular cirrhosis in LEW rat. The rAAVs were injected through the portal vein shortly before the induction; **B**: The histology and immunohistochemistry of the livers with or without treatment at the 10th week (H-E, original magnification ×40; MT: Masson's trichrome stiaining, the fibrotic elements were stained in green, original 100×; collagen 1 and fibronectin staining, original magnification ×100); **C**: The measurement of fibrotic area in the livers at the 10th week by the computer software as described in Materials & Methods. Data were shown as mean±SE, $n=5$, $^bP<0.001$; **D** and **E**: The systolic and portal pressure of rats after induction of liver cirrhosis with or without treatments. The data were shown as mean±SE, $n=10$, $^dP<0.001$.

staining, 4.4%±1.2% by fibronectin staining, $P<0.001$). In contrast, in the livers of non-treatment or rAAV/GFP treated groups, there was a higher level of fibrotic element deposition (22.2%±5.8% or 20.8%±3.8% by MT staining, 21.9%±5.7% or 24.5%±2.8% by collagen 1α staining, 24.1%±4.4% or 21.1%±3.6% by fibronectin staining, Figure 2C). In addition, the beneficial effects of HO-1 on the development of liver cirrhosis could be reflected by the reduction of portal hypertension (8.0±0.8 mmHg versus 13.3±0.4 mmHg in the non-treatment group and 13.8±0.6 mmHg in the rAAV/GFP treatment group, $P<0.001$), whereas there was no significant difference at the systolic blood pressure (81.7±0.3 mmHg) in comparison to non-treatment (81.8±0.6 mmHg) or vector controls (87.0±0.2 mmHg, $P>0.31$, Figures 2D and 2E).

Stable HO activity protected against CCl_4-mediated recurrent liver injury

Repeated administration of CCl_4 to adult rats led to chronic liver injury with elevated plasma level of total bilirubin (no treatment group: 57.25±6.95 μmol/L; rAAV/GFP group: 60.5±4.87 μmol/L) and ALT (non-treatment group: 4 522±334 IU/L; rAAV/GFP group: 6 552±1 363 IU/L, $n=5$). Increasing HO activity in the liver by rAAV/HO-1 significantly improved the liver function of rats under long-term CCl_4 toxicity, the amount of total bilirubin and ALT decreased dramatically in the rAAV/HO-1-treated rats in comparison to non-treatment or rAAV/GFP controls (total bilirubin: 6.20±3.49 μmol/L; ALT: 199.6±80.5 IU/L, $n=5$, $P<0.005$, Figures 3A and 3B). To further examine the protective effects of over-expressing HO-1 in the livers, we determined the transcript level of apoptotic genes and energy exchanges of livers under long-term CCl_4 toxicity. Rnase protection assay showed the transcript level of Fas, caspase 3, and BAX genes significantly diminished in the rAAV/HO-1-transduced livers ($n=3$, $P<0.05$,



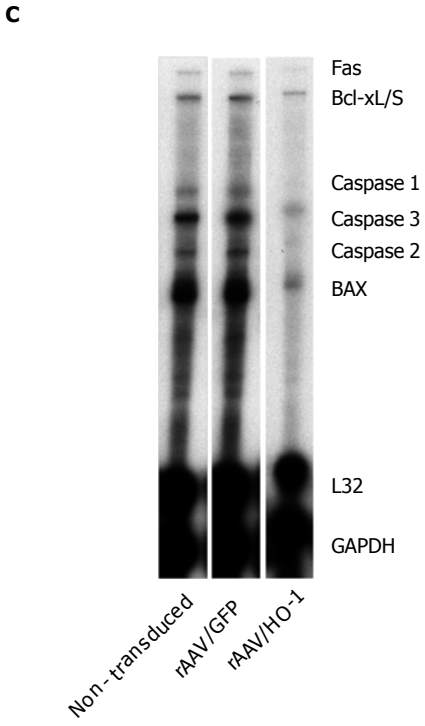


Figure 3 **A** and **B**: The liver function of rats with or with treatment under long-term CCl_4 toxicity was presented by the determination of plasma ALT and total bilirubin level. The data was shown as mean±SEM, n = 5-10, ^aP<0.001; **C** and **D**: The representative picture of the expression patterns of apoptotic genes in the livers at the 10th week. The mRNA level of genes was detected by RNase protection assay. The quantification of the expression level was measured by phosphoimager as described in Materials & Methods. The data were shown as mean ± SE, n = 3-5, ^bP<0.05; **E:** The ATP level of the livers of rats with or without treatments at the 10th wk, n = 5-10, ^dP<0.001.

Figures 3C and 3D), whereas these genes were strongly expressed in the non-transduced or rAAV/GFP-transduced livers. The beneficial effects of HO-1 on CCl_4-mediated chronic liver injury were also shown by the improvement of liver energy exchanges. Over-expression of HO-1 by rAAV significantly improved the intrahepatic ATP level in rAAV/HO-1transduced liver in comparison with non-treatment or rAAV/GFP-transduced livers of rats under long-term CCl_4 toxicity (n = 3-5, P < 0.05; Figure 3E).

Stable HO activity suppresses the pro-inflammatory and pro-fibrogenic responses in CCl_4-treated rats

To characterize the effects of HO-1 on the chronic inflammatory response followed by liver injury, we examined the histopathological parameters of liver tissues and transcript or protein level of pro-inflammatory cytokine-MIF. A large amount of ED1-positive (infiltrating) macrophages accumulated in the portal tract areas in the non-transduced or rAAV/GFP-transduced livers, whereas only a few ED1-positive macrophages were found in rAAV/HO-1-transduced livers (Figure 4A). In contrast, there was no significant change in the number of ED2-positive (tissue-residential) macrophages in the livers of CCl_4-treated rats. In consistent with the immunohistochemical stainings, detection of intrahepatic mRNA level by Rnase protection assay and serum levels of MIF by ELISA method showed significant reduction of the expression level of MIF in rAAV/HO-1-treated rats in comparison to non-treatment or rAAV/GFP controls (n = 3-5, P < 0.001, Figures 4B and 4C).

To examine the effects of rAAV/HO-1 on the fibrogenic process after liver injury, we next examined the

expression patterns of pro-fibrogenic cytokine-TGF-β1, which plays an important role in the activation and transition of HSCs to myofibroblast-like cells after liver injury. Immunohistochemistry showed that TGF-β1 was highly expressed in the portal tract areas in the livers of long-term CCl_4-treated rats. In contrast, only a minimal number of cells expressed TGF-β1 in rAAV/HO-1-transduced livers of rats under the same CCl_4 protocol (Figure 4A). Parallel to the immunohistochemical stainings, RNase protection assay also showed significant reduction in the transcript level of TGF-β1 in rAAV/HO-1-transduced livers (n = 3-5, P < 0.001, Figure 4B). This finding was associated with the decreased number of activated HSCs (desmin-positive) in rAAV/HO-1-transduced livers (Figure 4A).

DISCUSSION

There is a wide range of factors can result in hepatocellular injury. However, the primary response leading to subsequent pro-inflammatory and pro-fibrogenic responses in liver is extraordinary similar [14]. Analysis of clinical parameters based on the treatment of primary liver disease showed encouraging results in the decrease of the severity of liver fibrosis/cirrhosis [15,16]. However, none of anti-fibrotic treatment has been shown clinically effective. A number of therapeutic approaches targeting on anti-oxidant, anti-inflammatory response, suppression of HSC activation, induction of HSC apoptosis or increase of the degradation of extracellular matrix have been shown effective in the attenuation of the severity of liver fibrosis/cirrhosis in various animal models [14]. Most of them, however, cannot easily translate to the clinical setting, because the majority of approaches focus on the single step of disease progression and the clinical situation is far more complex than that in the experimental setting. Thus, it is still urged to find out a therapeutic target that has multiple effects on disease progression and can be applied potentially in the clinical setting.

HO-1-mediated anti-apoptotic and anti-inflammatory activities are among the most attractive mechanisms in cellular protection [17]. In fact, HO-1 only expresses in a subpopulation of Kupffer cells in normal liver, whereas a small amount of HO-2 is constitutively being expressed in hepatocyte [3,4]. None of them expresses in sinusoidal endothelial cells and hepatic stellate cells [3]. However, a significant increase in HO-1 expression was observed on the whole organ level under stress conditions [4]. In the liver with acute injury, HO-1 was expressed in the majority of Kupffer cells and infiltrating macrophages, which might function as a feedback loop to control the macrophage activation [18-20]. Although the whole HO activity increases dramatically, the need for the liver to overcome the insults of cellular injury seems to be relatively insufficient. Increasing HO activity by introduction of exogenous HO-1 by rAAV/HO-1 in our model that significantly improved the long-term outcomes of CCl_4-induced liver cirrhosis could be based on (1) the anti-apoptotic effects of HO-1 on hepatocyte; (2) the anti-inflammatory effects of HO-1 on the control of the cellular response of hepatocyte in the production of MIF under stress and/or injury condi-

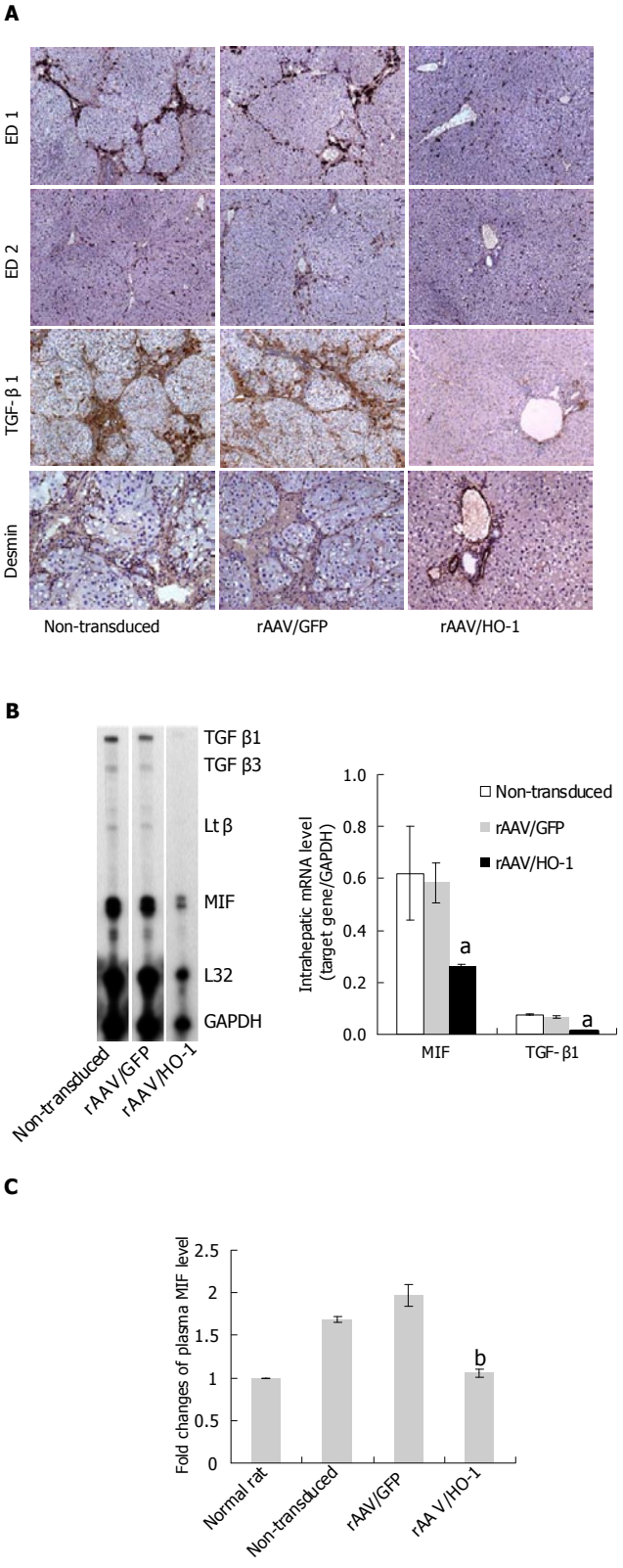


Figure 4 **A:** The representative pictures of pro-inflammatory and pro-fibrogenic responses in the livers of rats with or without treatment at the 10th week. Infiltrating macrophage (ED1), tissue residential macrophages (ED2), TGF-β1, and activated hepatic stellate cells (desmin) were detected by immunohistochemistry; original magnification, ×100; **B:** The profile of pro-inflammatory and profibrogenic cytokine expression. The level of mRNA was detected by RNase protection assay and was quantified by phosphoimager, n = 3-5, ^aP<0.05; **C**: The plasma level of macrophage migration inhibitory factor (MIF) was detected by ELISA. The data were shown as fold changes of over the level in normal rat, n = 3-5, ^bP < 0.001.

tions and the suppression of the macrophage activation; and (3) the anti-fibrogenic effects of HO-1 on the suppression of the collagen synthesis and/or proliferation of activated HSCs.

Improvement of liver function under long-term CCl_4 toxicity by rAAV/HO-1 may reflect the fact that the expression of HO-1 in the hepatocyte was able to prevent liver damage, which was supported by the down-regulation of pro-apoptotic genes and enhancement of liver ATP level in our model. In acute liver injury and ischemia/reperfusion injury of transplanted liver, recent data suggested that the resistance of HO-1 expressing cells to the pro-apoptotic stimuli might be directly through its enzymatic product CO and/or indirectly through the induction of the Fe^{2+}-sequestering protein ferritin. Exposure of CO to the primary hepatocyte could prevent the tumor necrosis factor-α-mediated and anti-CD95-mediated apoptotic events through the down-regulation of caspase-3 activity [21], whereas the induction of ferritin suppressed serum-deprived or oxidative stress-mediated hepatocyte apoptosis by modulation of intracellular Fe^{2+} level and inhibition of Fe^{2+}-mediated conversion of hydrogen peroxide into OH^- and OH through the Fenton reaction [7,22]. In addition to anti-apoptotic effects, HO-1 could also suppress the production of pro-inflammatory cytokine-MIF production in a human primary hepatocyte culture (Tsui TY, et al. unpublished data). This may suggest that HO-1 can function as homeostatic molecule in the prevention of apoptotic event and the control of cellular response.

HO-1 can be induced in various cell types including HSC through direct or indirect mechanisms. Our data are consistent with a recent finding in primary human HSC culture showing that the induction of HO-1 expression in HSC suppressed the transcript level of pro-collagen 1α and serum-mediated HSC proliferation [23]. This may suggest that HO-1 can serve as a negative regulator in the control of HSC activation and proliferation. Thus, less accumulation of fibrotic elements and prevention of the development of portal hypertension in rAAV/HO-1-transduced animals might reflect the outcomes of cellular protection, anti-inflammatory and anti-fibrogenic responses. Although further investigation is needed to clarify the mechanisms in more details, increased HO activity in liver and systemic effects of the products of HO enzymatic activity can be the key in the control of the development of cirrhosis and portal hypertension in our model.

In conclusion, our data demonstrated that enhancement the HO activity in a stable manner can suppresses the pathophysiological changes of cirrhosis. In addition, rAAV-mediated gene transfer may represent an attractive approach in controlling the development of cirrhosis. However, further studies should be carried out in order to answer the question of whether the rAAV approach is suitable for the hepatitis virus-mediated chronic liver injury and the subsequent outcome.

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BASIC RESEARCH

Rosuvastatin reduces rat intestinal ischemia-reperfusion injury associated with the preservation of endothelial nitric oxide synthase protein

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Abstract

AIM: To investigate the protective effect of rosuvastatin on ischemia-reperfusion (I-R)-induced small intestinal injury and inflammation in rats, and to determine the effect of this agent on the expression of endothelial nitric oxide synthase (eNOS) protein.

METHODS: Intestinal damage was induced in male Sprague-Dawley rats by clamping both the superior mesenteric artery and the celiac trunk for 30 min, followed by reperfusion for 60 min. Rosuvastatin dissolved in physiological saline was administered intraperitoneally 60 min before ischemia. The severity of the intestinal mucosal injury and inflammation were evaluated by several biochemical markers, as well as by histological findings. The protein levels of eNOS were determined by Western blot.

RESULTS: The levels of both intraluminal hemoglobin and protein, as indices of mucosal damage, were significantly increased in the I-R group compared with those in the sham-operated group. These increases, however, were significantly inhibited by treatment with rosuvastatin in a dose-dependent manner. The protective effects of rosuvastatin were also confirmed by histological findings. Exposure of the small intestine to I-R resulted in mucosal inflammation characterized by significant increases in thiobarbituric acid-reactive substances, tissue-associated myeloperoxidase activity, and the mucosal contents of rat cytokine-induced neutrophil chemoattractant-1 (CINC-1) and tumor necrosis factor-α (TNF-α). These increases in inflammatory parameters after I-R were significantly inhibited by pretreatment with rosuvastatin at a dose of 10 mg/kg. Furthermore, mRNA expression of CINC-1 and TNF-α was increased after I-R, and this increase was also inhibited by rosuvastatin. The mucosal protein levels of eNOS decreased during I-R, but were preserved in rats treated with rosuvastatin.

CONCLUSION: Rosuvastatin inhibits rat intestinal injury and inflammation induced by I-R, and its protection is associated with the preservation of eNOS protein.

Key words: Rosuvastatin; Rat; Intestine; Ischemia-reperfusion injury; Endothelium; Nitric oxide synthase

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INTRODUCTION

Intestinal ischemia-reperfusion (I-R) injury is a grave condition resulting from acute mesenteric ischemia, small bowel transplantation, abdominal aortic aneurysm, hemorrhagic, traumatic or septic shock, or severe burns[1,2]. Recent reports have demonstrated that neutrophil infiltration in the intestinal mucosa via neutrophil-endothelial cell interactions plays a significant role in the pathogenesis of I-R-induced intestinal injury because activated neutrophils generate tissue damaging products such as reactive oxygen species, protease, collagenase, and a ferrous iron-ferritin complex [3-5].

Leukocyte accumulation is a complex phenomenon that also involves endothelium-based adhesion molecules as well as leukocyte chemotaxis factors such as interleukin-8 (IL-8). Intercellular adhesion molecules (ICAMs) are normally expressed at a low basal level, but their expression can be enhanced by various inflammatory cytokines such as IL-1 and tumor necrosis factor-α (TNF-α). A variety of cytokines, including TNF-α, interferon-γ and IL-1β, are released from post-ischemic tissues.

Statins, which are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are widely used in the treatment of hyperlipidemia and coronary artery disease [6]. Recently accumulating evidence suggests that statins have anti-inflammatory and endothelial cell protective actions that are independent of their cholesterol-lowering effect [7]. Statins can increase the expression of endothelial nitric oxide synthase (eNOS) by blocking Rho geranylgeranylation [8], and they can also block the lymphocyte function-associated antigen-1 (LFA-1)-ICAM-1 interaction by binding the L-site [9]. Rosuvastatin, a new HMG-CoA reductase inhibitor, has exhibited a more potent affinity for the active site of HMG-CoA reductase than other statins. In addition, the cytoprotective action of rosuvastatin against ischemic injury has been clearly documented [10-14]. Ikeda *et al* [10], for example, demonstrated that rosuvastatin reduces neutrophil-induced cardiac contractile dysfunction in the isolated ischemic reperfused rat heart. However, to date, the effects of rosuvastatin on intestinal I-R injury have not yet been investigated. In this study, we show that rosuvastatin dramatically attenuates I-R-induced intestinal injury and inflammation. Moreover, rosuvastatin was found to reverse the decrease in eNOS expression after I-R in intestinal mucosa.

MATERIALS AND METHODS

Chemicals

All chemicals were prepared immediately prior to use. Rosuvastatin was a gift from AstraZeneca UK, Ltd. (London, UK). Thiobarbituric acid (TBA) and 3,3',5,5'-tetramethylbenzidine were obtained from Wako Pure Chemical Indust. (Osaka, Japan), and 1,1,3,3-tetramethoxypropane was obtained from Tokyo Kasei (Tokyo, Japan). Enzyme-linked immunosorbent assay (ELISA) kits for rat TNF-α and cytokine-induced neutrophil chemoattractant-1 (CINC-1) were obtained from BioSource International (Camarillo, CA) and Immuno-Biological Laboratories Co., Ltd. (Gunma, Japan), respectively. All other chemicals used were of reagent grade.

Preparation of rats for intestinal I-R

Male Sprague-Dawley rats weighing 180-200 g were obtained from Keari Co., Ltd. (Osaka, Japan). The animals were housed at 22℃ in a controlled environment with 12 h of artificial light per day. They were allowed access to rat chow and water ad libitum. The rats were anesthetized with urethane (1 mg/kg, ip). After a midline laparotomy, the celiac and superior mesenteric arteries were isolated near their aortic origins. Intestinal ischemia was induced by clamping both the superior mesenteric artery and the celiac trunk for 30 min, resulting in a total occlusion of these arteries, following our previously reported method [15, 16]. After this period of occlusion, the clamps were removed. The animals were randomized into groups receiving different concentrations of rosuvastatin or physiological saline alone by intraperitoneal injection 1 h before ischemia. The maintenance of the animals and the experimental procedures performed on them were carried out in accordance with National Institutes of Health (NIH) guidelines for the use of experimental animals. All procedures were approved by the Animal Care Committee of Kyoto Prefectural University of Medicine (Kyoto, Japan).

Assessment of intestinal mucosal injury induced by I-R

The animals were killed after reperfusion, and the intestine was removed and submitted for examination. After making a 30-cm proximal intestinal loop, saline (10 mL) was injected into the loop. The contents were aliquoted after centrifugation. Intestinal injury after reperfusion was evaluated by examining the luminal contents of hemoglobin and protein, and by histological examination. Intestinal bleeding was quantified indirectly as the hemoglobin concentration in luminal lavage fluid using a kit and following the manufacturer's protocol (Wako). Luminal protein levels were also determined using a kit according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, USA). For histological evaluation, formalin-fixed tissues were stained with hematoxylin and eosin and evaluated by light microscopy by a pathologist who was not informed of the experimental conditions for any given specimen.

Measurements of TBA-reactive substances and MPO activity

The concentrations of TBA-reactive substances were measured in the intestinal mucosa using the method described by Ohkawa *et al* [17] as an index of lipid peroxidation. The animals were killed by exsanguination from the abdominal aorta after the experiments, and their small intestines were removed. The intestinal mucosa was then scraped off using two glass slides, and homogenized with 1.5 mL of 10 mmol/L potassium phosphate buffer (pH 7.8) containing 30 mmol/L KCl in a Teflon Potter-Elvehjem homogenizer. The level of TBA-reactive substances in the mucosal homogenates was expressed as nanomoles of malondialdehyde per milligram of protein using 1,1,3,3-tetramethoxypropane as the standard. The total protein in the tissue homogenates was measured using a kit, according to the manufacturer's protocol (Bio-Rad).

Tissue-associated myeloperoxidase (MPO) activity was determined using a modification of the method described by Grisham *et al* [18] as an index of neutrophil accumulation. Briefly, two milliliters of mucosal homogenates were centrifuged at 20 000 g for 15 min at 4℃ to pellet the insoluble cellular debris. The pellet was then rehomogenized in an equivalent volume of 0.05-M potassium phosphate buffer (pH 5.4), containing 0.5% hexadecyltrimethylammonium bromide. The samples were centrifuged at 20 000 g for 15 min at 4℃ and the supernatants were saved. The MPO activity was assessed by measuring the H_2O_2-dependent oxidation of 3,3',5,5'-tetramethylbenzidine. One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance of 1.0/min at

655 nm and 25 ℃.

Determination of mucosal content of CINC-1 and TNF-α

The concentrations of the inflammatory cytokines TNF-α and CINC-1 in the supernatant of mucosal homogenates were determined by rat TNF-α and CINC-1-specific ELISA kits according to the manufacturer's instructions.

RT-PCR

The mRNA expression of intestinal TNF-α and CINC-1 was determined by reverse transciprion-polymerase chain reaction the (RT-PCR). Samples of intestinal tissue for mRNA isolation were removed from the intestine. The total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method using Isogen (Nippon Gene, Tokyo, Japan) and the concentration of RNA was determined by absorbance at 260 nm in relation to that at 280 nm. RNA was stored at –70 ℃ until it was used for the RT-PCR. Amplification was carried out in a 50-μL mixture containing 2 μL of the RT product, 0.6 μmol/L of both the sense and antisense primers, 0.4 mmol/L dNTP mix, and 0.5 μL Taq DNA polymerase (Takara Shuzo Co., Shiga, Japan). The reaction was performed as follows: 35 cycles of amplication (denaturation at 94 ℃ for 30 s, annealing at 50 ℃ for 30 s, and extension at 72 ℃ for 60 s), followed by a final extension step of 7 min at 72 ℃. The primers had the following sequences: for TNF-α, sense 5'-ATGAGCACAGAAAGCATGATC-3', and antisense 5'-TACAGGCTTGTCACTCGAATT-3'; for CINC-1, sense 5'-CTGTGCTGGCCACCAGCCGC-3', and antisense 5'-ACAGTCCTTGGAACTTCTCTG-3'; and for ß-actin, sense 5'-ATCGTGGGCCGCCCTAGGCA-3', and antisense 5'-TGGCCTTAGGGTTCAGAGGGG-3'. The PCR products were separated electrophoretically in a 25 g/L agarose gel and stained by ethidium bromide.

Western blotting

Intestinal cells were thawed on ice and homogenized at 4 ℃ in a solution of 50 mmol/L Tris–HCl (pH 7.6), 300 mmol/L NaCl, 0.5 g/L Triton X-100, 10 g/L aprotinin, 10 g/L leptin, 1 mmol/L phenylmethysulfonyl fluoride (PMSF), 1.8 g/L iodoacetamide, 50 mmol/L NaF, and 1 mmol/L DTT in order to extract the total cell protein. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose (Bio-Rad). Membranes were probed with specific antibodies against eNOS (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and immune complexes were visualized by Western blotting with a commercial kit (ECL, Amersham, Buckinghamshire, England), following the manufacturer's recommendations.

Statistics

All results are presented as mean ± SE. The data were compared by two-way analysis of variance (ANOVA), and differences were considered to be significant if the *P* value was less than 0.05 based on Scheffe's multiple comparison test. All analyses were performed using the Stat View 5.0-J program (Abacus Concepts, Inc., Berkeley, CA, USA) on a Macintosh computer.

RESULTS

Rosuvastatin inhibits intestinal reperfusion injury

We found that 60 min of reperfusion after 30 min of ischemia resulted in increases in luminal protein and hemoglobin concentrations (Figure 1). In contrast, pre-treatment with rosuvastatin 1 h before the ischemia was found to inhibit these increases in a dose-dependent manner (Figure 1). Neither vehicle alone nor rosuvastatin alone affected the luminal protein or hemoglobin concentrations (data not shown). Therefore, in the following experiments, we used rosuvastatin at a dose of 10 mg/kg and examined its pharmacological action. In rats treated with I-R, multiple erosions and bleeding developed in the small intestine; however, pretreatment with rosuvastatin at a dose of 10 mg/kg 1 h before the ischemia inhibited both the intestinal erosions and the bleeding. The protective effect of rosuvastatin was further confirmed histologically. Representative hematoxylin and eosin-stained sections of the ileum from sham, I-R, and I-R + rosuvastatin-treated animals are depicted in Figure 2. While the ileum of sham animals exhibited normal mucosal architecture with intact villi, I-R resulted in large areas of epithelial crypt loss, predominantly neutrophilic infiltrate throughout the mucosa, erosion, and mucosal bleeding. In contrast, pretreatment with rosuvastatin resulted in smaller erosions with few neutrophils. Rosuvastatin alone did not produce any macroscopic or microscopic lesions in the rat intestine.

Effect of rosuvastatin on TBA-reactive substances

The extent of lipid peroxidation was determined by measuring TBA-reactive substances in the small intestine. No significant differences were found in the intestinal TBA-reactive substance levels between vehicle- and rosuvastatin-treated rats in the sham-operated group. Sixty min of reperfusion caused a significant increase in TBA-reactive substances in the control rats. This increase in TBA-reactive substances in the intestinal mucosa was significantly inhibited by 10 mg/kg of rosuvastatin (Figure 3A).

Effect of rosuvastatin on MPO activity

A hallmark of intestinal reperfusion injury is the accumulation of neutrophils in the injured tissue. Therefore, we next evaluated neutrophil accumulation based on tissue-associated MPO activity. There were no differences in intestinal MPO activities between vehicle- and rosuvastatin-treated rats in the sham-operated group, while the MPO activity in the intestinal mucosa markedly increased from a basal activity of 0.23 ± 0.07 nkat/g protein to 5.20 ± 1.65 nkat/g protein after I-R in the control group. The increase in MPO activity in the intestinal mucosa after reperfusion was significantly inhibited by treatment with rosuvastatin (Figure 3B).

Effect of rosuvastatin on mRNA expression and mucosal protein contents of CINC-1

In order to determine whether or not treatment with rosuvastatin can modulate the inflammatory response through the regulation of cytokine production, we analyzed intestinal mucosal levels of CINC-1 and TNF-α. The intestinal concentrations of TNF-α and CINC-1 increased signifi-

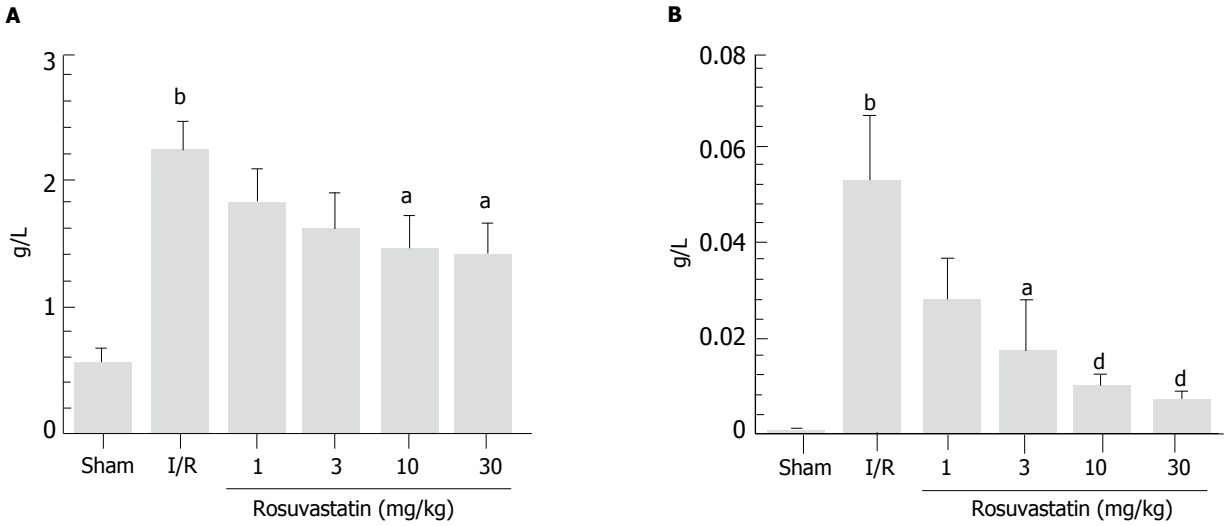

Figure 1 Dose-response of rosuvastatin on acute intestinal I-R injury in rats (mean ±SE). **A:** Intraluminal protein; **B:** Hemoglobin. $^{b}P<0.01$ *vs* Sham, $^{a}P<0.05$, $^{d}P<0.01$ *vs* Vehicle + I-R.

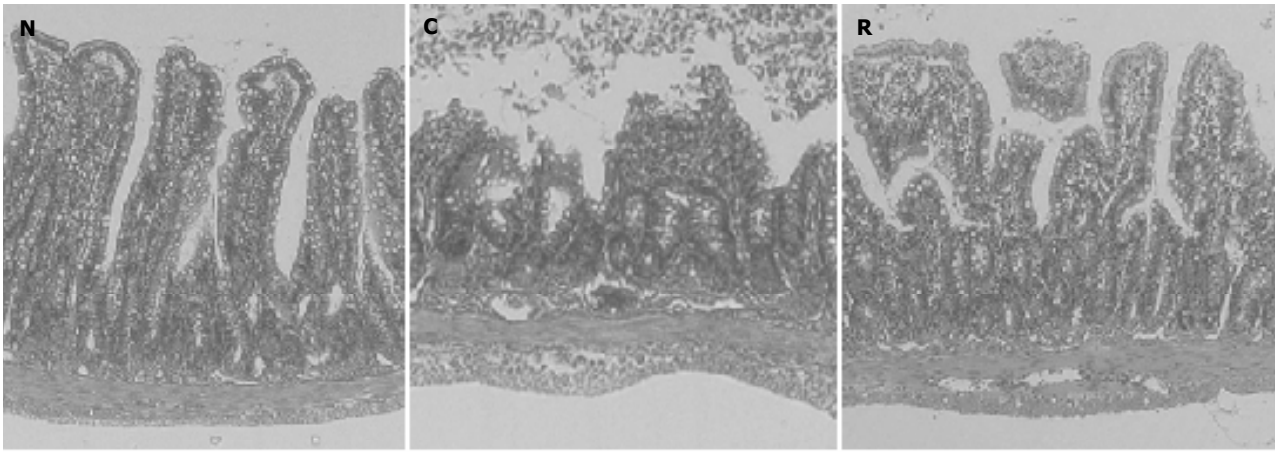

Figure 2 Histological appearance of the small intestine in rats (HE ×20). **N:** Sham. **C**: I-R. **R**: I-R plus rosuvastatin.

cantly in rats treated with I-R, and these increases were significantly inhibited by treatment with rosuvastatin at a dose of 10 mg/kg (Figures 4A, 4B). To further confirm the inhibitory effect of rosuvastatin on CINC-1 and TNF-α production, we analyzed the intestinal expression of CINC-1 and TNF-α using RT-PCR yielding 495 and 225 base pair products to identify CINC-1 and TNF-α gene expression, respectively. As shown in Figure 5, we found the expression of these genes in rats treated with sham-operation to be negligible or faint. In contrast, transcription was readily enhanced in I-R-treated rats. Treatment with rosuvastatin suppressed the level of mRNA expression for each gene.

Effect of rosuvastatin on eNOS protein expression

Since previous studies have shown that statins exerts beneficial effects on the endothelium through an increase in NO production from eNOS [19, 20], we investigated whether eNOS expression could be affected by I-R with or without rosuvastatin pre-treatment. Figure 6 was a representative of at least 3 experiments performed on different experimental days. The expression levels of eNOS protein were decreased during the I-R. However, pretreatment with rosuvastatin significantly reversed the decrease in eNOS protein. Rosuvastatin alone did not affect intestinal eNOS protein levels in sham-operated rats.

DISCUSSION

The present study clearly demonstrates that rosuvastatin, a new HMG-CoA reductase inhibitor, has a protective effect against reperfusion-induced intestinal injury and inflammation in rats. In this study, intestinal injury was assessed by a variety of methods including luminal protein/hemoglobin concentrations and histological evaluation. The results of each assessment showed that rosuvastatin treatment significantly inhibited intestinal injury. In addition, we demonstrated that, in I-R-induced intestinal inflammation, the expression of pro-inflammatory cytokines (CINC-1 and TNF-α) was enhanced in association with neutrophil accumulation, as determined by MPO activity in the homog-

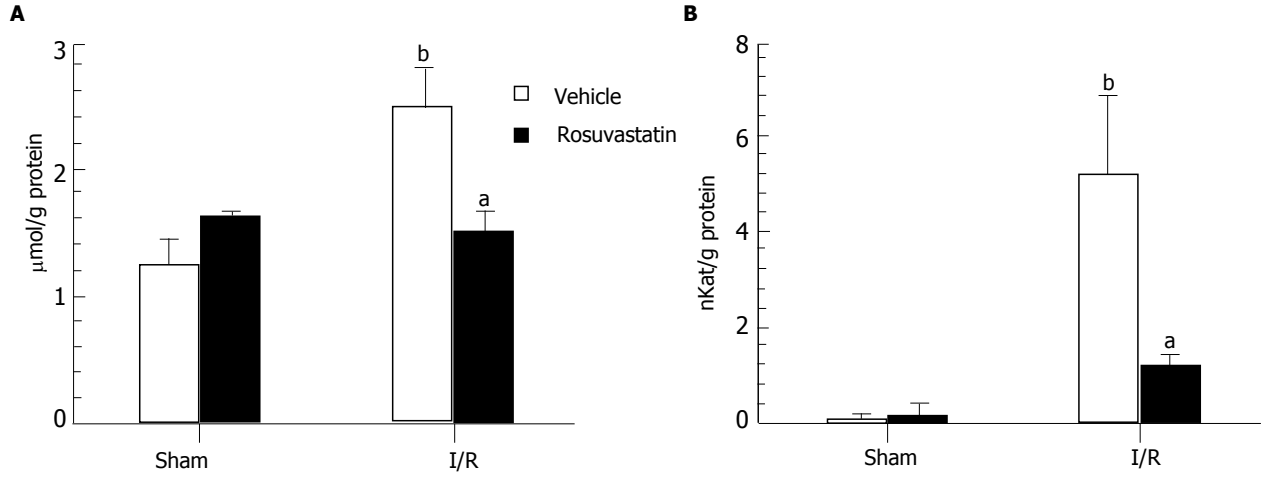

Figure 3 Effects of rosuvastatin on lipid peroxide concentrations and neutrophil accumulation in the intestinal mucosa of rats treated with I-R (mean±SE). **A:** TBA-reactive substance; **B:** Myeloperoxidase activity. $^{b}P<0.01$ *vs* Sham, $^{a}P<0.05$ *vs* Vehicle + I-R.

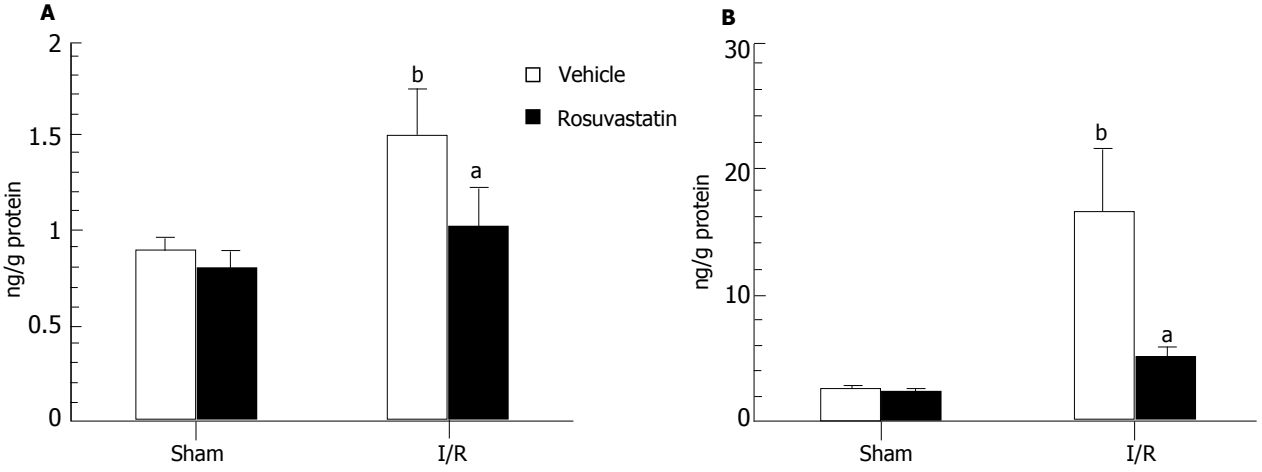

Figure 4 Effects of rosuvastatin on the intestinal CINC-1 and TNF-α after I-R in rats (mean±SE). **A:** CINC-1; **B:** TNF-α. $^{b}P<0.01$ *vs* Sham, $^{a}P<0.05$ *vs* Vehicle + I-R.

enate of the small intestine. Rosuvastatin treatment inhibited both the up-regulation of pro-inflammatory cytokine expression and the neutrophil infiltration, which prevents tissue injury. These results suggest that rosuvastatin has a protective action against I-R-induced intestinal mucosal injury as well as an anti-inflammatory action against reperfusion-induced inflammation.

Oxygen-derived free radicals produced during I-R are known to cause peroxidation of polyunsaturated fatty acids in cell membranes. The resulting increase in lipid peroxides can lead to changes in membrane fluidity and permeability, and finally to cell lysis. In the present study, TBA-reactive substances in intestinal mucosa, which are an index of lipid peroxidation, were significantly increased in I-R-treated rats compared to sham-operated rats, and rosuvastatin treatment significantly inhibited the increase in these substances. However, neither rosuvastatin nor other statins have been reported to have antioxidant activities *in vitro*, nor has the inhibition of lipid peroxidation been previously reported. Our recent study also showed that rosuvastatin had no effect on scavenging hydroxyl and superoxide radicals determined by electron spin resonance-spin trapping assay (data not shown). Therefore, the inhibitory effect of rosuvastatin on I-R-induced lipid peroxidation in rat intestinal mucosa may result from its anti-inflammatory activity, rather than from its direct antioxidant activity.

Recent reports hypothesize that neutrophil-mediated inflammation is involved in the pathogenesis of I-R-induced intestinal injury. This hypothesis has been supported by several studies; neutrophil depletion by intraperitoneal injection of anti-neutrophil serum has been found to significantly attenuate intestinal mucosal injury elicited by I-R [21, 22]; in addition, immunoneutralization of the CD11/CD18 adherence complex on neutrophils and CINC-1 is known to attenuate intestinal reperfusion injury [23, 24]. The present study showed that MPO activity, an index of tissue-associated neutrophil accumulation, increases in the intestinal mucosa after I-R, and that this increase is significantly inhibited by treatment with rosuvastatin. The inhibition of neutrophil-endothelial interaction by statins has been demonstrated in a number of previous reports. Honjo *et al* [25] have shown that both cerivastatin and pravastatin significantly reduce the number of rolling/accumulated leukocytes in the retinal veins after I-R. Naidu *et al* [26] have

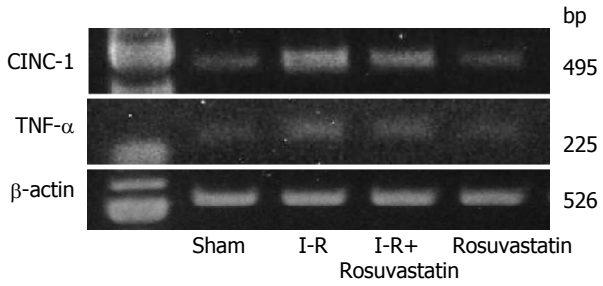

Figure 5 Effects of rosuvastatin on the expression of CINC-1 and TNF-α mRNA in the intestinal mucosa after I-R in rats.

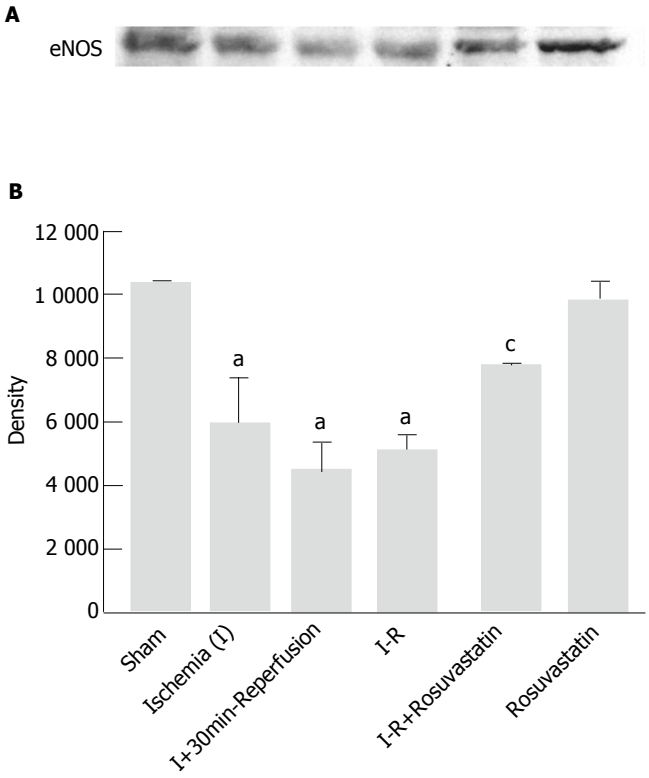

Figure 6 Effect of rosuvastatin on eNOS protein expression during I-R. $^{a}P<0.05$ *vs* Sham, $^{c}P<0.05$ *vs* Vehicle + I-R.

also demonstrated that pretreatment with simvastatin inhibits increases in tissue MPO content in lungs treated with I-R. In addition, the present study showed that the expression of the pro-inflammatory cytokines CINC-1 and TNF-α markedly enhanced in the intestine after I-R, and that these increases are also significantly inhibited by rosuvastatin. The inhibitory effect of statins against mRNA expression for inflammatory genes was also confirmed by a recent study using DNA microarray technology [27], in which it was demonstrated that the statins atorvastatin and pitavastatin directly affect the expression levels of genes involved in inflammation, including interleukin-8 (similar to rat CINC-1), in cultured human umbilical vein endothelial cell. These results, together with the present data, indicate that the inhibition of neutrophil accumulation as well as the inhibition of cytokine production by rosuvastatin may be one of the protective factors decreasing I-R-elicited intestinal mucosal injury.

The beneficial effects of statins on acute inflammation are abolished when it is administered together with an NOS inhibitor [20] or when it was administered to eNOS knockout mice [19], indicating that an enhanced release of NO from eNOS may be involved in the action of statins. Statins are known to up-regulate eNOS expression and activate this enzyme in the systemic vasculature by blocking Rho geranylgeranylation [8, 28] as well as by activating the PI-3 kinase/Akt pathway [20]. As shown in the present study, the intestinal levels of eNOS protein gradually decreased during 30-min ischemia and 60-min reperfusion, indicating that reperfusion may be associated with a posttranscriptional reduction in the expression of the eNOS enzyme. In addition, rosuvastatin-treated animals demonstrated preserved expression of eNOS protein compared to the I-R-treated group, although this agent did not affect the level of eNOS protein in normal rats. Therefore, the persistent expression of eNOS protein at 60 min of reperfusion in rosuvastatin-treated rats is likely to explain the reduction in tissue injury and neutrophil accumulation. In line with our observations, NO is reported to prevent leukocyte adhesion to the endothelium by repressing the up-regulation of cell adhesion molecules in the endothelial cells [29, 30].

Our results showed that I-R-induced intestinal inflammation is characterized by increased production of inflammatory mediators and decreased expression of eNOS. The blockade of these mediators and the preservation of eNOS expression by rosuvastatin were accompanied by significant suppression of intestinal inflammation *in vivo*. These data suggest that rosuvastatin may represent a novel therapeutic approach for the treatment of I-R-induced intestinal injury.

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BASIC RESEARCH

Usefulness of biopsying the major duodenal papilla to diagnose autoimmune pancreatitis: A prospective study using IgG4-immunostaining

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Abstract

AIM: To examine the histological and immunohistochemical findings of biopsy specimens taken from the major duodenal papilla of autoimmune pancreatitis (AIP) patients.

METHODS: The major duodenal papilla in the resected pancreas of 3 patients with AIP and of 5 control patients [pancreatic carcinoma (n = 3) and chronic alcoholic pancreatitis (n = 2)] was immunostained using anti-CD4-T cell, CD8-T cell and IgG4 antibodies. Forceps biopsy specimens taken from the major duodenal papilla of 2 patients with AIP and 5 control patients with suspected papillitis were prospectively taken during duodenoscopy and immunohistochemically examined.

RESULTS: Moderate or severe lymphoplasmacytic infiltration including many CD4-positive or CD8-positive T lymphocytes and IgG4-positive plasma cells ($\geqslant$10/HPF), was observed in the major duodenal papilla of all 3 patients with AIP. The same findings were also detected in the biopsy specimens taken from the major duodenal papilla of 2 patients with AIP, but in controls, there were only a few ($\leqslant$3/HPF) IgG4-positive plasma cells infiltrating the major duodenal papilla.

CONCLUSIONS: An abundant infiltration of IgG4-positive plasma cells is specifically detected in the major duodenal papilla of patients with AIP. Although this is a preliminary study, IgG4-immunostaining of biopsy specimens taken from the major duodenal papilla may support the diagnosis of AIP.

INTRODUCTION

Autoimmune pancreatitis (AIP) is a unique form of pancreatitis in which autoimmune mechanisms are involved in the pathogenesis. It is characterized by irregular narrowing of the main pancreatic duct, enlargement of the pancreas, increased levels of serum γ globulin or IgG, presence of autoantibodies, and responsiveness to steroid therapy[1,2]. Histological findings of the pancreas of AIP are also characteristic, and include dense lymphoplasmacytic infiltration with fibrosis[3]. Recently, it was reported that serum concentrations of IgG4 are significantly and specifically raised in patients with AIP[4]. However, the sensitivity of raised serum IgG4 concentrations is 67%[5]-68%[6] in some reports.

AIP occurs predominantly in elderly males and frequently presents as obstructive jaundice[7]. This presentation is also typical of pancreatic carcinoma. In typical cases of AIP that show diffuse change of the pancreas, the diagnosis can be easily made based on the combination of computed tomography (CT) and endoscopic retrograde cholangiopancreatography (ERCP) findings. However, in segmental mass-forming cases, the differentiation between AIP and pancreatic carcinoma remains difficult. The "double-duct sign", representing stricture in both the pancreatic and bile ducts, is often found in patients with pancreatic head carcinoma. This finding, however, is also frequently observed in AIP[7], because the inflammatory process compresses both the main pancreatic duct and the distal common bile duct[3]. As most patients with AIP respond to oral steroid therapy[2], an accurate diagnosis of AIP can avoid unnecessary

laparotomy or pancreatic resection. On the other hand, histopathological approach to the pancreas is sometimes difficult.

We previously reported that IgG4-positive plasma cells abundantly infiltrated various organs, as well as the pancreas of a patient with AIP, and that an abundant infiltration of IgG4-positve plasma cells was not observed in the organs of patients with pancreatic carcinoma or chronic alcoholic pancreatitis[3,8]. In order to find a useful new method to diagnose AIP on the basis of histological and immunohistochemical studies of the resected pancreas, we prospectively examined the histological and immunohistochemical findings of biopsy specimens obtained from the major duodenal papilla of AIP patients.

MATERIALS AND METHODS

The major duodenal papilla in the resected pancreas of 3 patients with AIP who underwent pancreatoduodenectomy due to suspected pancreatic carcinoma was histologically examined. The specimens were fixed in 10% formaldehyde. Serial sections were cut from paraffin-embedded tissue blocks, and immunostained using anti-CD4-T (Novocastra, Newcastle upon Tyne, UK), CD8-T (DakoCytomation, Glostrup, Denmark) cell subsets, as well as IgG4 (The Binding Site, Birmingham, UK) antibodies with avidin-biotin-peroxidase complex (ABC). The number of immunohistochemically identified cells per high power field (HPF) in each section was counted.

We recently treated 2 patients with AIP, a 73-year-old male and a 60-year-old female. Both patients had obstructive jaundice due to stenosis of the lower bile duct. Segmental narrowing of the main pancreatic duct was observed in the pancreatic head on ERCP, and focal enlargement of the pancreatic head was detected on CT. Serum IgG4 concentrations were elevated to 325 mg/dL in the male and 825 mg/dL in the female. The major duodenal papilla was normal on duodenoscopy during ERCP. Forceps biopsy specimens from the major duodenal papilla were taken during duodenoscopy. Histological and immunohistochemical studies were done on these biopsy specimens. After the biopsy was taken, steroid therapy was given to both patients, who showed marked responsiveness both morphologically and serologically. In 1 patient, rebiopsy from the major duodenal papilla was done after steroid therapy.

Histological and immunohistochemical studies were also done on control specimens. Controls consisted of the major duodenal papilla specimens of the pancreases resected by pancreatoduodenectomy for pancreatic head carcinoma ($n = 3$) and chronic alcoholic pancreatitis ($n = 2$), as well as endoscopically biopsied specimens taken from the major duodenal papilla of patients with suspected papillitis ($n = 5$).

RESULTS

Moderate or severe lymphoplasmacytic infiltration was observed in the major duodenal papilla of all 3 patients with AIP. Immunohistochemically an abundant infiltration of CD4- or CD8-positive T lymphocytes and IgG4-positive plasma cells (≥10/HPF) was observed in these 3 major duodenal papillae. Moderate or severe lymphoplasmacytic infiltration which included many CD4- or CD8-positive T lymphocytes and IgG4-positive plasma cells (≥10/HPF) was also observed in the biopsy specimens taken from the major duodenal papilla of 2 AIP patients (Figures 1 and 2). Although infiltration of CD4- or CD8-positive T lymphocytes was detected to some extent in the major duodenal papilla of controls, there were few IgG4-positive plasma cells infiltrating the major duodenal papilla of controls (≤3/HPF). The abundant infiltration of CD4- or CD8-positive T lymphocytes and IgG4-positive plasma cells disappeared in the biopsy specimen taken from the major duodenal papilla of 1 patient after steroid therapy.

Figure 1 Histology of biopsy specimen taken from the major duodenal papilla of a patient with autoimmune pancreatitis showing severe lymphoplasmacytic infiltration.

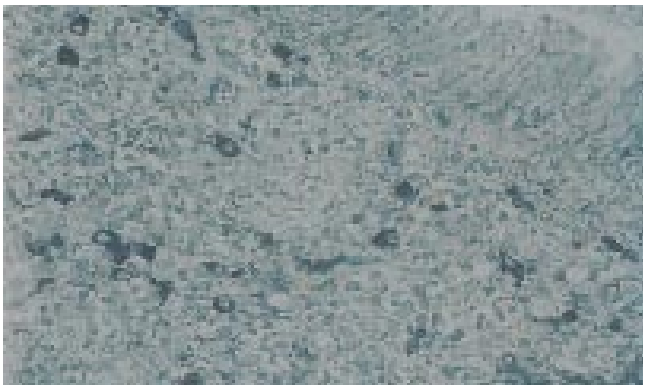

Figure 2 IgG4-immunostaining of biopsy specimen taken from the major duodenal papilla of a patient with autoimmune pancreatitis showing an abundant infiltration of IgG4-positive plasma cells (≥10/HPF).

DISCUSSION

Chronic pancreatitis and several other pancreaticobiliary diseases may be associated with inflammation of the major duodenal papilla, histologically showing infiltration of neutrophils or lymphocytes, or fibrosis[9]. In 2002, Unno *et al*[10] reported that a swollen major duodenal papilla was detected in 41% of 17 patients with AIP, and many infiltrating T lymphocytes were present in the biopsy specimens taken from the swollen papillary tissue. In 2004,

Sahin *et al*[11]reported that dense T-lymphocytic infiltration was present in the resected major duodenal papilla of 2 patients with AIP. In the present study, dense infiltration of CD4- or CD8-positive lymphocytes was also detected in the resected major duodenal papilla of 3 patients with AIP. Furthermore, an abundant infiltration of IgG4-positive plasma cells was detected in the major duodenal papilla of these patients and was not observed in the major duodenal papilla of controls.

Our previous immunohistochemical studies[3,8] of resected pancreases taken from AIP patients showed that the infiltrating inflammatory cells consisted of CD4- or CD8-positive T lymphocytes and IgG4-positive plasma cells, and that an abundant infiltration IgG4-positive plasma cells in the pancreas was not detected in other diseases. In addition, an abundant infiltration of IgG4-positive plasma cells was detected in various organs of patients with AIP, including peripancreatic retroperitoneal tissue, biliary tract, salivary glands, lymph nodes, and others. We therefore proposed the existence of a novel clinicopathological entity, an IgG4-related systemic disease characterized by extensive IgG4-positive plasma cell infiltration of organs together with CD4- or CD8-positive T lymphocytes[8]. Based on this concept, the dense infiltration of IgG4-positive plasma cells along with CD4- or CD8-positive lymphocytes that are detected in the major duodenal papilla of patients with AIP seems to be induced by the same mechanism as is operative in the pancreas.

These findings led us to do a prospective immunohistochemical study using an anti-IgG4 antibody to study the biopsy specimens taken from the major duodenal papilla of AIP patients. An abundant IgG4-positive plasma cell infiltration was detected in the biopsy specimens taken from the non-swollen major duodenal papilla of 2 AIP patients, and was not detected in the biopsy specimens taken from controls. Of note, the abundant infiltration of IgG4-positive plasma cells disappeared after steroid therapy. Although the number of examined cases is small, IgG4-immunostaining of biopsy specimens taken from the major duodenal papilla may be useful to support the diagnosis of AIP.

In conclusion, an abundant infiltration of IgG4-positive plasma cells was detected in the major duodenal papilla of patients with AIP. Although this is a preliminary study, IgG4-immunostaining of biopsy specimens taken from the major duodenal papilla may be useful to support the diagnosis of AIP.

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BASIC RESEARCH

Effects of *Aloe vera* and sucralfate on gastric microcirculatory changes, cytokine levels and gastric ulcer healing in rats

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Abstract

AIM: To compare the effects of *Aloe vera* and sucralfate on gastric microcirculatory changes, cytokine levels and gastric ulcer healing.

METHODS: Male Spraque-Dawley rats ($n=48$) were divided into four groups. Group1 served as control group, group 2 as gastric ulcer group without treatment, groups 3 and 4 as gastric ulcer treatment groups with sucralfate and *Aloe vera*. The rats from each group were divided into 2 subgroups for study of leukocyte adherence, TNF-α and IL-10 levels and gastric ulcer healing on days 1 and 8 after induction of gastric ulcer by 20% acetic acid.

RESULTS: On day 1 after induction of gastric ulcer, the leukocyte adherence in postcapillary venule was significantly ($P<0.05$) increased in the ulcer groups when compared to the control group. The level of TNF-α was elevated and the level of IL-10 was reduced. In the ulcer groups treated with sucralfate and *Aloe vera*, leukocyte adherence was reduced in postcapillary venule. The level of IL-10 was elevated, but the level of TNF-α had no significant difference. On day 8, the leukocyte adherence in postcapillary venule and the level of TNF-α were still increased and the level of IL-10 was reduced in the ulcer group without treatment. The ulcer treated with sucralfate and *Aloe vera* had lower leukocyte adherence in postcapillary venule and TNF-α level. The level of IL-10 was still elevated compared to the ulcer group without treatment. Furthermore, histopathological examination of stomach on days 1 and 8 after induction of gastric ulcer showed that gastric tissue was damaged with inflammation. In the ulcer groups treated with sucralfate and *Aloe vera* on days 1 and 8, gastric inflammation was reduced, epithelial cell proliferation was enhanced and gastric glands became elongated. The ulcer sizes were also reduced compared to the ulcer group without treatment.

CONCLUSION: Administration of 20% acetic acid can induce gastric inflammation, increase leukocyte adherence in postcapillary venule and TNF-α level and reduce IL-10 level. *Aloe vera* treatment can reduce leukocyte adherence and TNF-α level, elevate IL-10 level and promote gastric ulcer healing.

Key words: *Aloe vera*; Sucralfate; Gastric microcirculation; TNF-α; IL-10; Gastric ulcer healing

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INTRODUCTION

Gastric ulcer is produced by the imbalance between gastroduodenal mucosal defense mechanism and damaging force. Impaired mucosal defense is invoked in ulcer patients with normal levels of gastric acid and pepsin. Patients chronically using non-steroid anti inflammatory drugs (NSAIDs), including aspirin, can be pointed with some assurance at suppression of mucosal prostaglandin synthesis. Cigarette smoking impairs healing and favors recurrence, possibly by suppressing mucosal prostaglandin synthesis. Alcohol is another agent causing gastric mucosal lesion. It rapidly penetrates the gastroduodenal mucosa causing membrane damage, exfoliation of cells and erosion[1]. Corticosteroids at a high dose and repeated use promote ulceration. Personality and psychologic stress are important contribution factors as well[2].

Gastric ulceration results from the imbalance between gastrotoxic agents and protective mechanisms result in acute inflammation. Interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNFα) are the major proinflammatory cytokines, playing an important role in production of acute inflammation[3] accompanied with

neutrophil infiltration of gastric mucosa[4].

Aloe plants have been used medicinally for centuries. Among them, *Aloe barbadensis*, commonly called *Aloe vera*, is one of the most widely used healing plants in the history of mankind[5].

Two distinct preparations of Aloe plants are most used medicinally. The leaf exudate (aloe) is used as a laxative and the mucilaginous gel (*Aloe vera*) extracted from the leaf parenchyma is used as a remedy against a variety of skin disorders[6]. Aloe leaf exudate also possesses antidiabetic[7] and cardiac stimulatory activity[8].

Aloe vera is one of the few substances known to effectively decrease inflammation and promote wound healing[9,10]. *Aloe vera* gel could promote the healing of burns and other cutaneous injuries and ulcer[11, 12], thus improving wound healing in a dose-dependent manner and reducing edema and pain[9].

Aloe vera gel has been demonstrated to protect human beings[13-15] and rats[16-22] against gastric ulceration. This antiulcer activity is due to its anti-inflammatory[20], cytoprotective[16], healing[20, 23] and mucus stimulatory effects[24].

However, the effects of *Aloe vera* on gastric microcirculation, inflammatory cytokines in gastric ulcer patients have not yet been reported. Therefore, the aim of this study was to study the effects of *Aloe vera* and sucralfate on gastric microcirculatory changes, cytokine level and gastric ulcer healing.

MATERIALS AND METHODS

Animal preparation

Male Spraque Dawley rats weighing 200-280 g purchased from the International Animal Research Center, Salaya ($n = 48$), were used in this study. Group 1 served as control group, group 2 as gastric ulcer group without treatment, groups 3 and 4 as gastric ulcer treatment groups with sucralfate (200 mg/kg/dose, twice daily) and *Aloe vera* (200 mg/kg/dose, twice daily).

The animals were fasted but allowed only water 12 hours before experiment. On the day of experiment, the animals were weighed and anesthetised with intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). After tracheostomy, carotid artery and jugular vein were canulated for blood pressure measurement using polygraph and administration of fluorescent marker. The abdominal wall was incised and the stomach was extended and fixed. Then the leukocyte adherence in stomach was observed by *in vivo* microscopy.

Study of interaction between leukocytes and endothelial cells in postcapillary venule

For visualization of leukocytes, acridine orange was infused intravenously (0.5 mg/kg BW) as previously described[25]. The number of leukocyte adhesions was recorded using video recorder. Videotape of each experiment was played back and then leukocyte adherence was monitored. The leukocytes were markedly adhered to the postcapillary venule (about 15-35 μm in diameter). The location of leukocyte adherence in three areas was observed. Leukocytes were considered adherent to the vessel endothelium if they remained stationary for 30 or longer. Adherent leukocytes were expressed as the mean number of leukocyte adherences per field of view as previously described[26].

Mean number of leukocyte adherences = [the number of (area 1 + area 2 + area 3) cells/field]/3

Determination of serum cytokine levels

After the experiment, blood samples were taken by cardiac puncture, allowed to clot for 2 h at room temperature or overnight at 2-8 ℃ before centrifugation for 20 minutes at approximately 2000 r/min. Serum was separated and stored at about -80 ℃ for determining TNF α and IL-10 levels by ELISA kit (Quantikine, R&D systems).

Histological analysis

The stomach was fixed in 10% formalin and embedded in paraffin. Sections were cut at a thickness of 5 μm and stained with hematoxylin and eosin (H&E) as previously described[3]. Histopathological changes and maximum length of gastric ulcer were observed under light microscope with magnification × 20. Histopathological examination was performed by pathologists.

Statistical analysis

Data were expressed as mean ± SE. Statistical analysis was done using one-way analysis of variance and comparision of results between groups was made using post hoc test. $P < 0.05$ was considered statistically significant.

Ethical considerations

This study was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

RESULTS

Interaction between leukocytes and endothelial cells

After gastric ulcer was induced by administration of 20% acetic acid, leukocyte adherence to endothelial cells of postcapillary venules (15-35 mm in diameter.) was observed under intravital fluorescence microscopy. The number of leukocytes adhered to postcapillary venules for 30 s or longer was counted per each field of observation. The mean number of leukocyte adherences in the ulcer group without treatment (d1: 13.13 ± 1.19 cells/field; d8: 13.61 ± 1.99 cells/field) was significantly increased compared to the control group (d1: 1.69 ± 0.17 cells/field; d8: 5.53 ± 0.65 cells/field).

On days 1 and 8 after induction of gastric ulcer, the number of leukocyte adherences was significantly decreased both in the ulcer group treated with sucralfate (d1: 3.22± 0.76 cells/field; d8: 3.80 ± 0.79 cells/field) and in the ulcer group treated with *Aloe vera* (d1: 4.29 ± 0.39 cells/field; d8: 4.46 ± 0.27 cells/field) ($P < 0.05$) compared to the ulcer group without treatment (d1: 13.13 ± 1.19 cells/field; d8: 13.61 ± 1.99 cells/field). The number of leukocyte adherences in the ulcer group treated with *Aloe vera* was reduced as the ulcer group treated with sucralfate. The mean ± SE of leukocyte adherences on days 1 and 8 is shown in Table 1 and Figure 1.

Table 1 Leukocyte adherence on postcapillary venules indifferent groups (mean ± SE, $n = 6$)

Group	Mean leukocyte adherence (cells/field)	
	Day 1	Day 8
Control	1.69 ± 0.17	5.53 ± 0.65
Ulcer	13.13 ± 1.19[a]	13.61 ± 1.99[a]
Ulcer+sucralfate	3.22 ± 0.76[c]	3.80 ± 0.79[c]
Ulcer+*Aloe vera*	4.29 ± 0.39[c]	4.46 ± 0.27[c]

[a]$P < 0.05$ *vs* control group; [c]$P < 0.05$ *vs* ulcer groups.

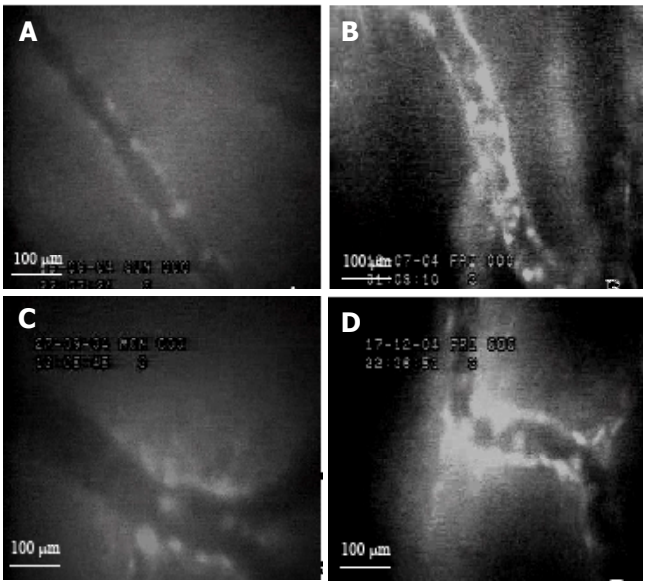

Figure 1 Intravital microscopic (´40) images of leukocyte adherence on vascular endothelium of postcapillary venules in control group (**A**), ulcer group without treatment (**B**), ulcer groups treated with sucralfate (**C**) and *Aloe vera* (**D**) on day 8.

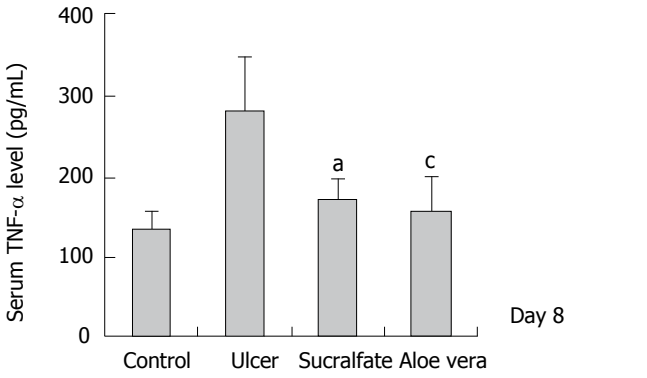

Figure 2 TNF-α level in different groups ($n = 6$) (mean ± SE), [a]$P < 0.05$ *vs* control group; [c]$P < 0.05$ *vs* ulcer groups.

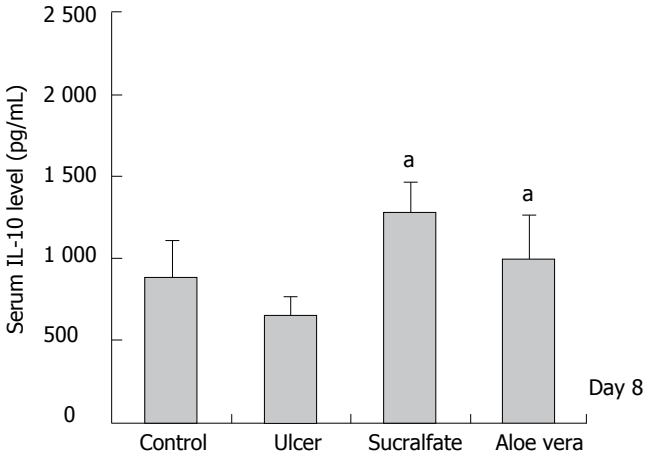

Figure 3 IL-10 level in different groups ($n = 6$) (mean±SD),[c]$P < 0.05$ *vs* ulcer groups.

Changes of TNF-α and IL-10 levels

After gastric ulcer was induced by administration of 20% acetic acid, the level of TNF-α (d_1: 151.40 ± 26.87 pg/mL; d_8: 280.44 ± 67.02 pg/mL) was significantly higher than that in the control group (d1: 12.51 ± 2.35 pg/mL; d8: 133.50 ± 20.95 pg/mL). However, the level of IL-10 after gastric ulcer was induced by administration of 20% acetic (d_1: 472.66 ± 167.75 pg/mL; d_8: 646.60 ± 118.92 pg/mL) was significantly lower than that in control group (d_1: 911.46 ± 230.81 pg/mL; d_8: 883.98 ± 227.62 pg/mL). The level of TNF-α in ulcer group treated with sucralfate (138.62 ± 47.45 pg/mL) and in ulcer group treated with *Aloe vera* (153.02 ± 26.90 pg/mL) was higher than that in control group (12.51 ± 2.35 pg/mL) on d 1. On d 8, the level of TNF-α in ulcer group treated with sucralfate (170.21 ± 23.82 pg/mL) and in ulcer group treated with *Aloe vera* (154.32 ± 43.55 pg/mL) was significantly ($P < 0.05$) lower than that in ulcer group without treatment (280.44 ± 67.02 pg/mL) and was different from that in control group (133.50 ± 20.95 pg/mL). TNF-α level was reduced in ulcer group treated with *Aloe vera* as in the ulcer group treated with sucralfate.

Furthermore, the level of IL-10 in ulcer group treated with sucralfate (d_1: 1 419.93 ± 359.81 pg/mL; d_8: 1 283.64 ± 179.72 pg/mL) and in ulcer group treated with *Aloe vera* (d_1: 1178.13 ± 159.87pg/mL; d_8: 984.02 ± 269.26 pg/mL) was higher than that in ulcer group (d_1: 472.66 ± 167.75 pg/mL; d_8: 646.60 ± 118.92 pg/mL) on days 1 and 8 after induction of gastric ulcer. The mean ± SE of TNF-α and IL-10 levels on d 1 and 8 is shown in Figures 2 and 3, respectively.

Histopathological changes

After administration of 20% acetic acid, histopathological examination revealed hemorrhage, congestion and edema in the gastric mucosa with mild to moderate leukocyte infiltration in gastric lesion. Gastric lesions were erosive and ulcerative. Congestion, edema and erosive lesion were found only in control group. Moreover, the mean maximum length of gastric ulcer in ulcer group without treatment (4.17 cm ± 0.11 cm) was significantly longer than that in control group (3.25 cm ± 0.11 cm) ($P < 0.05$). On day 8 after induction of gastric ulcer, control group and ulcer group without treatment had still mild congestion and edema, mild leukocyte infiltration and erosive lesion in gastric mucosa. The mean length of gastric ulcer in ulcer group without treatment (3.48 cm ± 0.10 cm) was larger than that in control group (3.20 cm ± 0.22 cm). On day 1, the mean maximum length of gastric ulcer in ulcer groups treated with sucralfate (3.73 cm ± 0.12 cm) and with *Aloe vera* (3.60 cm ± 0.18 cm) became shorter after treatment

Table 2 Maximum length of gastric ulcer (cm) in different groups ($n = 6$) (mean ± SE)

Group	Maximum length of gastric ulcer (cm)	
	Day 1	Day 8
Control	3.25 ± 0.11	3.20 ± 0.22
Ulcer	4.17 ± 0.11[a]	3.48 ± 0.10
Ulcer + sucralfate	3.73 ± 0.12[b]	3.33 ± 0.11
Ulcer + Aloe vera	3.60 ± 0.18[b]	3.43 ± 0.10

[a]P <0.05 *vs* control group, [b]P <0.05 *vs* ulcer groups

when compared to the ulcer group without treatment (4.17 cm ± 0.11 cm). On day 8, the mean maximum length of gastric ulcer in ulcer groups treated with sucralfate (3.33 cm ± 0.11 cm) and *Aloe vera* (3.43 cm ± 0.10 cm) was slightly reduced with no significant difference after treatment when compared to the ulcer group without treatment (3.48 cm ± 0.10 cm). The mean ± S.E of the maximum length of gastric ulcer is shown in Table 2.

DISCUSSION

In this study, after gastric ulcer was induced by administration of 20% acetic acid, gastric inflammation increased leukocyte adherence to the endothelial surface of postcapillary venules and was characterized by the migration of macrophages and PMNs in the ulcer area. The migrated macrophages then released proinflammatory cytokines such as TNF-α and interleukin-1β (IL-1β). Pro-inflammatory cytokines can up-regulate adhesion molecule expression on endothelial cells and leukocytes[27,28] and cause leukocyte recruitment[29]. Adhesion molecules on endothelial cells and leukocytes involve rolling, adhesion, and transmigration of leukocytes in gastric inflamed areas. It was reported that increment of PMNs may play an important role in the pathogenesis of NSAIDs-induced gastropathy[30]. On the other hand, NSAIDs may enhance the expression of cell adhesion molecules on the surface of endothelial cells[31]. Adhesion molecules play an important role in the recruitment of leukocytes to inflammation sites, leading to gastric mucosal injury[31, 32]. It was also reported that leukocyte adhesion and/or aggregation can occlude microcirculation, resulting in ischemic mucosal injury[31, 33]. Leukocyte infiltration in gastric mucosa can cause tissue damage leading the ulcerative lesion[34].

In this study, gastric ulcer induced by administration of 20% acetic acid had elevated TNF-α levels, demonstrating that elevated pro-inflammatory cytokine level induces interaction between leukocytes and endothelial cells. It was suggested that 20% acetic acid could stimulate macrophages to release proinflammatory cytokines. TNF-α can stimulate ICAM-1 expression on vascular endothelial cells. ICAM-1 is an adhesion molecule, which plays a pivotal role in inflammatory reaction by increasing leukocyte adhesion to endothelium and promoting transen-dothelial migration of leukocytes to inflammatory sites[3]. Moreover, it was reported that TNF-α can also stimulate expression of LFA-1 (CD11a/CD18)[35], an adhesion molecule on leukocytes. This might be the reason why of TNF-α and leukocytes-endothelium interaction is increased in the inflammatory area.

However, after gastric ulcer was induced by administration of 20% acetic acid IL-10 level was reduced on day 1, but elevated spontaneously on day 8 as compared to the control groups (Figure 3). This might be due to the fact that when the gastric mucosa was damaged by acetic acid, T and B lymphoctyes in submucosal beneath the damaged area that typically produce basal level of IL-10 were also damaged. The location of macrophages was actually beyond the damaged area, therefore, the macrophages were then survived. The survived macrophages are able to stim-ulate the release of TNF-α in response to acetic acid injury. Therefore, our findings suggest that TNF-α is synthesized more than IL-10. When inflammation occurs, IL-10 is synthesized. The increment of IL-10 level reduces gastric inflammation through its feedback inhibition of TNF-α production. Therefore, IL-10 is spontaneously elevated in chronic gastric inflammation. The elevation of IL-10 then reduces gastric tissue inflammation simultaneously.

Aloe vera and sucralfate could reduce leukocyte adherence after gastric ulcer was induced by 20% acetic acid. It has been reported that *Aloe vera* can decrease carrageenan- induced edema and neutrophil migration in rats[36]. In addition, *Aloe vera* has antiinflammatory effect on burn wounds by reducing leukocyte adhesion in rats[37, 38]. On the other hand, *Aloe vera* is able to inhibit prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and thromboxane B2 production in guinea pig with burn wounds[39]. Thromboxanes and prostaglandins (PGs) can elicit platelet aggregation, leukocyte adherence and vasoconstriction, thus enhancing ischemia. Moreover, *Aloe vera* possesses bradykininase activity and also decreases inflammation[9]. Bradykinin causes increase in vascular permeability and stimulates inflammation[40]. Therefore, these effects of Aloe vera might reduce the causes of inflammatory process, the effects and leukocyte adherence after gastric ulcer is induced by 20% acetic acid.

Aloe vera and sucralfate could reduce TNF-α level on days 1 and 8 after induction of gastric ulcer (Figure 2). *Aloe vera* has cytoprotective effect on gastric mucosa by stimulating endogenous prostaglandins production[20]. Sucralfate is a cytoprotective drug which also stimulates PG production. Prostaglandins (PGs), especially prostaglandin E2 (PGE2) can protect gastric mucosa from various irritants, promote mucus production and increase mucosal blood flow[41-43]. It was reported that PGE2 plays a role in modulating TNF-α production and is also a potent inhibitor of neutrophil adherence and chemotaxis[44]. Ding *et al*[45] reported that PGE2 inhibits TNF-α release in gastric mucosa and reduces in neutrophil activation and subsequently ischemia and mucosal damage. They also showed that inhibition of TNF-α by PGE2 could result in the reduction of neutrophil CD11b/CD18 and endothelial ICAM-1 expression directly or indirectly, thus subsequently reducing neutrophil adhesion on vascular wall.

In our study, *Aloe vera* and sucralfate could elevate IL-10 level on days 1 and 8 after induction of gastric ulcer. IL-10 is an anti-inflammatory cytokine produced by various cells including monocytes/macrophages and T lymphocyte. IL-10 can inhibit cytokine synthesis by macrophages[46]. In

addition, the mild anti-inflammatory effects of IL-10 may be due to the suppression of TNF-α production[47]. Bodger *et al*[48] showed that mucosal secretion of IL-10 and TNF-α is increased in *H pylori* gastritis. IL-10 may be protective and can limit tissue damage caused by inflammation. Therefore, elevation of IL-10 can down-regulate TNF-α production in macrophage.

Aloe vera and sucralfate could reduce inflammation and promote gastric ulcer healing, which has been confirmed by histopathological examinations. *Aloe vera* and sucralfate promote epithelial cell proliferation, elongation and dilatation of oxyntic grand. *Aloe vera* and sucralfate have a cytoprotective effect on gastric mucosa by stimulating PGE_2 production. PGE_2 plays an important role in the maintenance of mucosal integrity and mucus production. It was reported that *Aloe vera* could promote burn wound healing in rats[37, 38, 49]. In addition, *Aloe vera* could induce angiogenesis *in vivo*[5], which plays an important role in wound healing. *Aloe vera* can result in reduced vasoconstriction and improve perfusion of gastric mucosal capillaries, thus promoting ulcer healing[13, 50, 51]. Furthermore, gastric acid is considered as an important aggressive factor in the stomach and is known to produce gastric injury[52]. *Aloe vera* is able to decrease gastric acid secretion and increase mucus secretion[53].

The mechanism of *Aloe vera* could be explained by its action on inflammation and ulcer healing. The results of this study suggest that *Aloe vera* could decrease leukocyte adherence and TNF-α levels in inflammatory tissue. Our findings demonstrate that *Aloe vera* could act as an antiinflammatory agent on gastric ulcer. Our findings also indicate that ulcer healing effect of *Aloe vera* is mediated by increasing IL-10, an important cytokine for wound healing process.

In conclusion, *Aloe vera* acts on inflammation and promotes ulcer healing. *Aloe vera* might be used as a therapeutic agent for gastric ulcer patients.

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BASIC RESEARCH

Construction and evaluation of anti-gastrin immunogen based on P64K protein

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Abstract

AIM: To construct two kinds of anti-gastrin immunogen based on P64K protein from *Neisseria meningitids* and to compare their immunogenic effect.

METHODS: G17P64K gene was cloned and ligated into pET28a plasmid, then transformed into BL21(DE3). After inoculation of LB medium and IPTG induction, the recombinant protein was solubly expressed at a high level. The purification of G17P64K fusion protein was similar to that of P64K. An initial step of purification consisting of 30% saturated ammonium sulfate precipitation was done. Additional fine optimizations included phenyl-sepharose, G200 Sephadex gel filtration and Q-sepharose anion exchanger chromatography. Highly purified protein was obtained and sequenced at the N-terminal amino acid residues. Polypeptide was synthesized by Fmoc solid phase chemical method and cross-linked to carrier protein P64K and DT mutant by MBS method and then the rabbit anti-gastrin 17 antibody was prepared by immunizing rabbit with cross-linked and fused protein. The titer and the activity *in vitro* of antibody were assessed.

RESULTS: G17P64K gene and the recombinant bacteria were obtained. After four steps purification, protein sample that has the purity above 90% was achieved. At the 84th day after the first immunization, the titer of antibody against cross-linked protein reached 51 200. Evaluation of the antibody *in vitro* manifested that it had a high inhibitory activity on the growth of tumor cell SW480.

CONCLUSION: The P64K-polypeptide cross-linked immunogen immunized rabbit and achieved a higher titer antibody against gastrin 17 than the G17P64K fusion protein immunogen, which could inhibit the growth of the tumor cell SW480.

Key words: Gastrin; P64K protein; therapeutic vaccine

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INTRODUCTION

The peptide hormone gastrin, released from the G cells of the antrum and duodenum, is known to stimulate the secretion of gastric acid and act as a trophic factor within gastrointestinal tract[1-3]. In recent years evidence has accumulated that gastrin is a growth factor for gastrointestinal tumors, such as gastric cancer, colon cancer, and pancreatic cancer[3-6]. Therefore it has emerged as a potential anticancer target[7].

There are several forms of gastrin; the precursor molecule preprogastrin is cleaved by an endopeptidase to progastrin, which is further processed to glycine-extended gastrin 34 and to gastrin 34[8]. These 35- and 34-amino acid peptides can be cleaved to form glycine-extended gastrin 17 and gastrin 17 respectively. Cells with gastrin receptors can respond to mature amidated forms of gastrin, as well as precursor forms, particularly glycine-extended gastrin 17. It has become clear that in cancer cells originating in various parts of the gut, the gastrin gene and the genes that encode the gastrin receptors are switched on at a very early stage of oncogenesis. Scientists have demonstrated that the glycine-extended gastrin 17 and gastrin 17 can promote cancer cell division via an autocrine or endocrine pathway[9]. A unique B cell epitope to glycine-extended gastrin 17 and gastrin 17, which is not found on gastrin 34 and CCK, consisting of the 9-amino acid stretch at the amino-terminal end of the molecule, has been identified and mapped. The immunogens comprising this unique epitope result in high levels of anti-gastrin 17 antibodies

that do not cross-react with gastrin 34 and CCK, which share with gastrin 17 a common receptor to carry out their physiologic function.

P64K protein isolated from pathogenic bacterium *Neisseria* is found in the outer membrane of the cell and well recognised in sera from individuals convalescent from meningococcal disease or vaccinated with VA-MENGOC-BC®, a Cuban antimeningococcal vaccine based on outer membrane vesicles[10]. Its high molecular mass and strong immunogenicity have made it a suitable carrier protein for weak immunogens. Recent studies have manifested that P64K protein has a better immune enhancing effect than conventional carrier protein such as BSA, TT[11]. The B cell epitope of gastrin 17 was cross-linked or fused via a peptide spacer to P64K to comprise an immunogen against gastrin 17.

MATERIALS AND METHODS

Materials

P64K gene and protein were conserved in our laboratory. DH5α, BL21(DE3) were purchased from BioDev (Beijing China). Restriction endonucleases, polymerase, T4 DNA ligase and DL2000 were from Takara (Dalian,China). GENECLEAN II kit was ordered from Q.BIOgene (Morgan Irvine,USA). SV Minipreps DNA purification system was provided by Promega (Madison,USA). DT mutant (CRM197) was purchased from Sigma(Ronkonkoma, USA). Polypeptide was synthesized and cross-linked in Institute of Basic Medical Sciences (Academy of Military Medical Sciences, Beijing, China). Low molecular weight calibration kit for SDS electrophoresis was purchased from Amersham Biosciences(Beijing,China). Goat anti-rabbit IgG-HRP was from Bio-LAB(Beijing China). New Zealand white rabbits and BALB/C nude mice were supplied by Laboratory of Animal Center (Academy of Military Medical Sciences, Beijing, China).

SW480 is a human colonic epithelial tumor cell. It could be stimulated to proliferate by gastrin via an autocrine or endocrine pathway and inhibited by gastrin inhibitor[12,13]. It was obtained from The Cell Center of Basic Medical Sciences(Chinese Academy of Medical Sciences, Beijing, China). Cell lines were grown in DMEM (GIBCO-BRL) supplemented with 10% heat-activated fetal bovine serum(FBS) in a humidified incubator at 37 ℃ in an atmosphere of 5% CO_2.

Methods

Construction of recombinant expression plasmid: Cloning of gene, isolation of plasmid and all other molecular biology procedures were carried out according to the standard procedures published. P64K gene was cloned from *Neisseria meningitids* and gastrin17 B cell epitope was designed into 5' upperstream primer[14]. G17P64K gene was cloned by PCR amplification under the following conditions: 30 cycles of 94℃ for 1 min, 50 ℃ for 1 min and 72 ℃ for 2 min with one additional cycle for 10 min at 72 ℃. The reaction components were: 1μg of P64K DNA; 50 pmol of primer 1 (5'- CATGCCATGGAAGGCCCTTGGCTTGAAGAGGAAGAATCTTCACCCCCTCCGCCGCTTTAGTTGAATTGAAAGTG-3') and primer 2 (5'-GGGAATTCTTATTTTTTCTTTTGCGGAG-3'); 200 μmol/L of each deoxynucleotide triphosphates (dNTPs); PCR buffer (10 mmol/L KCl, 20 mmol/L Tris-HCl pH 8.8, 10 mmol/L $(NH_4)_2SO_4$, 2 mmol/L $MgSO_4$, 0.1%Triton X-100; double distilled water to a final volume of 50 μL and 1 unit per reaction of Pyrobest DNA polymerase. PCR product was purified by agarose gel electrophoresis, digested by *Nco*I and *Eco*RI, then ligated into the pET28a plasmid digested with the same enzymes. The ligation products were then transformed into *E.coli* DH5α. Positive clones were selected by PCR using the conditions described above and subjected to double-stranded DNA sequencing with T7 sequencing primer according to the manufacturer's specifications. Sequence processing was done with the DNAStar software.

Expression of recombinant protein in shake flask cultures: The strain *E.coli* BL21 (DE3) was transformed with G17P64K recombinant plasmid, which was also transformed with plasmid (negative control). A transformed colony from each construct was inoculated into 20 mL Luria Bertani (LB) cultures. Bacteria were grown in a shaker at 37℃. After measurement of the optical density at 600 nm (OD_{600}), IPTG was added to induce recombinant protein synthesis considering OD_{600} of 0.5. Cell culture was allowed to continue incubation for 4 h. Samples were collected and centrifuged at 10 000 g for 10 min at 4 ℃. The bacterial pellet was washed twice with 10 mmol/L Tris and 1mmol/L EDTA, pH8 (TE), and then resuspended in 1 mL TE. Cells were destructed by sonication for 15 min. After centrifugation of the lysate at 27 000 g for 30 min at 4℃, supernatants and pellets from destruction were collected. All protein samples were subjected to 15% SDS-PAGE according to the standard procedure of Laemmli. Protein bands were visualized by staining with Coomassie brilliant G-250. Gels were scanned and the purity was estimated by densitometry using the Molecular Analysis software.

Production of recombinant protein in bioreactors: The colony containing recombinant plasmid was inoculated into 5 mL cultures of LB containing kanamycin(30 μg/mL). Cells were grown in a shaker at 37 ℃ to an OD_{600} of 0.8. The sample was transferred into 200 mL LB culture and continued to grow for 12 h at 37 ℃ with shaking (200 rpm). This culture was used to inoculate 3 L of rich media supplemented with 30 μg/mL kanamycin. When OD_{600} reached 20, expression of G17P64K gene was induced by addition of IPTG to a final concentration of 1 mmol/L and allowed to take place at 37 ℃ for 7 h. One milliliter of the bacteria was taken out at 0, 1, 2, 3, 4, 5, 6 and 7 h after induction. Cells were harvested by centrifugation at 10 000 g for 20 min and stored at -20 ℃. Five microliters of bacteria were electrophoresed on a 15% SDS-PAGE for evaluation of protein expression.

Purification of the recombinant protein: The supenatant obtained after centrifugation of the lysate was precipitated by adding solid ammonium sulfate to 20%, 30%, 40%, 50% saturation respectively. And the condition of the most optimal purification effect was ascertained. The pellet was resuspended after centrifugation at 27 000

Table 1 The dosage of rabbit immunization

Polypeptide (mg/rabbit)	G17P64K protein (mg/rabbit)	G17(9) : P64K (mg/rabbit)	G17(9) : DT (mg/rabbit)	P64K protein (mg/rabbit)
0.25	2.0	0.5	0.5	0.25

g for 20 min at 4 ℃. The resuspended solution was applied to the following chromatography steps similar to P64K.

The first stage was a phenyl-sepharose hydrophobic interaction column. After the sample was loaded, the column was washed with loading buffer(2.0 M ammonium sulfate, 20 mmol/L Tris-HCl, 1 mmol/L EDTA, pH7.2) and eluted with a 1.5-0 mol/L ammonium sulfate gradient. G17P64K protein was eluted as a major peak in about 0.8M ammonium sulfate and G17P64K-containing fractions were pooled by adding solid ammonium sulfate to 100% saturation. The second step was a G200 Sephadex column. The concentrated sample was loaded on the column, re-equilibrated and eluted with PBS. G17P64K was eluted in the major peak and both sides contained only small amounts of contaminants. The last chromatography step was a Q-Sepharose anion exchange column. The sample from the previous G-200 sephadex column was diluted 10 times and loaded onto the anion exchange column. After washing the column with loading buffer (20 mmol/L KPB, 5mmol/L NaCl, 1mmol/L EDTA, pH6.0), G17P64K was eluted with 5-500 mmol/L NaCl gradient. In this step, the G17P64K-containing peak was the only major peak. The samples were subjected to 15% SDS-PAGE under denaturing conditions as previously described.

Chemical synthesis and crosslink of polypeptide: Polypeptide with the following amino acid sequences were synthesized: pyro-Glu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Gly-Gly-Gly-Gly-Ser-Cys. Synthetic polypeptide contained a B-cell epitope consisting of residues 1 to 9 of gastrin 17, a five amino acid peptide spacer, and an N-terminal cysteine. It was produced by Fmoc solid-phase synthetic chemical method and its purity was detected by high-performance liquid chromatography(HPLC) analysis method. The polypeptide was conjugated to amino group present on a carrier protein such as P64K and CRM-197(DT mutant) via the terminal peptide cysteine residue utilizing heterobifunctional linking agent-m-maleimidobenzoyl-N-hydroxysuccinimide ester(MBS). After desalting? and concentrating over a PM 10 ultrafiltration membrane, the conjugate was lyophilized and stored desiccatedly at -20℃ until use.

Preparation of rabbit anti-gastrin 17 antibody: New Zealand rabbits were immunized with the immunogens including synthetic polypeptide, G17P64K recombinant protein, polypeptide-DT conjugate and polypeptide-P64K conjugate. Four groups of 2 rabbits were immunized with one of the immunogens. Each animal was injected subcutaneously with 1.5 mL of immunogen. An additional group of 2 rabbits was immunized with P64K protein as a blank immunization control. Each group of rabbit was immunized as shown in Table 1. Rabbits were vaccinated by subcutaneous injection of immunogens emulsified with CFA/PBS for the first immunization and IFA/PBS for the subsequent 2 booster injections at 3-wk intervals. Blood samples were collected from rabbits at wk 0, 2, 5, 8 and 12.

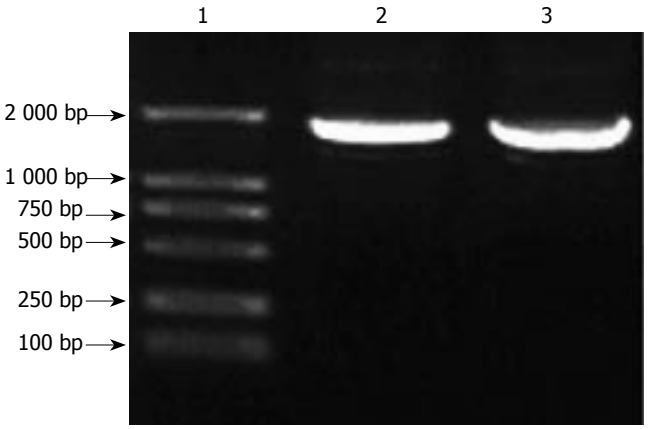


Figure 1 PCR products on agarose electrophoresis. Lane1: DL2000 marker; Lane 2, 3: PCR products.

Detection of antibody titer*:* Antibodies levels in sera were determined by enzyme linked immunosorbent assay (ELISA). To detect anti-gastrin 17 antibodies titer, 96-well plates were coated with 200 μL/well of corresponding peptide(10 μg/mL) in carbonate buffer(0.05 mol/L $NaCO_3$, pH 9.6) for 2 h at 37 ℃. Bovine serum albumin (2%) was used as a blocking reagent. After three washes in /0.05% PBS Tween 20 (PBST), 100 μL of serial dilution of each serum (starting dilution 1:100) was added to the plates, which were incubated for 2 h at 37 ℃. All sera were analyzed in four replicates. Serum anti-gastrin 17 antibody levels were expressed as their absorbance (492 nm) values in ELISA and used for statistical analysis.

Evaluation of the antibody in vitro: SW480 cells were seeded into 96 flat bottomed well plates(Costar) at a concentration of 5×10^4 per well in a 100 μL volume in the serum free medium. Affinity-purified immunoglobulins(IgG) raised against gastrin 17 and normal rabbit control IgG were normalized to standard protein concentration of 0.5 mg/mL. IgG dilutions from 1/2 to 1/256 were prepared in serum free medium and added to the cells in 100 μL volume per well. Each condition was performed with four replicates. Cell proliferation was assessed after 72 h by the methylthiazoyl tetrazolium(MTT) assay.

Statistical analysis

Statistics evaluation of *in vitro* data was performed by a one way analysis of variance on the SPSS package.

RESULTS

Construction of recombinant expression plasmid

After amplification of G17P64K gene by PCR, there was a band of about 1.8kb by agarose gel electrophoresis(Figure 1). The fragment was purified, digested by *Nco*I and *Eco*RI

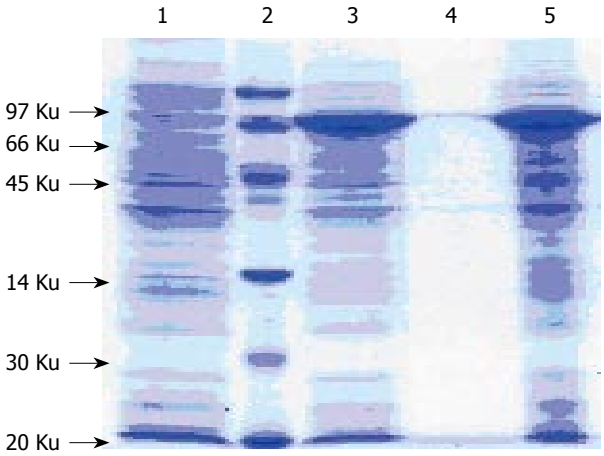


Figure 2 15% SDS-PAGE analysis of expression products. Lane 1: Non-recombinant bacteria; Lane 2: Low molecular protein marker; Lane 3: Recombinant bacteria; Lane 4: Deposition after sonication; Lane 5: Supernatant after sonication.

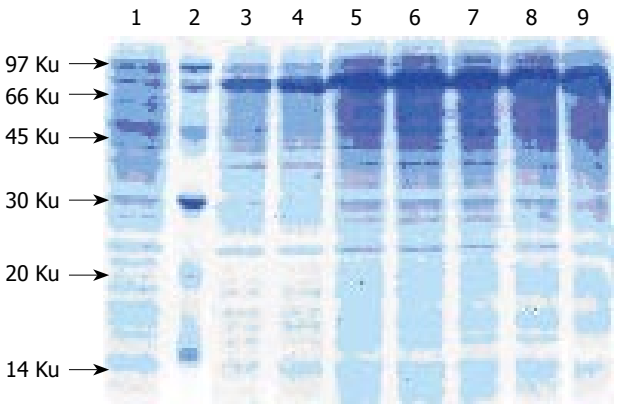


Figure 3 The expression of G17P64K protein in bioreactor. Lane 1: Low molecular protein marker; Lane 2: Sample before IPTG induction; Lanes 3-9: Sample after induction 1-7 h.

and cloned into the pET28a. The resulting plasmid was designated as pET28a-G17P64K. Sequencing revealed that the G17P64K gene was fused to the correct reading frame with the coding sequence.

Expression of recombinant protein in shake flask cultures

SDS-PAGE was carried out with the sample from BL21(DE3), which transformed with pET28a- G17P 64K (Figure 2). An approximate 70KD protein was solubly expressed in BL21(DE3) and accounted for about 30% of the total cellular protein.

Production of recombinant protein in bioreactors

Recombinant bacteria were grown in 5-L fermentors to investigate whether the G17P64K protein could be expressed and the yields could be amplified using a scale-up cultivation. The 5-L fermentors strategy allowed cultures to grow to final OD_{600} of 33. At 5-L fermentors culture of 5 h, induction of the T7 promotor and production of G17-P64K protein was initiated. The G17P64K protein was gradually increased per OD_{600} until the end of fermentation (Figure 3).

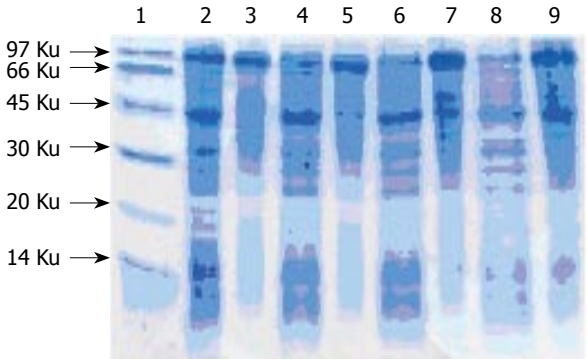


Figure 4 15% SDS-PAGE analysis of sample after ammonium sulfate deposition. Lane 1: Low molecular protein marker; Lanes 2, 4, 6, 8: Supernatant after ammol/ Lonium sulfate deposition; Lanes 3, 5, 7, 9: Deposition after 20%, 30%, 40%, 50% ammonium sulfate deposition.

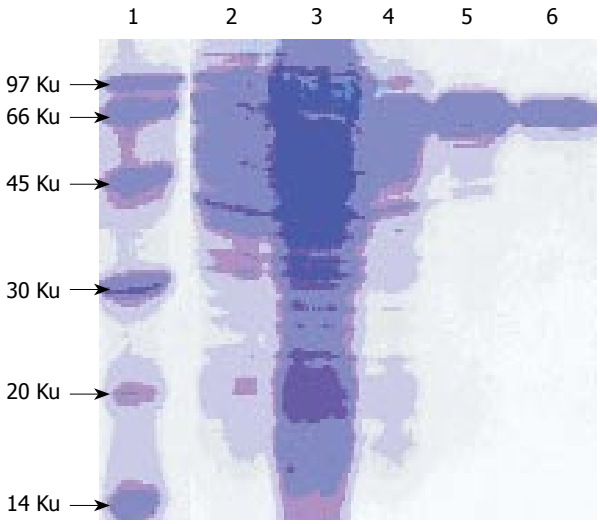


Figure 5 15% SDS-PAGE analysis of purified G17P64K fusion protein. Lane 1: Low molecular protein marker; Lane 2: Total protein after induction; Lane 3: Supernatant after sonication; Lane 4: Sample after hydrophobic chromatography; Lane 5: Sample after gel filtration chromatography; Lane 6: Sample after anion exchanger chromatography.

Purification of the recombinant protein

The G17P64K production system described in this work may permit the protein to be purified using commercially available, conventional method. SDS-PAGE manifested that 30% saturation of ammonium sulfate could most efficiently remove the contaminants (Figure 4). The first step consisted of a phenyl-sepharose hydrophobic interaction column chromatography and used a high con-centration to enhance G17P64K capture. A G200 Sep-hadex column chromatography was used next to further minimize host cell protein contaminants, followed by a Q-Sepharose anion exchanger column chromatography to help eliminate the residual contaminants. The results of this purification scheme are shown in Figure 5. After four steps of purification, the final product was judged to be more than 90% of purity. And the result of N-terminal amino acid sequencing (MEGPW) was in accordance to

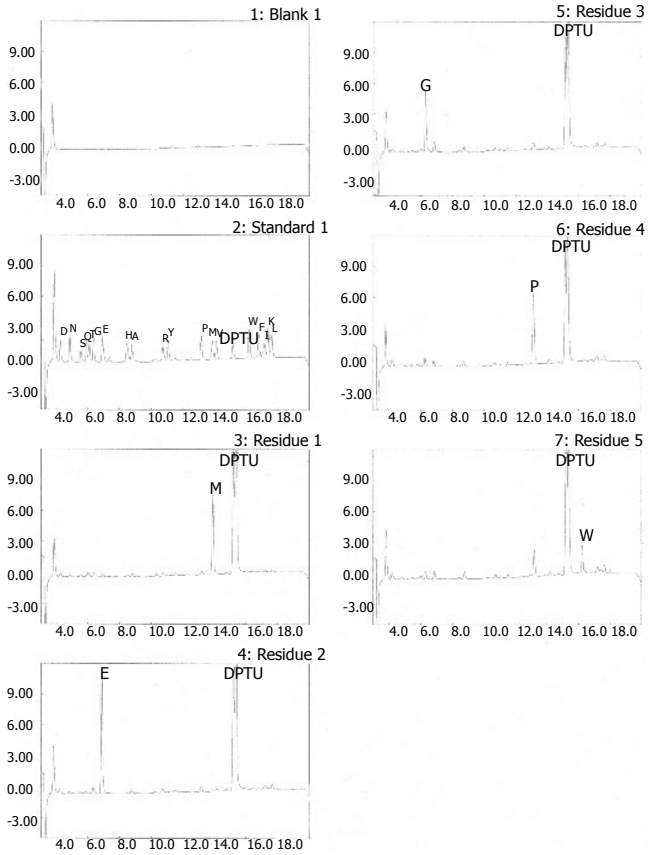
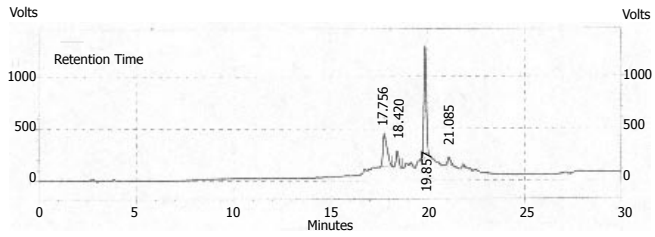


Figure 6 N-terminal amino acid sequence of G17P64K protein.



200 UV Results

Retention Time	Area	Area (%)	Height	Height (%)
17.756	4493678	27.37	312380	19.19
18.420	1530656	9.32	158889	9.76
19.857	9748167	59.37	1087743	66.84
21.085	646928	3.94	68440	4.21
Totals	16419429	100.00	1627452	100.00

Figure 7 HPLC identification of the purity of synthetic polypeptide.

the anticipated sequence (Figure 6).

Chemical synthesis and crosslink of polypeptide

Polypeptide was synthesized by Fmoc solid-phase synthetic chemical methods. The total weight of synthetic polypeptide was determined to be 60mg and the purity was detected to be 66.84% by HPLC (Figure 7). The synthetic peptide was conjugated to carrier protein via MBS cross-linking reagent. The conjugate was lyophilized and stored desiccatedly at -20 ℃ until use.

Table 2 Rabbit anti-gastrin 17 antibody levels

	Polypeptide	G17P64K protein	G17(9) : DT	G17(9) : P64K
D 0	0	0	0	0
D14	100	100	800	800
D35	800	800	6400	6400
D54	3200	3200	25600	25600
D84	6400	6400	51200	51200

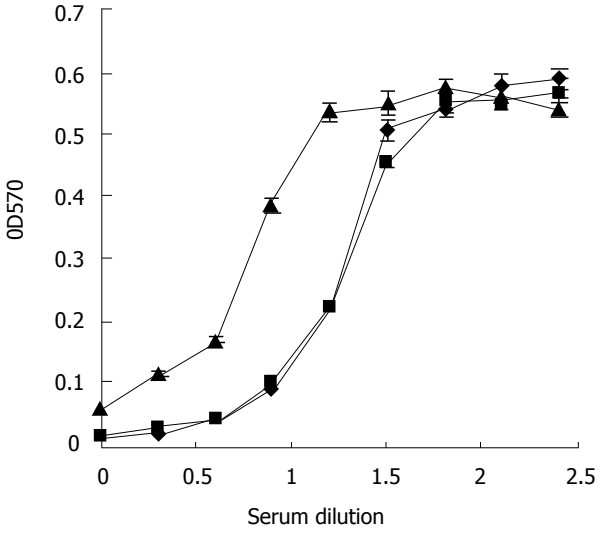


Figure 8 Effects of rabbit anti-gastrin 17 antibody on the *in vitro* growth of SW480 ◆: anti-gastrin 17(9): P64; ■Red: rabbit anti-gastrin 17(9): DT; ▲ : rabbit control IgG

Detection of antibody titer

Anti-gastrin 17 antibody levels in rabbit sera were determined by ELISA (Table 2). High levels of antibody were obtained against synthetic polypeptide conjugated to protein carriers DT and P64K. The result also revealed that the immunogenicity of G17P64K fusion protein was as weak as the single synthetic peptide.

Assessment of the activity of antibody in vitro

MTT uptake was used to measure *in vitro* proliferation which assesses the oxidative ability of the cell to convert a soluble tetrazolium compound into an insoluble colored crystalline product which can be dissolved and assessed colorimetrically. This has previously been shown to correlate with direct cell counts in many cases. The characterization of SW480 has revealed that endocrine/autocrine gastrin–mediate pathways may be operational in the growth of the cells. Figure 8 shows the MTT uptake of SW480 in the presence of increasing dilutions of rabbit ani-gastrin 17 IgG. In experiment, the growth of SW480 was significantly reduced by treatment with rabbit anti-gastrin 17(9) : DT and anti-gastrin 17(9) : P64K from 1/1 to 1/16 dilution, and modestly inhibited at dilutions of 1/32 and 1/64 ($P < 0.05$, one way analysis of variance). The effect of rabbit anti-G17(9) : P64K treatment on the *in vitro* growth of SW480 was not significantly different from rabbit anti-G17(9) : DT from 1/1 to 1/64.

DISCUSSION

Immunization against specific cancer promoting hormones may be useful in the treatment and prevention of cancer. Gastrin has been identified as the central trophic factor for gastrointestinal cancers and has therefore emerged as a potential anticancer target. There are many optional inhibitors of gastrin production[15]. Gastrin-receptors antagonists have been evaluated, but failed to produce any survival benefit in clinical trials[16-18]. Anti-gastrin antibody was demonstrated to be able to bind to gastrin peptides and prevent their interaction with gastrin receptors in preclinical research. And *in vivo* animal studies showed that infusion of anti-gastrin antibodies could inhibit the growth of human colorectal xenografts[19-22]. However, passive infusions of antibodies have serious drawbacks for the patient. Antibodies given passively to patients are not long-lasting and 1 or 2-h infusion would be required every week. In addition, antibody titer would have to be in excess of those required to neutralize normal gastrin secretion so that tumor-associated gastrin would be targeted. Active immunization was likely to work better. A novel immunotherapy has now completed phase III clinical trials of "Gastrimmune" as monotherapy in patients with pancreatic cancer and reported positive results[23-25]. Aphton Corp. has filed for marketing approval in Australia, Canada and New Zealand in 2004. Therefore targeting gastrin as an active-immunotherapeutic approach is appropriate.

There are several forms of gastrin. The glycine-extended gastrin 17 and gastrin 17 are the central trophic factor of cancer cell, while gastrin 34 and CCK are necessary for human body to maintain normal operation. A unique B cell epitope to glycine-extended gastrin 17 and gastrin 17 that is not found on gastrin 34 and CCK, consisting of the 9-amino acid stretch at the amino-terminal end of the molecule, has been identified and mapped. The immunogens comprising this unique epitope result in high levels of anti-gastrin 17 antibodies that do not crossreact with gastrin 34 and CCK, and could effectively decrease the side-effect of vaccine.

An important drawback for the use of the conventional carrier protein in different conjugate vaccines is carrierinduced hapten-specific suppression. It has been demonstrated when mice, presensitized with keyhole limpet hemocyanin (KLH) and subsequently vaccinated with KLH-dinitrophenol(DNP) conjugates, elicited a reduced anti-DNP IgG response[26]. Being a novel carrier protein, P64K protein has its superiority to BSA, TT and DT. This study evaluated the effect of P64K presensitization on its ability as a carrier, by comparing the hapten-specific humoral immune response elicited in rabbit. It proved that P64K protein can help to activate humoral immune system and enhance immune reaction against gastrin 17 B cell epitope as DT.

There are two problems for the immunogen comprising G17 recombinant protein associated with the low level of anti-gastrin 17 antibody because the immunogen is made up of single synthetic polypeptide. First, the excess methionine at the N-terminal of the G17P64K recombinant protein remodels the conformation of the B cell epitope of gastrin 17, and thereof disturbs the recognition of the B cell. Second, polypeptide could provide more B-cell epitope than the G17P64K recombinant protein in the experiment of preparation of anti-gastrin 17 antibody.

A series of tests should be done to assess the inhibitory effect and specificity of the P64K-gastrin immunogen, including the *in vivo* experiment and the adjustment between the inhibitory effect and the gastrin 17 levels. Furthermore, if there were a suitable cell line, an *in vivo* experiment could be done directly, but not human-rabbit-mice? conversion. Another test is the quality control of the crosslink immunogen of P64K and synthetic peptide. Other kinds of cancer cell line should be considered.

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BASIC RESEARCH

Effect of Chinese medicine Qinggan Huoxuefang on inducing HSC apoptosis in alcoholic liver fibrosis rats

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Abstract

AIM: To investigate the effect of Qinggan Huoxuefang (QGHXF) on improvement of liver function and pathology in rats, and to analyze the mechanism.

METHODS: Wistar rats were divided into three groups at random: normal control group (12), micro-amount carbon tetrachloride group (CCl_4)(12) and model group A (60). The model group A was ingested with the mixture (500 mL/L alcohol, 8 mL/kg per day; corn oil, 2 mL/kg per day; pyrazole, 24 mg/kg per day) once a day and intraperitoneal injections of 0.25 mL/kg of a 250 mL/L solution of CCl_4 in olive oil twice a week for 12 wk. The CCl_4 group received intraperitoneal injections only. At the end of 8 wk the model group A (60) was divided into 5 subgroups: model group, Xiaochaihu Chongji (XCH) group, QGHXF high dose group, moderate dose group and low dose group, and were given the drugs respectively. At the end of 12 wk, all the rats were killed and blood samples collected, as well as liver tissue. Blood samples were used for evaluation of alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyltransferase (γ–GT). Liver specimens were obtained for routine HE, apoptosis gene array and flow cytometry analysis.

RESULTS: A liver fibrosis animal model was successfully established. Fibrosis was obviously reduced in QGHXF high dose group, and no fibrosis formed in CCl_4 group. Compared with model group the QGHXF group and XCH group could obviously decrease the level of ALT, AST, ALP, and GGT ($P<0.05$). QGHXF high dose group was better than XCH group in ALT (615±190 *vs* 867±115), and AST(1972±366 *vs* 2777±608). Moreover, QGHXF could reduce liver inflammation, fibrosis-induced hepatic stellate cell (HSC) apoptosis and regulate apoptosis gene expression. The HSC apoptosis rates of QGHXF groups were 22.4±3.13, 13.79±2.26 and 10.07±1.14, higher than model group, 6.58±1.04 ($P<0.05$). Compared to model group, 39 genes were up-regulated, 11 solely expressed and 17 down-regulated in high dose group.

CONCLUSION: QGHXF can improve liver fibrosis and induce HSC apoptosis.

Key words: Qinggan Huoxuefang; Alcoholic liver fibrosis; Apoptosis; Gene array

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INTRODUCTION

Alcohol abuse and dependence have been a public problem all over the world. In the United States, 45 billion dollars were spent on alcoholic intemperance and its related problems[1,2]. In recent years morbidity rate of alcoholic liver diseases (ALD) has risen rapidly. It has become the second liver disease after viral hepatitis in China[3,4]. Liver disease in the alcoholics is due not only to malnutrition but also to ethanol's hepatotoxicity linked to its metabolism by means of the alcohol dehydrogenase and cytochrome P450 2E1 (CYP2E1) pathways and the resulting production of toxic acetaldehyde[5-8]. Alcoholic liver disease is a major source of alcohol-related morbidity and mortality. Heavy drinkers and alcoholics may progress from fatty liver to alcoholic hepatitis to cirrhosis, and it is estimated that 10 to 15 percent of alcoholics will develop cirrhosis[9]. Alcoholic liver fibrosis (AF) is one of the alcoholic liver diseases. It is the excessive accumulation of extracellular matrix (ECM) proteins including collagen that occurs in chronic liver diseases. Advanced liver fibrosis results in cirrhosis, liver failure, and portal hypertension and often requires liver transplantation[10]. However, recent evidence indicates that even ad-

focus of researches. Fibrogenesis in human ethanol injury is due to the activity of stellate cells, Kupffer cells, and to a lesser extent, to endothelial cells[14]. Above all of the factors, hepatic stellate cell (HSC) is the key to fibrosis. Activated HSCs are the source of collagen deposition in the liver[15]. This study aimed to explore the mechanism of QGHXF (a traditional Chinese herb) on AF through examination of liver function, and histopathology, detection of HSC apoptosis and gene array, in an effort to search for the experimental basis for traditional Chinese medicine in AF therapy.

MATERIALS AND METHODS

Materials

Jianzhuang Alcohol (52°) and corn oil were purchased from Lianhua Supermarket. Formaldehyde, 400 g/L, olive oil, carbon tetrachloride, hematoxylin and eosin were supplied by Shanghai Chemicals Company. The test kit of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (γ-GT), and alkaline phosphatase (ALP) were purchased from Shanghai Rongsheng Biotech Co. Ltd. QGHXF (bupleurum root 9 g, scutellaria root 9 g, red sage root 15 g, carapax trionycis 9 g, *Radix puerariae* 15 g), concentrated to 2.6 kg/L were processed by Department of Pharmacy, Longhua Hospital, Shanghai University of Traditional Chinese Medicine. XCH was from Shanghai Shikang Technology Co. Ltd (ZZ-3484 No.081006). In situ cell death detection kit (Cat. No. 1684817) and DAB substrate (Cat. No.1718096) were purchased from Roche Diagnostics Ltd.

Animal preparation[16-20]

Eighty-four specific pathogen free (SPF) male Wistar rats weighing 150 ± 20g were purchased from Shanghai Experimental Animal Co. Ltd. All the rats were randomly assigned into three groups: normal group (12), micro-amount CCl_4 group (12) and model group A (60). The model group A was ingested the mixture (500 mL/L alcohol, 8 mL/kg per day; corn oil, 2 mL/kg per day; pyrazole, 24 mg/kg per day) once a day and intraperitoneal injections of 0.25 mL/kg of a 25% solution of CCl_4 in olive oil twice a week for 12 wk. The CCl_4 group received intraperitoneal injections only. Normal group was ingested saline (10 mL/kg per day). At the end of 8 wk the model group A (60) was divided into 5 subgroups: model group, XCH group, QGHXF high dose group, moderate dose group and low dose group, and drugs were given respectively. Model group was given saline (5 mL/kg per day); QGHXF high dose group was given QGHXF 2.6 g/kg, 5 mL/kg; moderate dose group was given 1.3 g/kg, 5 mL/kg; low dose group was given 0.65 g/kg, 5 mL/kg; XCH group was given XCH (3 g/kg per day,); and model group and CCl_4 group were given saline. At the end of 12 wk all the rats were anaesthetized and killed. Blood sample and liver tissue specimens were collected. A portion of liver was fixed for histopathology. Another portion was for flow cytometry assay and the remaining tissue stored at -80°C until assayed.

Serum ALT, AST, ALP and GGT determination

ALT, AST, ALP and GGT were evaluated in samples of serum obtained at the end of the experiment. The activity was evaluated by using a commercial clinical test kit (Shanghai Rongsheng Biotech Co. Ltd.) according to instructions of the kit.

Histopathology and estimation criterion

Liver tissue was instantly fixed in 40 g/L phosphate buffered formaldehyde, processed by routine histology procedures, embedded in paraffin, cut into 5 μm section and mounted on the slide. The samples were stained with hematoxylin and eosin (HE) for histopathological examination[21]. Histopathological criteria refer to the report[22].

Flow cytometry assay[23,24]

Six liver specimens were obtained randomly from each group. The liver tissue was rapidly removed, weighed, and placed into 10 mL of ice-cold PBS containing 2 g/L bovine serum albumin (BSA) (Sigma), 0.01 mol/L EDTA, and 10 g/L deoxyribonuclease I (Sigma), and then the tissue was broken in a glass homogenizer and passed through a 40 μm nylon cell stainer (Becton Dickinson). The suspension was centrifuged at 500 g for 10 min at room temperature. The pellet was resuspended in 500 μL of PBS with BSA and transferred into a fresh tube. The cells obtained by the above method were fixed with ice-cold 700 mL/L ethanol in PBS at 4°C for 8 h, then incubated with RNase (20 mg/L) for 30 min at 37°C and labeled with propidium iodide (50 mg/L). DNA contents were measured by an FACSCalibur cytometer (Becton Dickinson). Multicycle software (CellQuest software, Becton Dickinson) was used to produce histograms of DNA content frequency. Sub-diploid DNA peaks were quantified from the DNA content data.

The tests were completed in Flow Cytometry Laboratory of Shanghai Institute for Biological Sciences, Chinese Academy of Sciences.

Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay

Tissue sections were dewaxed and rehydrated according to standard protocols. They were incubated for 15-30 min at 21-37 ℃ with proteinase K working solution (10-20 mg/L in 10 mmol/L Tris/HCl, 7.4-8). Slides were rinsed twice with PBS. Fifty microliter TUNEL reaction mixture was added to the sample tissue and incubated for 60 min at 37 ℃ in a humidified atmosphere in the dark. Slides were rinsed 3 times with PBS. Fifty microliter Converter-POD was added to the sample. Slides were incubated in a humidified chamber for 30 min at 37 ℃, rinsed 3 times with PBS, then DAB substrate was added and counterstained with hematoxylin, mounted under glass coverslip and analyzed under light microscope.

Apoptosis gene array[25-29]

Three liver specimens were taken from each group of QGHXF high dose group, model group and normal group, then homogenized and mixed for apoptosis gene array, including RNA isolation, RNA yield and quality assessment, probe synthesis, hybridization, chemiluminescent detection, image and data acquisition and analysis. The

Table 1 Impact of QGHXF on ALT, AST, ALP, and -GT in AF rats (mean ± SD)

Group	*n*	ALT (nkat/L)	AST (nkat/L)	ALP (nkat/L)	GGT (nkat/L)
Normal	12	464 ± 90 [b]	1156 ± 250[b]	1065 ± 315[b]	360 ± 98[b]
CCl_4	12	674 ± 172	1678 ± 293[a]	1695 ± 406[a]	748 ± 242[a]
Model	10	926 ± 154	3344 ± 330	2806 ± 639	1376 ± 215
Low dose	11	747 ± 113	2552 ± 388[b]	1748 ± 462[a]	982 ± 236[a]
Moderate dose	10	718 ± 156	2215 ± 650[b]	1632 ± 502[a]	869 ± 303[a]
High dose	10	615 ± 190[bc]	1972 ± 366[bc]	1570 ± 497[a]	770 ± 240[b]
XCH	10	867	2777 ± 608[a]	2413 ± 609[a]	768 ± 292[b]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* model group; [c]$P < 0.05$ *vs* XCH.

data were analyzed and calculated by Gearray Analyzer software.

Statistical analysis

Data were expressed as mean ± SD and analyzed by the ANOVA and post-hoc test. $P < 0.05$ was regarded as statistically significant.

RESULTS

Rat condition

When the rats were given alcohol they became excited and ran around the cage. After that they could not walk, and at last fell into a profound sleep. As the time prolonged spoor time changed from 1~2 h to about 5 h. The weight of rats fell obviously and rats maintained cachexia. Conditions of the rats of QGHXF groups and XCH group were better than those of model group and CCl_4 group.

Liver function

Serum levels of ALT, AST, ALP, and γ-GT in model group were higher than those in normal group. All the indexes were improved in each medicine control group ($P < 0.05$ or 0.01), especially in QGHXF high dose group each index was better than that in XCH group. Details of the data are shown in Table 1.

Effects of QGHXF on liver pathology

The normal group showed normal lobular architecture with central veins and radiating hepatic cords (Figure 1A). The establishment of the model group was successful with marked fatty degeneration, slight confluence, inflammatory infiltrates of monocytes, portal inflammation and necrosis, obvious collagen deposition, and decreased density of hepatocytes (Figure 1B). The CCl_4 group only developed micro- and moderate steatosis. Inflammation and fibrosis could not be found (Figure 1C). The treatment group (QGHXF high dose group) could markedly improve those pathological parameters. Inflammation and collagen deposition decreased obviously, and steatosis could nearly not be found (Figure 1D).

Apoptosis of HSC

Apoptosis was measured in the liver tissue with FC-AS (Figure 2). All QGHXF groups had increased apoptosis rate of HSC, and decreased proliferation compared with the model group. The differences were significant (Table 2).

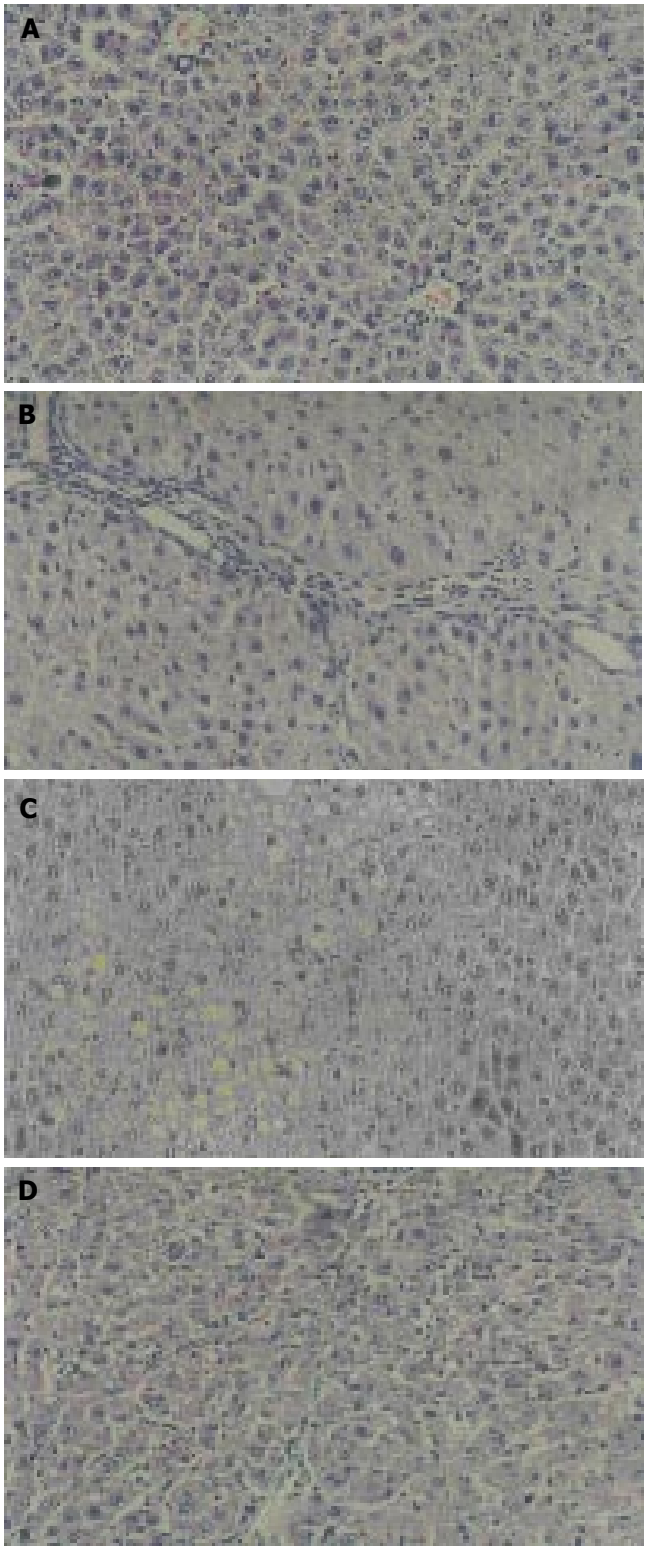

Figure 1 Analysis of liver pathology of each group (HE × 200). **A**: normal group; **B**: model group; **C**: CCl_4 group; **D**: high dose group.

TUNEL assay

TUNEL assay demonstrated standard apoptotic HSC in space of Disse and collagenous fibers in high dose group (Figure 3A), and in model group apoptotic hepatocytes

Table 2 Effect of QGHXF on proliferation and cell cycle of HSC ($n = 6$; mean ± SD)

Group	Apoptotic rate (%)	Cell cycle			
		G_0/G_1	S	G_2/M	PI value (%)
Normal	4.47 ± 0.8	79.30 ± 1.03	1.69 ± 0.73	19.02 ± 1.67	20.71 ± 1.03
Low dose	10.07 ± 1.14[a]	37.47 ± 0.68[b]	1.46 ± 0.46[a]	61.07 ± 0.33[b]	62.53 ± 0.68[b]
Moderate dose	13.79 ± 2.26[a]	68.31 ± 0.92[a]	2.11 ± 0.46[a]	29.58 ± 1.38	31.69 ± 0.92[a]
High dose	22.4 ± 3.13[b]	71.25 ± 1.05[b]	2.70 ± 0.46	26.06 ± 1.47[b]	28.75 ± 1.05[b]
Model	6.58 ± 1.04	64.55 ± 1.53	3.69 ± 0.66	31.76 ± 0.87	35.45 ± 1.53

Proliferative index = 100 % × (S+G_2/M)/(G_0/G_1+S+G_2/M). [a]$P < 0.05$, [b]$P < 0.01$ *vs* model group.

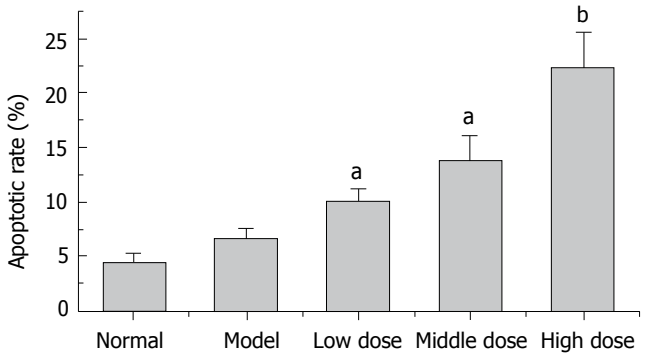

Figure 2 QGHXF induced apoptotic rate of HSC ($n = 6$, mean ± SD). There were significant differences between model group and QGHXF groups. [a]$P < 0.05$ *vs* model group. [b]$P < 0.01$ *vs* model group.

could be seen (Figure 3B). There were no obvious apoptotic cells in normal group (Figure 3C).

Gene array analysis

There were 112 genes in the apoptosis chip, and difference was defined when the same gene ratio was more than 2 or less than 0.5 between different groups. Compared to model group 39 genes were up-regulated (ratio > 2) in high dose group, 11 solely expressed and 17 down-regulated (ratio < 0.5). Compared with normal group 16 genes were up-regulated (ratio > 2) in model group, 6 solely expressed and 35 down-regulated(ratio < 0.5). Compared with normal group 38 genes were up-regulated (ratio > 2) in high dose group , 9 solely expressed and 21 down-regulated (ratio < 0.5). These different expression genes were mainly of TNF receptor family, Bcl-2 family, caspase family, and p53 and ATM pathway. Moreover, in model/normal group chip, only a few genes were consistent with those of high/model group chip, and they were Bad, Bak, Bik, NAIP1, 4-1BBL, TRIP (up-regulated) and TRAF2 (down-regulated). However, in high/model group compared with high/normal group, the up-regulated genes were nearly the same. The unique expressive genes in high/model group were Bar, Bcl-x, Bid, Casper, Caspase-7, chk1, DAPkinase, TNFb Mcl-1, OPG, FASL, and CD30L. In high/normal group they were Apaf-1, ATM, Bax, Bim, Bruce RAD53/chk2, TNFR1, TNFS, F11, and P53. Among down-regulated genes one fourth

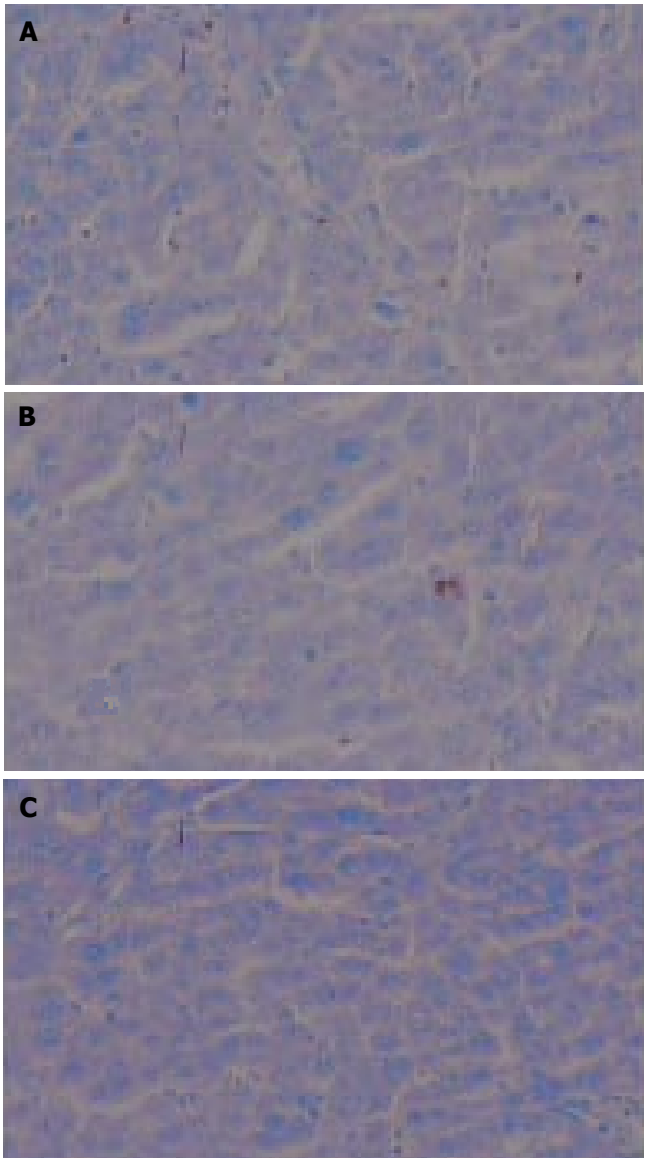

Figure 3 TUNEL assay of each group. **A**: High dose; **B**: Model; **C**: Normal; Counterstained with hematoxylin (original magnification, ×400).

were similar. They were Caspase-1, NOP30-like, RIP, TNFRF11A, and TRAF2. In comparison of model/normal group with high/normal group we found the up-regulated gene expression trend resembled the former result. The same genes were Bcl-2, hrk, IAP2, IAP3 Caspase-11, Caspase-14, and CD30. Down-regulated genes only expressed in high/normal group were CRAF1, CD27, TNFRF11A, RIP, NOP30-like, and Caspase-1 (Figure 4).

DISCUSSION

In contrast with the traditional view that cirrhosis is an irreversible disease, recent evidence indicates that even advanced fibrosis is reversible[11] . It has been demonstrated that apoptosis is the major mechanism by which activated HSCs are removed during recovery from fibrosis[30-32]. As a prophase of AC the reversibility of AF has been reported[33]. The toxicity of ethanol, acetaldehyde, reactive oxygen and other metabolic products are the main causes of AF. These factors lead to hepatocyte inflammation,

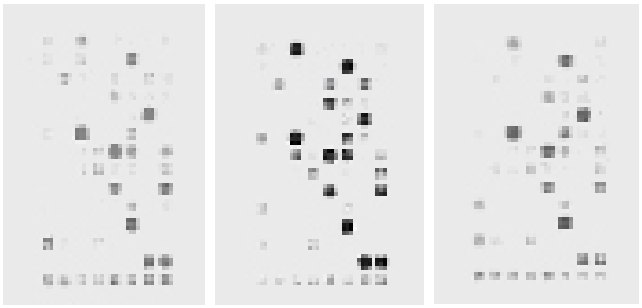

Figure 4 Gene expression profile of each group.

necrosis and apoptosis. AF was historically thought to be a passive and irreversible process due to the collapse of the hepatic parenchyma and its substitution with a collagen-rich tissue. Currently, it is considered a model of the wound-healing response to chronic liver injury. HSCs comprise 15% of the total number of resident liver cells, but they play a very important role in the course of AF[34]. They have been identified as the main collagen-producing cells in the liver. Following chronic injury, HSCs activate or transdifferentiate into myofibroblast-like cell, produce large amounts of extracellular matrix (ECM) and prevent ECM degradation[34,35]. So it is beneficial to induce HSC apoptosis in order to reduce its proliferation and ECM accumulation.

The aim of this study was to explore the mechanism of QGHXF on AF through induction of HSC apoptosis. According to TCM theory, "alcohol is pungent and hot". "Jingyue Quanshu" has already pointed out: if a man drinks too much he will be "Shuigu". Through our long-term clinical practice and epidemiological research we find dampness, heat and gore are the major pathogenesis of ALD, which is in consistent with TCM theory. Therefore, we used QGHXF to clear dampness and heat of the liver and eliminate the gore of the blood. There are five components in QGHXF: bupleurum root, scutellaria root, red sage root, *Carapax trionycis*, and *Radix puerariae*. Bupleurum root (bitter and pungent in flavor, slightly cold in property, acting on liver and gallbladder channel) can expel pathogenic factors from the exterior to reduce fever, sooth the depressed liver and invigorate the spleen-*yang*. Scutellaria root (bitter in flavor, cold in property, acting on the lung, gallbladder, stomach and large intestine channels) can clear away heat and dampness, purge fire, remove toxins from the body, stop bleeding, and prevent miscarriage. Red sage root (bitter in flavor, slightly cold in property, acting on the heart, pericardium and liver channel) can invigorate blood circulation, remove blood stasis, cool the blood, treat carbuncles and tranquilize the disturbed mind by nourishing the blood. *Radix puerariae* (sweet and pungent in flavor, cool in property, acting on the spleen and stomach channels) can dispel pathogenic factors from the superficial muscles to allay fever, promote the production of fluid to quench thirst, promote the eruption of measles and invigorate the spleen-yang to stop diarrhea. carapax trionycis (salty in flavor, slightly cold in property, acting on the liver and kidney channels) can nourish *yin* and suppress hyperactive *yang*, reducing fever and resolving hard lumps. So the QGHXF can clear dampness and heat of the liver and eliminate the gore of the blood.

In this experiment the levels of ALT, AST, GGT, and ALP were obviously decreased in XCH group and each of QGHXF groups($P < 0.05$) and QGHXF high dose group had the best effect. Moreover, QGHXF high dose group had better effect than XCH group on ALT and AST($P < 0.05$). In addition, histopathology proved the effect of the drug. In model group we could see marked fatty degeneration, slight confluence, inflammatory infiltrates of monocytes, portal inflammation and necrosis, obvious collagen deposition, fibrosis and hepatocyte loosening. The treatment group (QGHXF groups) could markedly improve those pathological changes. Inflammation and collagen deposition decreased obviously, and steatosis could nearly not be seen. To clarify that the fibrosis was caused by alcohol but not CCl_4, we established the CCl_4 group in which only micro- and moderate steatosis were found, while inflammation and collagen deposition were not found. It suggested that alcohol was the real reason for fibrosis. To analyse mechanism of the effect we used FCAS and TUNEL assay. The results showed QGHXF groups could not only increase apoptosis rate of HSC but also inhibit it from proliferation. It partly proved our hypothesis that QGHXF could induce HSC apoptosis to reverse liver fibrosis. However, the HSC proliferation rate of low dose group was higher than model group, the reason for which needs to be further studied.

What excited us was, from the FCM analysis, the double regulating function of Chinese medicine. Considering the multi- target effect of Chinese medicine we selected a gene chip for further investigation. According to gene chip result, the gene expression of *p53* and ATM pathway, Caspase family, Bcl-2 family, TNF receptor family, death effector domain family all changed in each group. It meant either alcohol or drug could affect the apoptosis gene expression of liver. As a mixed cell tissue of liver, the types and apoptosis pathway of apoptotic cells were so different that both up-regulated and down-regulated genes in model/normal groups were quite different from that of high/model groups. As we all know, alcohol can induce hepatocytic apoptosis so many apoptotic genes expressed in model/normal group, but these genes were not expressed in high/model group. Thus we deduced the types of apoptotic cells were not the same, and the result of TUNEL also supported this point.

By combination of the TUNEL results with that of FCM analysis (apoptotic rate of HSC was the lowest in model group, while it was the highest in high dose group), we could conclude that QGHXF can induce HSC apoptosis, prevent hepatocytic apoptosis and necrosis. As regards which pathway plays the key role in HSC apoptosis more profound studies are needed. QGHXF has been proven effective on AF from this experiment.

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CLINICAL RESEARCH

Changes in growth factor and cytokine expression in biliary obstructed rat liver and their relationship with delayed liver regeneration after partial hepatectomy

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Abstract

AIM: To study the effects of obstructive jaundice on liver regeneration after partial hepatectomy.

METHODS: Hepatocyte growth factor (HGF), its receptor, c-Met, vascular endothelial growth factor (VEGF) and transforming growth factor-β1 (TGF-β1) mRNA expression in both liver tissue and isolated liver cells were investigated after biliary obstruction (BO) by quantitative reverse-transcription polymerase chain reaction (RT-PCR) using a LightCycler. Immunohistochemical staining for desmin and α-smooth muscle actin (α-SMA) was also studied. Regenerating liver weight and proliferating cell nuclear antigen (PCNA) labeling index, and growth factor expression were then evaluated after 70% hepatectomy with concomitant internal biliary drainage in BO rats or sham-operated rats.

RESULTS: Hepatic TGF-β1 mRNA levels increased significantly 14 days after BO, and further increased with duration of cholestasis. Meanwhile, HGF and VEGF tended to increase, but was not significant. In cell isolates, TGF-β1 mRNA was found mainly in the hepatic stellate cell (HSC) fraction. Immunohistochemical studies revealed an increased number of HSCs (desmin-positive cells) and activated HSCs (α-SMA-positive cells) in portal areas after BO. In a hepatectomy model, liver regeneration was delayed in BO rats, as compared to sham-operated rats. TGF-β1 mRNA was significantly up-regulated up to 48 h after hepatectomy, and the earlier HGF mRNA peak was lost in BO rats.

CONCLUSION: BO induces HSCs proliferation and activation, leading to up-regulation of TGF-β1 mRNA and suppression of HGF mRNA in livers. These altered expression patterns may be strongly involved in delayed liver regeneration after hepatectomy with obstructive jaundice.

Key word: Biliary obstruction; Liver regeneration; Hepatocyte growth factor; Transforming growth factor-β; Hepatic stellate cells; Hepatectomy

Makino H, Shimizu H, Ito H, Kimura F, Ambiru S, Togawa A, Ohtsuka M, Yoshidome H, Kato A, Yoshitomi H, Sawada S, Miyazaki M. Changes in growth factor and cytokine expression in biliary obstructed rat liver and their relationship with delayed liver regeneration after partial hepatectomy. *World J Gastroenterol* 2006; 12(13): 2053-2059

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INTRODUCTION

Recently, major hepatectomy has been performed for treatment of advanced hepatic and biliary carcinomas [1]. However, major hepatectomy associated with obstructive jaundice is often complicated by hepatic failure [2, 3], suggesting that biliary obstruction (BO) may influence liver regeneration and cause hepatic failure after major hepatectomy. Although several previous studies regarding liver regeneration after hepatectomy with obstructive jaundice have been reported [4-6], it is not clear how BO affects liver regeneration. Aronson *et al* [4] reported that extrahepatic cholestasis inhibits liver regeneration after hepatectomy, whereas Mizuno *et al* [5], demonstrated that it has no effects on liver regeneration. Thus, the effect of BO on liver regeneration after hepatectomy is still open to discussion even in an experimental model. In clinical cases, whether preoperative biliary drainage before surgery is beneficial or not is also a matter of debate.

In the liver regeneration process, several growth factors are reported to play a crucial role in regulation of regeneration by providing either stimulatory or inhibitory signals for hepatocytes [7]. Epidermal growth factor (EGF),

transforming growth factor-α (TGF-α), and hepatocyte growth factor (HGF) stimulate DNA synthesis in hepatocytes *in vivo* and in culture, but HGF is known to be the most powerful mitogen of hepatocytes [8, 9]. In liver, HGF is produced by nonparenchymal cells, mainly hepatic stellate cells (HSCs), and acts on hepatocytes in a paracrine manner via its receptor, c-Met [10-12]. Vascular endothelial growth factor (VEGF) is also reported to be the only angiogenic factor that stimulates proliferation of sinusoidal endothelial cells (SECs) [13-15]. On the other hand, transforming growth factor-β1 (TGF-β1) is a potent growth inhibitor of hepatocytes [16-19]. TGF-β1 mRNA levels are very low or undetectable in normal liver, but increase significantly after partial hepatectomy [20-22]. In cell isolates from regenerating normal liver, the TGF-β1 mRNA was relatively abundant in SECs, Kupffer cells, and HSCs [23]. Meanwhile, in a liver injury model, induced by carbon tetrachloride or D-galactosamine administration, TGF-β1 mRNA expression was up-regulated mainly in HSCs [23-25].

HSCs are known to be located in the space of Disse, below the SECs lining, in close contact to and partially intercalated between hepatocytes, with their long processes extending along sinusoids [26]. HSCs express desmin, a cytokeletal intermediate filament characteristic of muscle cells [27], but once HSCs are activated, they transform into myofibroblast-like cells, and express α-smooth muscle actin (α-SMA). Furthermore, myofibroblast-like cells derived from HSCs produce large amounts of TGF-β1, which stimulates activated HSCs to produce more TGF-β1 in an autocrine manner [28, 29]. On the other hand, activated HSCs lose HGF productivity, although HGF is primarily produced from non-activated HSCs [10].

At present, the influence of BO on HSCs phenotype, especially their TGF-β1 expression during cholestasis and their influence after hepatectomy, is not yet determined. In this study, HGF, c-Met, VEGF and TGF-β1 mRNA expression were investigated after BO in both liver tissue and isolated liver cells, by means of *in situ* collagenase perfusion and counterflow elutriation, to determine potential cellular sources of these growth/inhibitory factors. Immunohistochemical staining with desmin and α-SMA antibody was also studied to evaluate the number of HSCs and their activation status. To determine the effect of BO on liver regeneration, we also investigated changes in hepatic HGF, c-Met, VEGF and TGF-β1 mRNA expression after 70% hepatectomy with concomitant internal biliary drainage in BO rats, and compared them to those in sham-operated rats. Regenerating liver weight and PCNA labeling index were also studied to evaluate the relationship between growth factor expression and liver regeneration after hepatectomy.

MATERIALS AND METHODS

Animals

Male Wister rats (SLC, Inc. Shizuoka, Japan), weighing 200 to 300 g were used in this study. All animals were housed in a temperature- and humidity-controlled environment with a 12-h light dark cycle, and allowed to drink water and eat *ad libitum*. All surgical procedures were performed under light diethyl ether anesthesia. The operative procedure was carried out using clean, but not sterile technique. Experiments with the animals followed our institution's criteria for the care and use of laboratory animals in research, which conform to National Institutes of Health guidelines.

Experiment 1

The rats were subjected to BO or sham surgery (sham-operated control). In the BO rats, the extrahepatic bile duct was isolated and a polyethylene tube with an outer diameter of 0.8 mm (Natsume, Tokyo, Japan) was inserted into the extrahepatic bile duct, according to the cut-down technique. The other end of the tube was then ligated to induce BO. In the sham-operated rats, the extrahepatic bile duct was isolated but was not occluded. The liver was carefully excised before surgery, and 14 and 21 d ($n = 10$ at each time point) after surgery. HGF, c-Met, VEGF and TGF-β1 mRNA levels in liver tissue were investigated by quantitative reverse-transcription polymerase chain reaction (RT-PCR), using a LightCycler (Roche Diagnostics, Mannheim, Germany). Furthermore, to determine potential cellular sources of these growth factors, liver cells (hepatocytes, SECs, Kupffer cells and HSCs) were isolated from liver tissue 14 d after BO, by means of *in situ* collagenase perfusion and counterflow elutriation. The levels of HGF, c-Met, VEGF and TGF-β1 mRNA in each cell fraction were then investigated by quantitative RT-PCR. Immunohistochemical staining with anti-desmin and anti-α-SMA antibody was also performed, to evaluate the number and activation status of HSCs.

Experiment 2

Fourteen days after BO, rats were subjected to 70% hepatectomy with concurrent internal biliary drainage. The tied end of the polyethylene tube was released and embedded in the duodenum. 70% of the liver was then resected according to the method of Higgins and Anderson [30]. In the sham-operated group, rats underwent internal biliary drainage with concurrent 70% hepatectomy 14 d after sham operation. For the assessment of the HGF, c-Met, VEGF and TGF-β1 expressions, the right inferior lobe of the liver was carefully excised before and 6, 12, 24, 48, 72, 120, 168, and 240 h ($n = 10$ at each time point) after hepatectomy. The levels of HGF, c-Met, VEGF and TGF-β1 mRNA in liver tissue were investigated by quantitative RT-PCR. In addition, remnant liver weight ratio and PCNA labeling index were also evaluated and compared between BO and sham-operated rats.

Quantitative RT-PCR analysis of HGF, c-Met, VEGF, and TGF-β1 mRNA expression

Total RNA was extracted from liver tissues or freshly isolated liver cells by the acid guanidium-thiocyanate/phenol /chloroform method, and 1 mg of extracted total RNA was subjected to a reverse transcription reaction, using Ready To Go™ T-primed 1st strand cDNA synthesis kit (Amersham Pharmacia Biotech, Buckinghamshire, England). The cDNA from 33 ng of total RNA was used as a template. HGF, c-Met, VEGF, and TGF-β1 mRNA levels were quantified by means of a LightCycler (Roche Diagnostics, Mannheim, Germany), using the double-strand-specific dye SYBE Green I. Details of the primers used in this study are summarized in Table 1. The PCR condition was as fol-

Table 1 Primer Sequences for RT-PCR

Gene		Primer Sequence	T (℃)
HGF	Sense	5′-TTATGGGGAATGAGAAATGC	60
	Antisense	5′-TCGAACAAAAATACCAGGAC	
c-Met	Sense	5′-CAGACGCCTTGTATGAAGT	60
	Antisense	5′-CATAAGTAGCGTTCACATGG	
TGF-β1	Sense	5′-ATGACATGAACCGACCCTTC	60
	Antisense	5′-TGTGTTGGTTGTAGAGGGCA	
VEGF	Sense	5′-AATTGAGACCCTGGTGGACA	56
	Antisense	5′-TAGTGACGTTGCTCTCCGAC	
GAPDH	Sense	5′-TGAACGGGAAGCTCACTGG	60
	Antisense	5′-TCCACCACCCTGTTGCTGTA	
β-actin	Sense	5′-CCTGTATGCCTCTGGTCGTA	60
	Antisense	5′-CCATCTCTTGCTCGAAGTCT	

T : annealing temperature .

lows: initial denaturation at 95 ℃ for 10 min, followed by 45 cycles of denaturation at 95 ℃ for 15 s, annealing for 10 s, and extension at 72 ℃ for 20 s. The expression level of each angiogenic factor was adjusted using the level of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA, and expressed as ratio to GAPDH mRNA.

Isolated liver cells from liver tissue

Liver cells 14 d after BO were isolated, to determine potential cellular sources of TGF-β1 and HGF mRNA. Rat hepatocytes were isolated according to the methods of Gumucio *et al* [31]. The SECs, Kupffer cells and HSCs were also isolated by means of in situ collagenase perfusion and counterflow elutriation, as described by Knook *et al* [32], with minor modification. A JE-5.0 elutriator rotor (Beckman Instruments, Palo Alto, CA) was used in a J6-MI Beckman centrifuge. The separation process was started by adding the nonparenchymal cell suspension to a sample-mixing chamber. The HSCs were eluted at flow rates of 16 to 18 mL/min, and at a speed of 3 200 r/min. The SECs were then eluted at flow rates of 23 to 26 mL/min, and Kupffer cells at flow rates of 36 to 39 mL/min, at a speed of 2500 r/min. The purity of HSCs was > 90%, as assessed by their positive staining for desmin [33]. The purity of SECs and Kupffer cells was > 90% and > 92%, respectively, as assessed by typical cobblestone morphology and positive staining for ED-1 [34], respectively. To evaluate expression of HGF and TGF-β1 mRNA in each cell fraction, freshly isolated cells were used for total RNA extraction.

Immunohistochemical staining

Resected liver tissue specimens from rats were fixed in 4% paraformaldehyde (Wako Chemical Co. Osaka, Japan) in phosphate-buffered saline, washed with phosphate-buffered saline, dehydrated with 30%, 70%, 95%, and 100% ethanol and xylene, and then embedded in paraffin. Four-micrometer sections were cut and mounted onto superfrosted slide glass (Matsunami Glass Ind., Ltd., Osaka, Japan). Sections were incubated with methanol-1% hydrogen peroxide to destroy endogenous peroxidase, and blocked with nonspecific staining blocking reagent (Dako, Glostrup, Denmark). After overnight incubation at 4 ℃ with mouse monoclonal anti-desmin antibody (diluted 1 ∶ 100; Dako), or mouse monoclonal anti-α-SMA antibody (diluted 1 ∶ 100; DAKO) sections were processed according to the standard immunoperoxidase method, using a streptavidin biotin peroxidase complex kit (Dako LSAB + Kit/HRP; Dako). The peroxidase reaction was then developed with diaminobenzidine (Dako).

PCNA labeling index

Immunohistochemical staining for PCNA was performed on formalin-fixed and paraffin-embedded liver tissue with anti-PCNA antibody as previously described [35, 36]. A three-step immunoperoxidase method using strept-avidin biotin complex (Dako, Copenhagen, Denmark) was performed, according to the procedure described by Hall *et al* [36]. PC-10 monoclonal antibody (Dako, Copenhagen, Denmark) was used at a dilution of 1 ∶ 100, with overnight incubation at 4 ℃. Evaluation of PC-10 immunostaining was performed based on the percentage of positive nuclei of 500 hepatocytes at high power (400 ×), and was expressed as a PCNA labeling index.

Statistical analysis

The results were expressed as mean ± SD. The Mann Whitney test was used for statistical analysis of unpaired data, and differences were considered significant at $P < 0.05$.

RESULTS

HGF, c-Met, VEGF and TGF-β1 mRNA expression after biliary obstruction

The expression of TGF-β1 mRNA (Figure 1D) was at low levels before BO, but increased significantly at 14 d ($P < 0.05$ *vs* sham) and further increased at 21 d ($P < 0.03$ *vs* sham) after BO. Meanwhile, the expression of HGF (Figure 1A) and VEGF (Figure 1C) mRNA tended to increase at 14 and 21 d after BO, but no significant differences were found, as compared to the sham-operated control. The expression of c-Met mRNA (Figure1B) was lower at 14 and 21 d after BO, but was not significantly different from the sham-operated control.

HGF and TGF-β1 mRNA expression in isolated specific cell populations

To determine cellular sources of TGF-β1 mRNA during extrahepatic cholestasis, liver cells were isolated at 14 d after BO, because the expression of TGF-β1 mRNA was strongly induced by that time. In cell isolates, the mRNA for TGF-β1 was found mainly in the HSC fraction (Figure 2). On the other hand, the HGF mRNA expression was found in the nonparenchymal cell fraction, especially in the SEC fraction.

Immunohistochemical staining for desmin and α-smooth muscle actin

In the sham-operated control, several desmin-positive cells, presumed to be HSCs, were seen around the portal area (Figure 3A). But 14 d after BO (Figure 3B), the number

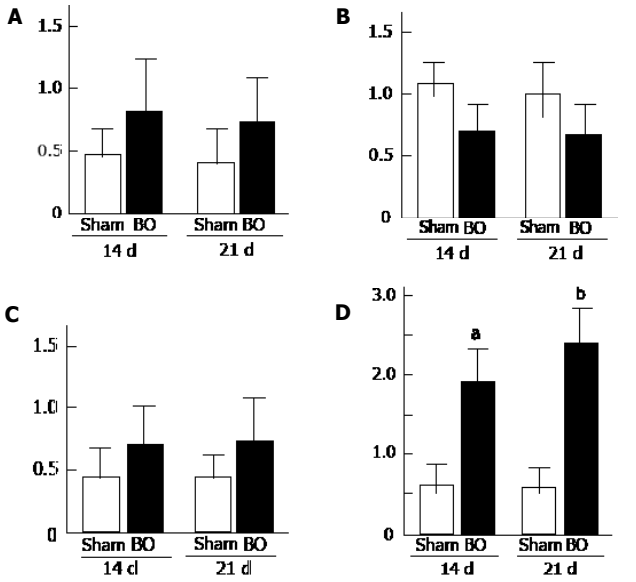

Figure 1 **A:** Hepatocyte growth factor (HGF); **B:** c-Met; **C:** vascular endothelial growth factor (VEGF); **D:** transforming growth factor-β1 (TGF-β1) mRNA expression in rat liver at 14 and 21 d after biliary obstruction (BO) and sham-operation (sham). ($^aP < 0.05$ *vs* sham-operated control, $^bP < 0.03$ *vs* sham-operated control). Results are expressed as mean ± SD of $n = 10$ for each period in each group.

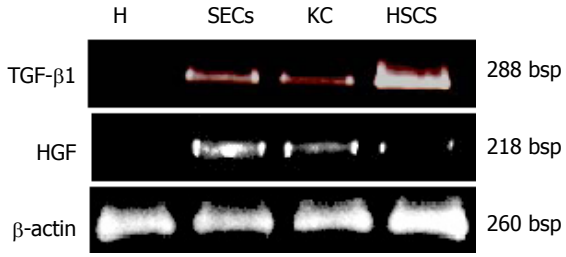

Figure 2 Hepatocyte growth factor (HGF) and transforming growth factor-β1 (TGF-β1) mRNA expression in isolated rat liver cells (H: hepatocytes; SECs: sinusoidal endothelial sells; KC: Kupffer cells; HSCs: Hepatic stellate cells) 14 d after biliary obstruction (BO).

of positive cells increased, and further increased at 21 d (Figure 3C). On the other hand, α-SMA-positive cells were hardly seen in the sham-operated controls (Figure 3D), whereas α-SMA-positive cells, presumed to be activated HSCs, were prominent in the surrounding portal areas 14 d after BO (Figure 3E).

Remnant liver weight and PCNA labeling index

The ratio of remnant liver to whole liver weight after 70% partial hepatectomy (PH) was approximately 30%. In the sham + PH group, the remnant liver weight ratio started to increase 24 h after hepatectomy (Figure 4A). A significant increase was found from 48 h to 120 h after surgery, as compared with the BO+ PH group. Thereafter, no significant differences were found between the two groups. The remnant liver weight in the BO+ PH group reached the same levels as that ~~in~~ attained by the sham + PH group 240 h after hepatectomy, but required a longer period.

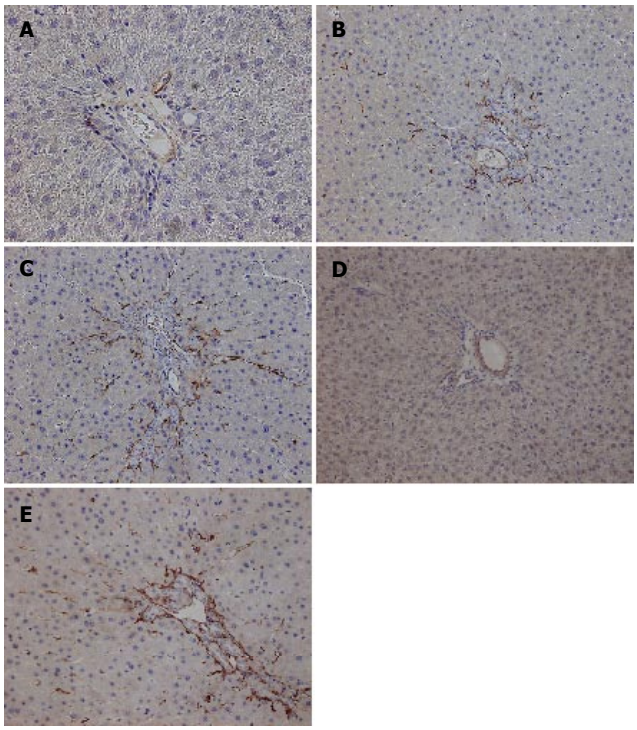

Figure 3 Immunohistochemical staining for desmin **A:** 14 d after sham operation; **B:** 14 d after biliary obstruction (BO); **C:** 21 d after biliary obstruction. Immunohistochemical staining for α-smooth muscle actin (α-SMA); **D:** 14 d after sham operation; E: 14 d after biliary obstruction.

Figure 4B shows changes in PCNA labeling index after hepatectomy. In the sham+ PH group, the PCNA labeling index was less than 5% in hepatocytes before hepatectomy. However, a dramatic increase in the PCNA labeling index of hepatocytes was observed 12 h after hepatectomy, reaching a peak 24-48 h after surgery ($P < 0.03$ *vs* BO+ PH group). In the BO + PH group, the PCNA labeling index was higher than in the sham+ PH before hepatectomy, and was then increased gradually, with a peak 120 h after hepatectomy. Thereafter, the PCNA labeling index decreased to the baseline value 240 h after surgery.

HGF, TGF-β1, VEGF, and c-Met mRNA expression after 70% hepatectomy

The expression of HGF mRNA in the sham + PH group started to increase 6 h after hepatectomy, with a peak at 12 h and 24 h (Figure 5A). Meanwhile, in the BO+ PH group, a small increase of HGF mRNA was observed up to 72 h after surgery, but no earlier peak was observed. The expression of c-Met mRNA in the BO+ PH group was lower, when compared with the sham+ PH control between 0 and 72 h after hepatectomy, but there was no significant difference between the groups (Figure 5B). The expression of VEGF mRNA in the BO+ PH group was somewhat higher up to 24 h after surgery, as compared to the sham+ PH (Figure 5C), but no significant differences were found at any point in this experiment. Meanwhile, the expression of TGF-β1 mRNA (Figure 5D) in the BO+ PH group was significantly up-regulated up to 48 h after hepatectomy, as compared to the sham+ PH group ($P < 0.05$). Thereafter, TGF-β1 mRNA expression was not significantly different between the groups.

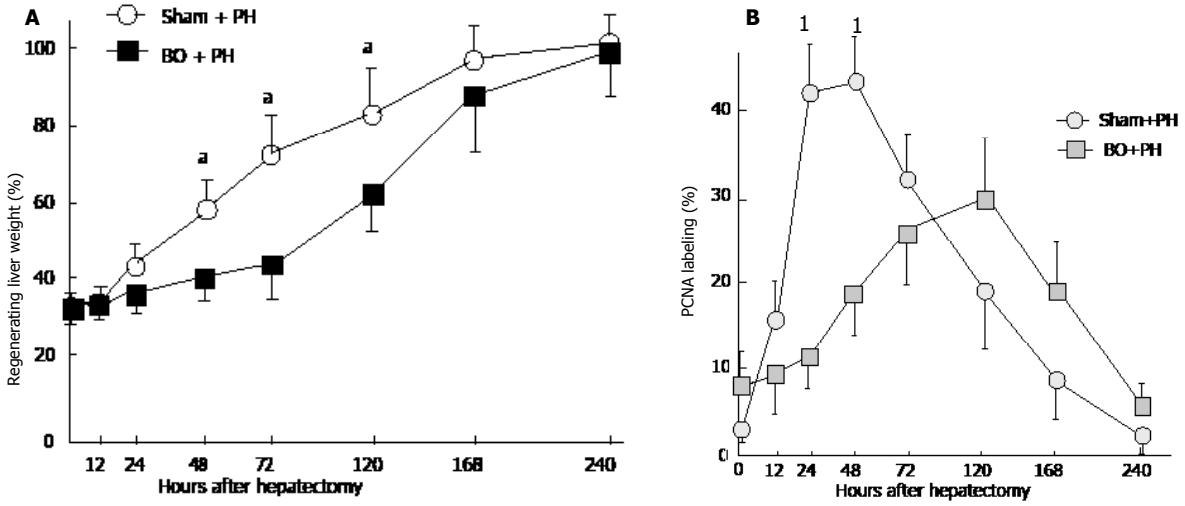

Figure 4 A: Changes in regenerating liver weight ratio after 70% partial patectomy (PH) in the sham-operated (sham) rats and the biliary obstructed (BO) rats (^aP <0.05 between the BO + PH group and the sham + PH group). Results are expressed as mean ± SD of n = 10 for each period in each group. B: Changes in the hepatocyte PCNA labeling index after 70% hepatectomy (PH) in the sham-operated (sham) rats and the biliary obstructed (BO) rats (^1P < 0.03 between the BO + PH group and the sham + PH group). Results are expressed as mean ± SD of n = 10 for each period in each group.

DISCUSSION

In the present study, we have clearly shown that hepatic TGF-β1 mRNA levels increased with length of cholestasis. Moreover, in cell isolates from BO liver tissue, TGF-β1 mRNA expression was found mainly in the HSC fraction. Immunohistochemical study also revealed an increased number of HSCs (desmin-positive cells) and activated HSCs (α-SMA-positive cells) in portal areas in proportion to the length of cholestasis. In the hepatectomy model, liver regeneration rate in the BO rats was delayed, as compared to sham-operated rats. TGF-β1 mRNA was also significantly up-regulated up to 48 h after hepatectomy in the BO rats, and no earlier peak of HGF mRNA expression was observed, despite a small increase in HGF mRNA during extrahepatic cholestasis. These findings suggest that increased TGF-β1 secreted from activated HSCs, and earlier suppression of HGF production, may greatly contribute to delayed liver regeneration in a paracrine manner. Earlier suppression of HGF after hepatectomy may be also related to HSCs activation, because activated HSCs reportedly lose their ability to express HGF mRNA [37].

Although several previous studies regarding liver regeneration after hepatectomy with obstructive jaundice have been reported, it is not clear how BO affects liver regeneration. Aronson *et al* [4] reported that extrahepatic cholestasis inhibits liver regeneration after hepatectomy, but they studied liver regeneration in the presence of postoperative BO. In this study, restoration of bile flow was performed simultaneously with hepatectomy, as in clinical cases. According to our results, the PCNA labeling index of hepatocytes, HGF and VEGF mRNA were somewhat higher in BO livers than in sham-operated livers. These results are consistent with previous studies [4, 6, 38, 39] indicating that BO induces DNA replication of hepatocytes. Furthermore, we clearly demonstrated that TGF-β1 mRNA was significantly up-regulated after BO. This phenomenon might be related to the hepatic repair process that compensates for hepatocyte injury caused by BO. This is also suggested by the fact that serum AST levels increased shortly after BO (data not shown). In cell isolates from BO liver, TGF-β1 producing cells were found to be mainly HSCs. Furthermore, an increased number of desmin-positive cells (HSCs) were found in portal areas, and α-SMA-positive cells, presumably activated HSCs, were progressively extended around portal areas in proportion to the length of BO. Activated HSCs reportedly lose their ability to express HGF mRNA, but produce a large amount of TGF-β1. These findings suggest that HSCs in portal areas were gradually activated into myofibroblast-like cells during BO. Activated HSCs produce TGF-β1, and TGF-β1 stimulates activated HSCs to produce more TGF-β1 in an autocrine manner, whereas activated HSCs hardly produce any HGF in the BO liver. HGF mRNA increased, to some extent, after BO, but HGF producing cells were mainly SECs, rather than HSCs. This may be related to the proliferative response of hepatocytes and bile duct cells during cholestatic liver injuries.

We clearly demonstrated that liver regeneration was significantly delayed after hepatectomy in the BO rats, as compared to the sham-operated rats. Moreover, TGF-β1 mRNA was significantly up-regulated up to 48 h after hepatectomy in the BO rats, and high levels of HGF mRNA were not induced in the earlier phase of hepatectomy. Meanwhile, c-Met expression in the BO liver was not significantly different from the sham-operated rats. These results suggest that delayed regeneration may be associated with the initial high expression of TGF-β1, and the suppression of HGF induction after hepatectomy. It is obvious that initial up-regulation of TGF-β1 has a non-beneficial effect on liver regeneration, because TGF-β1 is the most important cytokine controlling the inhibition of hepatocyte proliferation. Furthermore, although HGF mRNA increased to some extent in BO rats after hepatectomy, the amount may be insufficient to induce the initial stimulus for DNA synthesis of in hepatocytes for adequate

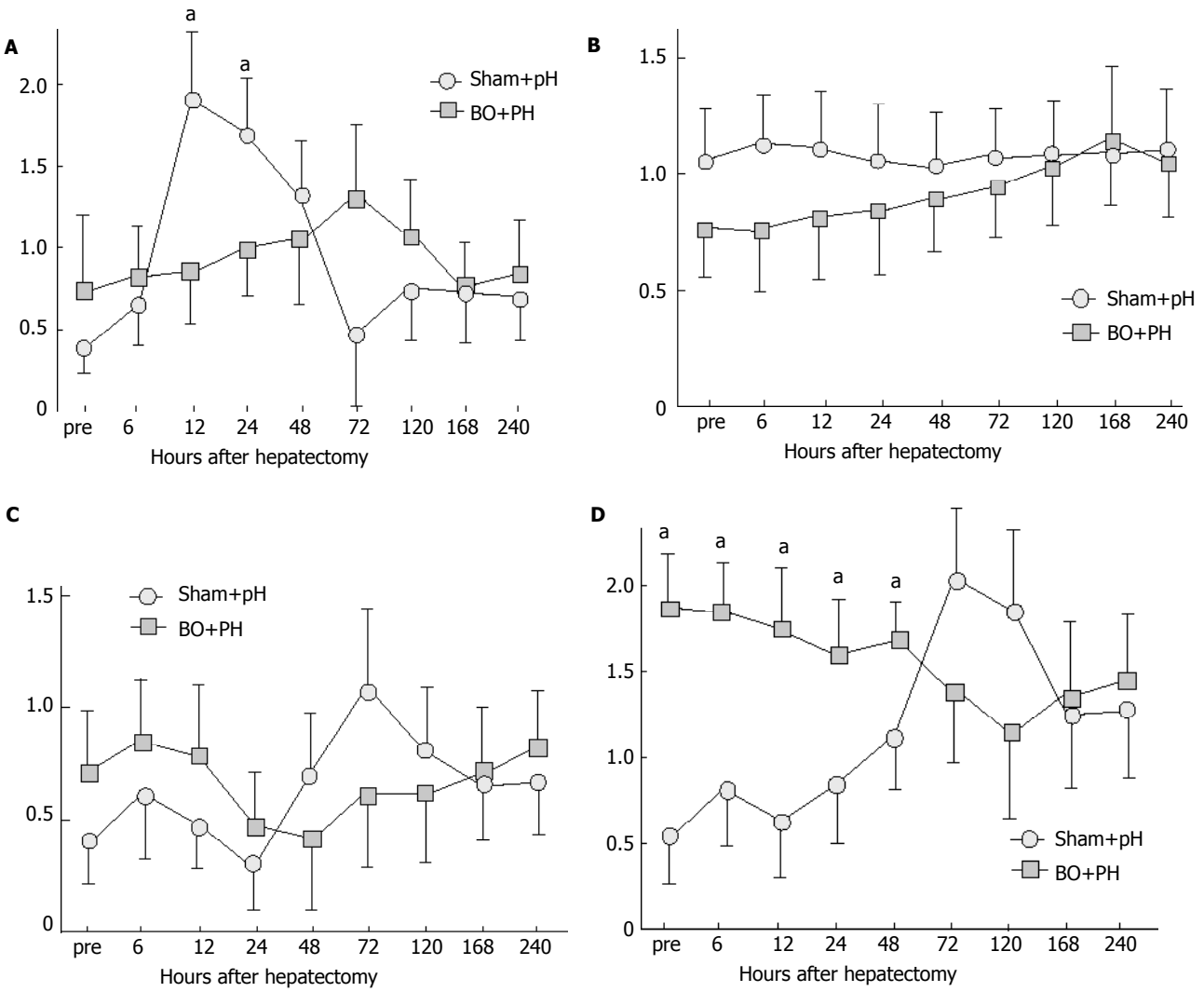

Figure 5 Changes in **A:** hepatocyte growth factor (HGF); **B:** c-Met; **C:** vascular endothelial growth factor (VEGF); **D:** transforming growth factor-β1 (TGF-β1) mRNA expression after partial hepatectomy (PH) in the sham-operated (sham) rats and the biliary obstructed (BO) rats (^aP <0.05 between the BO + PH group and the sham + PH group). Results are expressed as mean ± SD of n = 10 for each period in each group.

liver regeneration.

In conclusion, BO induces proliferation and activation of HSCs, resulting in up-regulation of TGF-β1 and negative regulation of HGF expression. The altered expression patterns may be involved to a considerable degree in delayed liver regeneration after hepatectomy in rats with obstructive jaundice. These findings may provide clues for the treatment of impaired hepatic regeneration after major hepatectomy with obstructive jaundice. That is, regulation of HSCs activation or restoration of an altered growth/inhibitory expression pattern might have beneficial effects on liver regeneration following major hepatectomy with obstructive jaundice.

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CLINICAL RESEARCH

Advanced gastrointestinal stromal tumor patients with complete response after treatment with imatinib mesylate

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Abstract

AIM: Most gastrointestinal stromal tumors (GISTs) express constitutively activated mutant isoforms of kit kinase or platelet-derived growth factor receptor alpha (PDGFRA), which are potential therapeutic targets for imatinib mesylate (Glivec). Partial response occurred in almost two thirds of GIST patients treated with Glivec. However, complete response (CR) after Glivec therapy was sporadically reported. Here we illustrated advanced GIST patients with CR after Glivec treatment.

METHODS: Between January 2001 and June 2005, 42 advanced GIST patients were treated with Glivec. Patients were administered 400 mg of Glivec in 100-mg capsules, taken orally daily with food. The response of the tumor to Glivec was evaluated after one month, three months, and every three months thereafter or whenever medical need was indicated. Each tumor of patients was investigated for mutations of kit or PDGFRA.

RESULTS: The median follow-up time of the 42 ad-vanced GIST patients treated with Glivec was 16.9 months (range, 1.0 - 47.0 months). Overall, 3 patients had complete response CR (7.1%), 26 partial response (67.8%), 5 stationary disease (11.9%), and 3 progressive disease (11.9%). The median duration of Glivec administration for the three patients was 36 months (range, 23-36 months). The median time to CR after Glivec treatment was 20 months (range, 9-26 months). Deletion and insertion mutations of c-kit exon 11 and insertion mutation of c-kit exon 9 were found in two cases and one case, respectively.

CONCLUSION: Complete response (CR) can be achieved in selected advanced GIST patients treated with Glivec. The median time to CR after Glivec treatment was 20 months. Deletion and insertion mutations of kit exon 11 and insertion mutation of kit exon 9 contribute to the genetic features in these selected cases.

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are soft-tissue sarcomas primarily arising from mesenchymal tissue in the gastrointestinal (GI) tract and abdomen. They are rare neoplasms, estimated to represent 0.1 to 3% of all GI tract tumors[1]. However, GISTs are the most common mesenchymal malignancy of the GI tract with precise incidence unknown[2]. GISTs appear to be related to the interstitial cells of Cajal of the mesenteric plexus[3]. These cells are considered GI pacemaker cells, from the interface between the automatic innervation of the bowel wall and its smooth muscle[4,5]. GISTs express the cell-surface transmembrane receptor kit with a tyrosin kinase activity and is the protein product of the kit proto-oncogene. There are frequent gain-of-function mutations of kit in GISTs. These mutations result in constitutive activation of kit signaling, which leads to uncontrolled cell proliferation and resistance to apoptosis. It has been recently reported that kit activation occurs in all cases of GISTs, regardless of the mutation status of kit.

Surgical resection remains the mainstay of therapy for GIST. However, recurrence is common; the 5-year survival rates after complete resection range from 40 to 65%[6-10]. Unresectable or metastatic GIST is a fatal disease that resists conventional chemotherapy. In a recently reported series, the response rate to doxorubicin therapy was less than 5%[11]. The effectiveness of radiation therapy for unresectable or metastatic GIST has not been proved. The median length of survival for patients with a metastatic GIST is approximately 20 months, and 9 to 12 months for patients with local recurrence[3]. Before the development of Glivec, the outlook for patients with advanced GIST was extremely poor. A significantly large number of patients with initial resection of GIST eventually experience

Imatinib mesylate (formerly STI571, now referred to as Gleevec in the United States and Glivec in Europe [Novartis]) selectively inhibits certain protein tyrosin kinases: intracellular ABL kinase, chimeric BCR-ABL fusion oncoprotein of chronic myeloid leukemia, transmembrane receptor kit, and platelet-derived growth factor (PDRGF) receptors[12-15]. Glivec induced a sustained objective response in more than half of patients with advanced GISTs[16]. However, complete response (CR) induced by Glivec on GIST patients has been sporadically reported. We report herein our experience on three GIST patient treated with Glivec achieving complete response.

MATERIALS AND METHODS

Patients

During January 2001 to May 2005, 42 histologically confirmed, unresectable or metastatic GIST patients ex-pressing CD117 (a marker of kit-receptor tyrosine kinase) and CD34 treated at Department of Surgery, Chang Gung Memorial Hospital, Taiwan were enrolled in this study. Metastatic disease was defined as that occurring at structures noncontiguous with the primary tumor site. Criteria for inclusion were as follows: at least one measurable tumor; adequate hepatic, renal, and cardiac function; an adequate platelet count; and an Eastern Coo-perative Oncology Group (ECOG) performance status of 3 or less. Patients could have previously received che-motherapeutic regimens (the last chemotherapy treatment must have been at least four weeks before the study entry) and undergone radiotherapy, or surgery, or both. R0 resection means curative resection without microscopic evidence of tu-mor. R2 resection means resection with macroscopic evidence of tumor. The study was approved by the Local Institutional Review Board of Chang Gung Memorial Hospital and written informed consent for drug administration and the analysis of tumor-associated genetic alteration was obtained from each patient.

Study design

A prospective, non-randomized, and single center trial was conducted to evaluate the role of Glivec in inducing objective response in GIST patients. Patients were administered 400 mg of Glivec in 100-mg capsules, taken orally daily with food. Patients had regular physical examinations and evaluations of performance status, body weight, complete blood count, and serum chemistry. The administration of each dose and any adverse events were recorded for each patient. Standard computed tomography (CT) was performed on each patient every three months to assess patient response. Standard [18F] fluoro-2-deoxy-D-glucose positron-emission tomography (PET) scanning was performed on selected patients to complement standard CT and assess changes in the metabolic profiles of the tumors.

Efficacy and safety evaluation

The response of the tumor to Glivec was evaluated after one month, three months, and every three months thereafter or whenever medical need was indicated. Assessments were performed according to the standard Southwest Oncology Group (SWOG) criteria and based solely on CT or PET[17]. Responses were classified as follows: complete response (CR) (disappearance of all disease that could be measured and evaluated); partial response (PR) (> 50% decrease in the sum of the products of the perpendicular diameters of all measurable lesions, the absence of progression, and the absence of new lesions); stationary disease (SD) (a response that did not qualify as a complete response, a partial response, or disease progression); and disease progression (DP) [> 50% increase or an increase of 10 cm (whichever was smaller) in the sum of the products of the perpendicular diameter of all measurable lesions, worsening of a lesion that could be evaluated, the reappearance of any lesion or the presence of a new lesion, or failure of the patients to return for evaluation because of disease progression]. Toxic effects were recorded in accordance with the National Cancer Institute Common Toxicity Criteria[18].

Analysis of KIT and PDGFRA mutations

Sections were prepared from formalin-fixed, paraffin-embedded pretreatment specimens trimmed to enrich tumor cells. Polymerase chain reaction amplification of genomic DNA for KIT and PDGFRA was performed and amplification was analyzed for mutations as previously described[19].

RESULTS

Age and sex

The investigation comprised one male patient and two female patients with ages ranging from 45 to 56 years (median: 51 years) (Table 1). All three patients had grade 0 ECOG status.

Tumor location, size, and treatment

Table 1 summarizes the size and location of each tumor. One patient underwent laparotomy with excisional biopsy and the other two had curative segmental resection of jejunal GIST previously. Tumors of all three patients di-splayed strong positive kit staining with the tumor size ranging from 10 cm to 20 cm (median: 10 cm). The inter-val between diagnosis of GIST and tumor recurrence ranged from 0 to 15 months (median 7 months). All three patients displayed peritoneal carcinomatosis and two had liver metastasis.

Treatment

All three patients were administered 400 mg Glivec after diagnosis of metastasis was made. The duration of Glivec administration ranged from 24 to 36 months (median: 36 months). The side effect of Givec treatment was grade II to III edema.

Genetic investigations of tumors from GIST

The sequencing analysis of the tumor from the three patients exhibited mutation in c-kit gene. Two displayed deletion and insertion mutation in exon 11 and one insertion mutation in exon 9 (Figures 1, 2, and 3).

Time to response and follow-up outcome

Since 2000, Glivec has been administered to advanced GIST patients. Forty-two patients with advanced stages

Table 1 Clinicopathological and mutational status of three advanced and metastatic GIST patients treated with imatinib meslyate with CR

Patient	1	2	3
Age (yr)	57	45	51
Gender	F	M	F
ECOG	Grade 0	Grade 0	Grade 0
Tumor origin	Jejunum	Stomach	Jejunum
Tumor size (cm)	20	10	10
Previous treatment	Operation	Laparotomy and excisional biopsy	Operation
Resection	R0	R2	R0
Site of tumor recurrence	Liver, locoregional, and peritoneum	Liver, peritoneum, and retroperitoneum	Peritoneum
Interval between previous treatment and recurrence (mo)	15	0	18
Glivec dose/ duration (mo)	400/36	400/23	400/36
Side effect	Grade II edema	Grade III edema	Grade II edema
Mutation status	Deletion and insertion mutation at codons 563-572 in exon 11	Deletion and insertion mutation at codons 556-557 in exon 11	Insertion AY at codons 502-503 in exon 9
Time to CR (mo)	20	9	26
CT	CR	CR	CR
PET	CR without activity	CR without activity	CR without activity
Duration of response (mo)	16	14	10
Overall survival (mo)	40	24	54
Status	Free of disease	Free of disease	Free of disease

ECOG: Eastern Cooperative Oncology Group; mo: months; CR: complete response; CT: computed tomography; PET: positron emission tomography.

of the disease were given 400 mg Glivec per day. The median follow-up duration was 16.9 months (range, 1.0-47.0 months). Overall, 3 (7.1%) patients had complete response (CR), 26 (67.8%) partial response, 5 (11.9%) stationary disease, 3 progressive disease, and 3 (7.2%) patients were unavailable to evaluate. The time to CR after Glivec treatment ranged from 9 to 26 months (median, 20 months) as illustrated by CT first and confirmed by PET without any metabolic activity (Figures 1, 2 and 3). The median follow-up period of the three advanced GIST patients treated with Glivec with CR was 40 months (range, 24 - 54 months).

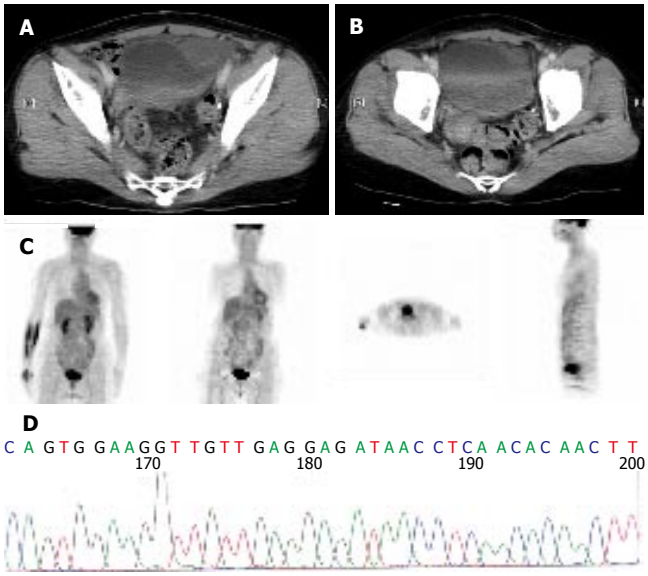

Figure 1 (**A**) Abdominal CT showing a tumor located near the urinary bladder (arrow); (**B**) abdominal CT showing complete response without tumor at the same level as Figure 1A; (**C**) [18F] fluoro-2-deoxy-D-glucose positron-emission tomography (PET) scanning PET revealing no tumor with metabolic activity in the whole body; (**D**) Direct sequencing analysis of DNA from patient 1 showed deletion and insertion mutation at codons 563-572 in exon 11 (arrow).

DISCUSSION

Before the introduction of Glivec, poor responses to radiation and chemotherapy made surgery the only realistic treatment to cure the primary lesion[5,10,20-22]. A substantial number of patients with initial resection of GISTs eventually experience recurrence. There has been no effective treatment for advanced GISTs and the outlook for patients is extremely poor.

Therapeutic responses to targeted inhibition of activated tyrosine kinases have been demonstrated for certain types of leukemia, sarcoma, and breast cancer[19]. The mechanisms of kinase activation vary considerably among these cancers, but there is little information available in literature about the influence of these mechanisms on drug response[19]. The GISTs, in particular, present a variety of genomic mutations across two different receptor tyrosine kinase genes. The KIT or PDGFRA mutation in Asian clinically advanced small bowel GIST patients was examined in this study. The kit kinase oncoproteins were intrinsically sensitive to Glivec, accounting for the excellent overall clinical response to Glivec, and coincident with results obtained by Heinrich *et al*[19]. Similar to the report by Demetri's *et al*[16], the CR and PR rates for Glivec in this

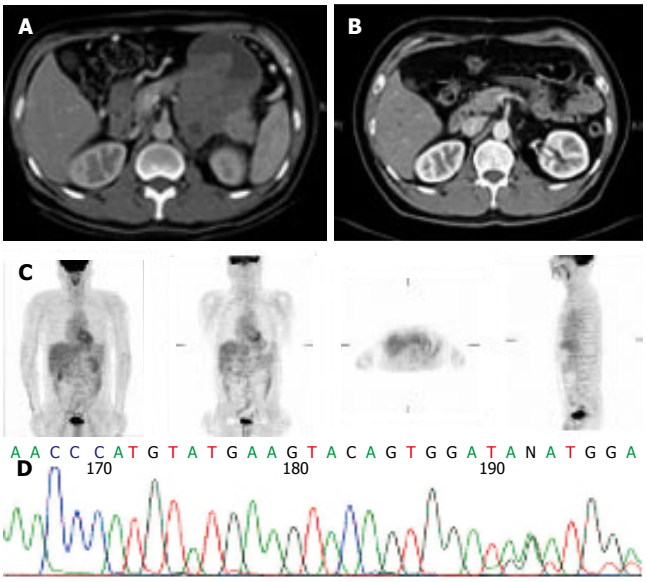

Figure 2 (**A**) Abdominal CT showing a huge retroperitoneal tumor invading the pancreas (arrow); (**B**) abdominal CT showing complete response without tumor at the same level as Figure 2A; (**C**) PET showing no tumor with metabolic activity in the whole body; (**D**) direct sequencing analysis of DNA from patient 2 showed deletion and insertion mutation at codons 556-557 in exon 11 (arrow).

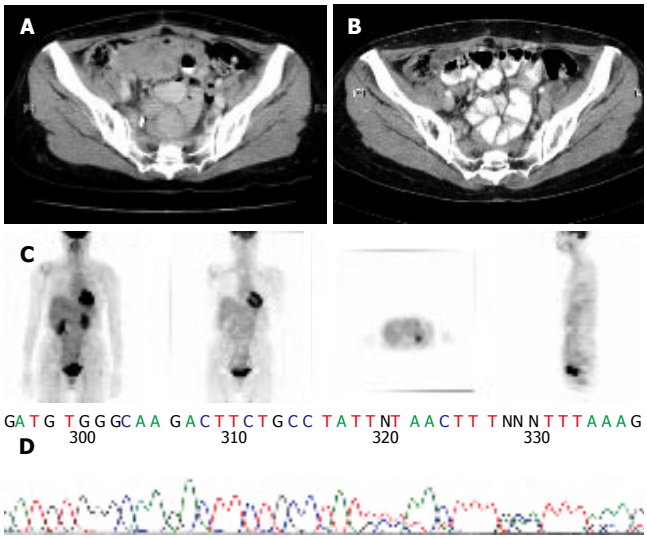

Figure 3 (**A**) Abdominal CT showing a tumor located near the ileum (arrow); (**B**) abdominal CT revealing complete response without tumor at the same level as Figure 3A; (**C**) PET showing no tumor with metabolic activity in the whole body; (**D**) direct sequencing analysis of DNA from patient 3 showed insertion AY at codons 502-503 in exon 9 (arrow).

study was 68.0%. Glivec induced a sustained objective response in more than half of the patients with advanced GISTs[16]. However, CR induced by Glivec on GIST patients was sporadically reported. In US Intergroup S0033 phase III study on 751 metastatic or unresectable GIST patients receiving 400 or 800 mg Glive per day[23], CR rate was 3%. Moreover, in the EORTC 62005 phase III study, the CR rate was 4.76% for 923 metastatic or unresectable receiving 400 or 800 mg Glive per day. Contrast to the aforementioned two studies, the CR rate in this study was 7%. The experience on CR after Glivec treatment for advanced or metastatic GIST patients in this study may justify the use of Glivec as neoadjuvant or adjuvant treatment in the future. FDG PET has been proven to be highly sensitive in detecting early response[24]. Stroobants *et al*[24] demonstrated that the CR rate increased to 52.3% (11/21), however, discrepancy was noted between the CT and PET results. In this study, CR was diagnosed according to SWOG criteria by CT scan first. PET scan was used to confirm its metabolic activity by FDG uptake on PET scan thereafter.

Regarding further use of Glivec for GIST patients with CR after Glivec treatment, no consensus was made. A recently reported randomized trial has shown that Glivec interruption after 1 year is associated with a high risk of relapse, even for patients with CR[25]. So, Glivec might be administered in the three patients until intolerance or patient refusal. The further use of Glivec for GIST patients with CR after Glivec treatment needs investigation.

In conclusion, CR can be achieved in selected patients with advanced GIST treated with Glivec. Deletion and insertion mutations of kit exon 11 and insertion mutation of kit exon 9 contribute to the genetic features in these selected cases.

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RAPID COMMUNICATION

Oxidative damage, pro-inflammatory cytokines, TGF-α and c-myc in chronic HCV-related hepatitis and cirrhosis

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Abstract

AIM: To assess whether a correlation exists between oxidative DNA damage occurring in chronic HCV-related hepatitis and expression levels of pro-inflammatory cytokines, TGF-α and c-myc.

METHODS: The series included 37 patients with chronic active HCV-related hepatitis and 11 with HCV-related compensated cirrhosis. Eight-hydroxydeoxyguanosine in liver biopsies was quantified using an electrochemical detector. The mRNA expression of TNF-α, IL-1β, TGF-α and c-myc in liver specimens was detected by semi-quantitative comparative RT-PCR.

RESULTS: TNF-α levels were significantly higher in hepatitis patients than in cirrhosis patients ($P = 0.05$). IL-1β was higher in cirrhosis patients ($P = 0.05$). A significant correlation was found between TNF-α and staging ($P = 0.05$) and between IL-1β levels and grading ($P = 0.04$). c-myc showed a significantly higher expression in cirrhosis patients ($P = 0.001$). Eight-hydroxydeoxyguanosine levels were significantly higher in cirrhosis patients ($P = 0.05$) and in HCV genotype 1 ($P = 0.03$). Considering all patients, 8-hydroxydeoxyguanosine levels were found to be correlated with genotype ($P = 0.04$) and grading ($P = 0.007$). Also multiple logistic regression analysis demonstrated a significant correlation among the number of DNA adducts, TNF-α expression and HCV genotype ($P = 0.02$).

CONCLUSION: In chronic HCV-related liver damage, oxidative DNA damage correlates with HCV genotype, grading and TNF-α levels. As HCV-related liver damage progresses, TNF-α levels drop while IL-1β and c-myc levels increase, which may be relevant to liver carcinogenesis.

Key words: Oxidative DNA damage; Chronic HCV-related hepatitis; Inflammatory mediators

Farinati F, Cardin R, Bortolami M, Guido M, Rugge M. Oxidative damage, pro-inflammatory cytokines, TGF-α and c-myc in chronic HCV-related hepatitis and cirrhosis. *World J Gastroenterol* 2006; 12(13): 2065-2069

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INTRODUCTION

Oxidative damage may affect a number of cell targets, including DNA[1-2]. Eight-hydroxydeoxyguanosine (8OHdG), a modified DNA base generated by genomic material interacting with reactive oxygen species, is a mutation that causes G-C to T-A transversion at DNA replication[3]. This adduct is a marker of oxidative DNA damage and one of the most widely-investigated lesions, since its consequences may well be linked to carcinogenic mechanisms[4-6].

Oxidative damage in general and 8OHdG accumulation in particular, have been described in experimental and clinical HCV infection, with HCV-related oxidative damage playing a major part in the induction of liver diseases[7-9]. Although it is well known that reactive oxygen species induction lies at the center of a complex network of tissue and inflammatory responses involving the expression of cytokines, growth factors and oncogenes, this network has not been thoroughly investigated in HCV-related liver diseases.

Liver injury is reportedly associated with a chronic inflammatory response involving tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), etc. The former plays a central role in liver injury, triggering the production of other cytokines that in turn recruit inflammatory cells, promote fibrogenesis and further activate oxidative burst[10]. The initiation of a number of intracellular signal pathways involving apoptotic and/or anti-apoptotic signals should also be included amongst the effects of TNF-α[11] and HCV infection is indeed associated with an increase in TNF-α production, and the expression of viral proteins apparently results in more severe liver injury and hepatocyte death[12-14].

On the other hand, IL-1β gives rise to the cascade of

the inflammatory response and recent reports have shown that its levels are higher in HCV-related liver diseases than in other forms of liver damage[15]. Its polymorphisms behind are related to the risk of progression to HCC[16-17].

Our hypothesis is that oxidative DNA damage prompted by pro-inflammatory cytokines and/or by a specific effect of HCV core protein[18-19], and associated with an imbalance between apoptosis and cytoproliferation[20], is a fundamental event in HCV-related liver carcinogenesis.

Since a number of additional mediators are involved in liver carcinogenesis, including oncogenes such as c-myc that controls hepatocyte proliferation[21] and growth factors such as TGF-α involved in controlling liver regeneration and tumoral progression[22], particularly when they are co-expressed[23], the present study was to seek any correlations between oxidative DNA damage and the levels of pro-inflammatory cytokines like TGF-α and of c-myc in chronic HCV-related liver damage.

MATERIALS AND METHODS

Patients

Forty-eight patients consecutively recruited (34 M / 14 F, mean age 42 ± 12 years) with liver disease characterized by abnormal serum transaminase levels for more than 6 months, were admitted to the Division of Gastroenterology for diagnostic liver biopsy. Informed consent was obtained from all patients. Patients taking medication or vitamins capable of interfering with oxidative balance or liver damage were excluded from the study. The study was approved by the Human Research Committee of the University of Padova. Thirty-seven patients (24 M / 13 F, mean age 40.5 ± 11 years) were assigned to chronic active HCV-related hepatitis (CAH) group and 11 patients (7 M / 4 F, mean age 50.5 ± 14 years) were assigned to HCV-related compensated (Child class A) cirrhosis (CIRR) group. Before biopsy, each patient was tested to measure HCV antibodies using a second-generation ELISA and all positive sera were confirmed by RIBA II assay. In all patients, anti-HCV seropositivity was confirmed by positive HCV-RNA levels using the Amplicor HCV test (Amplicor PCR Diagnostic, Hoffman-La Roche, Basel Switzerland). A standardized genotyping assay (Inno-Lipa HCV III, Innogenetics, Gent, Belgium) was used. HCV genotypes were classified as genotype 1, subtypes 1a and 1b, genotypes 2, 3 and 4 and their subtypes. All the following studies were performed prior to any treatment.

Morphological evaluation

Biopsies (one per patient) were taken using a 16-17 gauge modified Menghini needle under ultrasound guidance and local anesthesia. Only patients whose biopsy material was adequate (i.e. about 4 cm long) were included in the study to avoid taking a second biopsy. At least 2 cm of biopsy material was cut, fixed in 10% buffered formaldehyde and handed over to the pathologists. The tissue was embedded in paraffin, cut and routinely stained with H&E and PAS for routine evaluation. Together with the overall diagnosis, the pathologist (who was unaware of the clinical diagnosis) also gave a semi-quantitative score (0-3) for the presence and extent of macro- and micro-vesicular steatosis and the Knodell index[24], as modified by Ishak *et al*[25], including both a grading and a staging of hepatic disease.

Biochemical findings

Serum levels of ferritin, transaminases and γ-glutamyl transpeptidase (γGT) were determined as part of the routine clinical procedure. The tissue for biochemical determination was around 15 mg wet weight. Samples were processed immediately and stored at -80℃.

Quantification of 8OHdG from hepatic biopsies

Liver biopsy specimens obtained at endoscopy were stored at -80℃ for no longer than 3 wk. Preliminary experiments indicated that storage under these conditions could not affect the results of the assessment obtainable with unfrozen samples and the samples might remain stable for as long as 1 month (data not shown).

After thawing, the specimens were homogenized in separation buffer (75 mM NaCl, 10 mM Tris/Cl pH 7.5, 5 mM EDTA pH 6, 0.5% sodium dodecyl sulfate) and proteinase K at 55℃ overnight. After treatment with ribonuclease A, the DNA was purified according to Fraga *et al*[26]. Following nuclease P1 and alkaline phosphatase hydrolysis, samples were filtered through 0.22 μm nylon filter units (Scientific Resources, Inc., Alfatech, Genova - Italy), and approximately 20 μg of DNA per sample was injected into the HPLC (Shimadzu, Kyoto, Japan). 8OHdG and normal deoxynucleosides were separated in a 3 μm Supelcosil LC-18-DB analytical column (7.5 cm × 4.6 mm, Supelco, Bellefonte, PA) equipped with a 5 μm SupelguardTM LC-18-DB guard column cartridge. The solvent system consisted of an isocratic mixture of 90% 50 mmol/L potassium phosphate (pH 5.5) and 10% methanol at the 1 mL/min flow rate. 8OHdG was detected using an electrochemical detector (ECD; ESA Coulochem II 5200A, Bedford, MA) equipped with a high-sensitivity analytical cell model 5011 with the oxidation potentials of electrodes 1 and 2 adjusted to 0.15 V and 0.35 V, respectively. 8OHdG levels were referred to the amount of deoxyguanosine (dG) detected in the same sample by UV absorbency at 254 nm. The amount of DNA was determined according to a calibration curve versus known amounts of calf thymus DNA. 8OHdG levels were expressed as the number of 8OHdG adducts per 10^5 dG bases. An 8OHdG standard (Sigma) prepared immediately before determination, was injected before any set of samples. The coefficient of variation was <10% and the amount of DNA required for the assay (expressed in μg of DNA) was 100 μg. Samples with lower amounts of DNA were rejected, since the risk of methodological error was only acceptable above this cut-off.

TNF-α, IL-1β, TGF-α and c-myc determination

The mRNA expression of TNF-α, IL-1β, TGF-α and c-myc in liver specimens was detected by semi-quantitative comparative RT-PCR. Total RNA extracted from frozen liver tissue (stored at -80℃) by acid guanidium thiocyanate-phenol-chloroform according to the Chomczynski and Sacchi method[27], was quantified spectrophotometrically. Integrity of the RNA sample was assessed by electrophoresis on 2% agarose gel (FMC Bio Product, Rockland,

Table 1 Eight-hydroxydeoxyguanosine levels in chronic HCV-mediated liver damage (mean ± SD)

	n° 8OHdG/10^5 dG		*n*° 8OHdG/10^5 dG
CAH	42.3±25.3	**HCV genotype 1**	74±36
CIRR	73.64±28.2	**Other genotypes**	46.9±23
P	0.05		0.03

Table 2 TNF-α, IL-1β, TGF-α and c-myc expression in chronic HCV-mediated liver damage (mean ± SD)

	TNF-α/β-actin	IL-1β/β-actin	TGF-α/β-actin	c-myc/β-actin
CAH	0.7 ± 0.2	1.1 ± 0.3	0.41 ± 0.1	0.09 ± 0.05
CIRR	0.5 ± 0.2	1.4 ± 0.6	0.43 ± 0.2	0.46 ± 0.2
P	0.05	0.05	N.S.	0.001

MC, USA). One μg of RNA was reverse transcribed in cDNA in the presence of 1× PCR buffer, 1 mM each of dNTPs, 1 U RNase inhibitor, 2.5 μM random exomers and 2.5 U of murine leukemia virus. cDNA was amplified in a final volume of 50 μL of PCR buffer, 2 U Amplitaq DNA polymerase, 0.056 μmol/L of Taq Start antibody, 0.2 mM of each of the dNTPs, 0.4 μmol/L of each primers for TNF-α, IL-1β, TGF-α, c-myc and β-actin. PCR products underwent a vertical electrophoresis on polyacrilamide gel. Electrophoretic bands were stained with silver nitrate and scanned on a densitometer image analyzer system (Quantity-one Biorad, Hercules, CA, USA). The results were expressed as the optical density ratio of TNF-α, IL-1β, TGF-α and c-myc to control β-actin.

Statistical analysis

The data were examined statistically by one-way ANOVA and Student's *t* - test, Kruskal-Wallis and linear regression. Multiple logistic regression analysis was also used by including the following variables: 8OHdG levels, diagnosis, age, expression of TNF-α, IL- 1β, TGF-α and c-myc, genotype.

RESULTS

Patient characteristics

No difference in the patients' age or gender distribution was observed between the CAH and CIRR groups. ALT levels were significantly higher in patients with chronic hepatitis than in patients with cirrhosis (87.9 ± 49 *vs* 50.5 ± 14, $P = 0.002$ by *t*), while AST levels were significantly higher in CIRR group than in CAH group (178 ± 95 *vs* 52 ± 23, $P = 0.002$ by *t*) . Serum ferritin and γGT levels did not differ significantly between the two groups of patients.

According to the classification Ishak *et al*[25], the stages of disease were, by definition, significantly higher in CIRR than in CAH patients (5.25 ± 0.4 *vs* 2.5 ± 0.8, $P = 0.0001$ by *t*), while grading was similar in the two groups. All patients were HCV-RNA positive. Type 1 (1a/1b) infection was the most prevalent (45%), followed by subtype 3a (29%), type 2 (17%) and finally type 4 (3%).

Oxidative DNA damage, TNF-α, IL-1β, TGF-α and c-myc expression

8OHdG levels were significantly higher in CIRR patients ($P = 0.05$ by *t*) and when oxidative damage was correlated with different HCV genotypes, 8OHdG levels were higher in HCV genotype 1 hepatitis than in the other genotypes ($P = 0.03$ by *t*). The results of 8OHdG are shown in Table 1. Considering all patients, 8OHdG levels correlated significantly with genotypes ($P = 0.04$ Spearman's rank correlation) and grading ($P = 0.007$ Spearman's rank correlation). The results for pro-inflammatory cytokines, TGF-α and c-myc are shown in Table 2. TNF-α expression was significantly higher in CAH group than in CIRR group (0.7 ± 0.2 *vs* 0.5 ± 0.2, $P = 0.05$ by *t*), whereas IL-1β expression was significantly higher in CIRR group than in CAH group (1.4 ± 0.6 *vs* 1.1 ± 0.3, $P = 0.05$ by *t*). The previously mentioned higher oxidative DNA levels in genotype 1 HCV infection correlated with TNF-α ($P = 0.04$). A significant correlation was also found between IL-1β levels and grading ($P = 0.04$), and between TNF-α and staging ($P = 0.05$). No significant correlations were found between pro-inflammatory cytokine levels, steatosis score or genotype. TGF-α levels were similar in the two groups of patients (0.41 ± 0.18 *vs* 0.43 ± 0.21, $P =$ NS by *t*), while c-myc expression was significantly higher in patients with cirrhosis (0.46 ± 0.2 *vs* 0.09 ± 0.05, $P = 0.001$ by *t*). No significant correlations were found between c-myc, steatosis score or genotype. Finally, multiple logistic regression analysis confirmed the previously reported significant correlation among the number of DNA adducts, TNF-α expression and genotype ($P = 0.02$).

DISCUSSION

We have previously reported that oxidative DNA damage in the liver is, at least to some degree, a specific feature of HCV infection, in which it reaches its maximal levels[7]. Even though it occurs in the early stages too, 8OHdG accumulation parallels the progression of the disease and is more striking in subjects with HCV genotype 1b infection[28].

This paper provides data on patients with HCV-related liver damage, partly describing the complex network of relationships between DNA oxidative damage, cytokine synthesis and release, c-myc and TGF-α expression that may both be strongly involved in liver cancerogenesis[29-31]. Numerous data link oxidative damage (and the parameters considered here) with the progression of liver disease and the onset of liver cancer. In primary murine hepatocyte cultures, TNF-α expression causes 8OHdG formation and an increase in cell cycle progression indicates a possible role of TNF-α in early malignant transformation of hepatocytes[32].

The first set of our results was related to TNF-α and IL-1β which plays a direct role in causing growth arrest and a chronic role in inducing TNF-α expression[10-11]. This effect was not confirmed in our series, since a correlation between IL-1β and TNF-α was not detected. On the other hand, IL-1β expression was higher in the later stages of HCV-related liver disease, as previously dem-

onstrated by Gramantieri *et al*[33], while the opposite was true of TNF-α, whose levels of expression were higher in CAH patients. We have previously reported that the balance between cytoproliferation and apoptosis is disrupted in HCV infection[20]. It is worth stressing that both TNF-α and IL-1β are involved in controlling the above described balance, thus taking part in determining the liver cell's fate and progression to liver cancer. In fact, the binding of TNF-α and IL-1β to their receptors leads to the activation of transcriptional factors, such as NFkB and AP-1, again involved in controlling cell proliferation[10]. What role does persistent oxidative stress play in this scenario? The overproduction of oxidative species, linked to over-expression of inflammatory cytokines (as shown by the positive correlation between TNF-α and 8OHdG levels in the liver), might be responsible for inhibiting the apoptotic process, most likely by activating the NFkB-dependent pathway[34].

Last but not the least, oxidative damage may be related to the expression of proto-oncogenes, such as c-myc[35]. In our study, c-myc transcript levels were significantly higher in cirrhotic than in non-cirrhotic tissues, indicating that tissue damage progression from hepatitis to cirrhosis, with the related cell growth changes, may be mediated to some degree by c-myc, which indeed is considered one of the activators of cell proliferation[36]. In this series, we could detect no relationship between 8OHdG and c-myc, suggesting that they have different and independent proto-oncogene activation mechanisms.

It was reported that TGF-α /c-myc double transgenic mice exhibit enhanced cell proliferation and build up extensive oxidative DNA damage which possibly accounts for massive DNA damage and accelerated neoplastic development in the liver[37]. In the present study, all liver samples with or without cirrhosis, expressed low levels of TGF-α mRNA and revealed no correlation with any of the other parameters investigated. This may not be totally surprising, since a strong and prominent localization of TGF-α in ground-glass hepatocytes of HBV-related liver disease in association with HBV pre-S1 antigen has been reported and this may mean that TGF-α is more involved in HBV than in HCV liver disease[38].

In our study DNA oxidative damage correlated with TNF-α over-expression in chronic HCV-mediated liver damage. Evolution to cirrhosis was characterized by an increased oxidative DNA damage, c-myc expression and IL-1β release. When disease activity was severe, it was paralleled by an increased expression of IL-1β and c-myc associated with genotype 1b infection and accumulation of 8OHdG. The above findings suggest that chronic HCV-mediated oxidative DNA damage in the liver may have an impact not only on hepatocyte proliferation rate through c-myc activation but also on cell proliferation and apoptosis through TNF-α activation.

In conclusion, HCV infection is associated with increasing cell proliferation unaccompanied with any substantial increase in apoptosis[20], while TNF-α activation in this scenario has more to do with cell proliferation rather than with cell apoptosis.

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RAPID COMMUNICATION

Prevention of *de novo* HBV infection by the presence of anti-HBs in transplanted patients receiving core antibody-positive livers

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Abstract

AIM: To analyze whether the presence of anti-HBs in liver transplant recipients is effective in preventing HBV infection.

METHODS: Twenty-three patients receiving anti-HBc positive liver were studied. Nine recipients were anti-HBc positive as a result of previous HBV infection. Of them, one also received HBV vaccine during the pre-liver transplantation period. Fourteen recipients were anti-HBs positive due to HBV vaccine administered during the pre-transplant period. Liver biopsy was obtained in 10/14 anti-HBc negative/anti-HBs positive recipients and in 4/9 anti-HBc positive recipients.

RESULTS: After a mean follow-up period of 46 months, 1 recipient with protective serum anti-HBs levels developed *de novo* HBV infection as a consequence of immune escape HBV mutants. Among the 14 vaccinated anti-HBc negative/anti-HBs positive recipients, 1/10 patients with available liver biopsy (10%) had liver HBV-DNA at 13 mo post-liver transplantation without serum viral markers and did not develop *de novo* HBV infection. The vaccinated anti-HBc positive recipient without HBV vaccine response was HBV-DNA positive in serum and liver, viral DNA was continuously negative in the following tests, so a spontaneous seroconversion was diagnosed.

CONCLUSION: The presence of anti-HBs as a result of HBV vaccine or past HBV infection seems to be effective at protecting patients receiving livers from anti-HBc positive donors. However, the emergence of immune escape HBV mutants, which can evade the anti-HBs protection, should be considered as a risk of HBV infection.

Key words: HBV vaccine; Liver transplantation; De novo HBV infection; Hepatitis B core antibody

Barcena R, Moraleda G, Moreno J, Martín MD, de Vicente E, Nuño J, Mateos ML, del Campo S. Prevention of *de novo* HBV infection by the presence of anti-HBs in transplanted patients receiving core antibody-positive livers. *World J Gastroenterol* 2006; 12(13): 2070-2074

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INTRODUCTION

It has been reported that the incidence of hepatitis B virus (HBV) infection is high in recipients after liver transplantation (LT) from hepatitis B surface antigen (HBsAg) negative but anti-core antibody (anti-HBc) positive donors[1,2]. The frequency of HBV transmission depends on the HBV serological recipient status, while the presence of anti-HBc and anti-HBs in organ recipients may confer resistance to HBV infection. Their absence results in *de novo* HBV infection[3-5]. To avoid the occurrence of *de novo* HBV infection in recipients without serum HBV markers, use of passive immunization with hepatitis B immune globulin (HBIG) in combination with lamivudine is necessary[6,7]. Another possibility to make use of these anti-HBc positive organs is to direct liver grafts to patients with anti-HBc and/or anti-HBs as a consequence of past HBV infection or HBV vaccination[8].

It is possible that HBV transmission from anti-HBc

Table 1 Features of donors and liver recipients

Pt.	Age (yr)	Sex	Liver transplant indications	Donor HBV status		Recipient pre-LT HBV status		Liver HBV-DNA	Latest	Follow-up
				anti-HBc	anti-HBs	anti-HBc	anti-HBs	(mo after LT)	anti-HBs levels	(mo)
1	58	M	HCV cirrhosis	+	-	-	+ V (>10)	13 + (RC)	ND	66
2	46	F	Cryptogenic cirrhosis	+	-	-	+ V (>10)	1 -	-	69
3	62	M	Alcoholic cirrhosis	+	+	+	+	ND	ND	70
4	66	M	HCV cirrhosis	+	+	+	+	0 -	ND	71
5	55	F	HCV cirrhosis	+	+	-	+ V (>10)	0 - 12 - 36 -	-	51
6	44	M	HCV cirrhosis	+	-	-	+ V (>100)	4 - 12 -	+	63
7	63	M	HCV cirrhosis	+	+	-	+ V (>100)	1 -	ND	74
8	54	M	HCV cirrhosis	+	-	+	+	2 - 28 -	+	76
9	41	M	HCV/alcoholic cirrhosis	+	+	+	- V (<10)	23 + (RC)	-	66
10	52	F	Alcoholic cirrhosis	+	+	-	+ V (>50)	ND	-	52
11	40	M	HCV/alcoholic cirrhosis	+	-	-	+ V (>50)	0 -	-	56
12	65	M	Alcoholic cirrhosis/ HCC	+	+	-	+ V (>10)	0 -	-	61
13	50	F	Cryptogenic cirrhosis	+	+	-	+ V (>10)	ND	-	39
14	67	F	HCV cirrhosis/HCC	+	+	-	+ V (>10)	14 -	+	36
15	67	M	HCV cirrhosis	+	+	-	+ V (>10)	ND	ND	36
16	60	M	Alcoholic cirrhosis	+	+	-	+ V (>10)	1 -	ND	32
17	62	M	HCV/HCC	+	ND	+	-	6 -	-	31
18	67	M	Alcoholic cirrhosis	+	-	-	+ V (>10)	ND	ND	28
19	46	F	HCV cirrhosis	+	+	+	-	ND	ND	24
20	58	M	Alcoholic cirrhosis	+	-	+	+	ND	ND	18
21	49	M	HCV/alcoholic cirrhosis/HCC	+	+	-	+ V (>10)	5 -	ND	18
22	56	M	Alcoholic cirrhosis	+	+	+	+	ND	ND	17
23	57	M	Alcoholic cirrhosis	+	+	+	+	ND	+	16

HCV: hepatitis C virus; HCC: hepatocellular carcinoma; HBV: hepatitis B virus; V: HBV vaccine; RC: partially double-stranded HBV DNA genome; ND: not determined.

donors to recipients via LT may be due to the persistence of HBV in tissue in a state of replication-competent that can be reactivated to form infectious particles. During initiation of HBV infection, the partially double-stranded HBV DNA genome (rcDNA) becomes a covalently closed circular DNA molecule (cccDNA) that serves as a template for viral transcription[9] and is regulated and amplified by an intracellular pathway[10]. This process establishes a pool of nuclear cccDNA, which persists in the nuclei of infected cells as long as hepatocytes survive, explaining the requirement for long-term antiviral therapies[11]. This fact could explain the reactivation of HBV under certain conditions such as administration of immunosuppressive drugs[12]. The genomic organization of the HBV direct repeat region (DR) provides a strategy to distinguish rcDNA from cccDNA using PCR primers flanking the DR region.

The purpose of this study was to analyze whether past HBV infection or positive response to HBV vaccine is effective in preventing *de novo* HBV infection in patients receiving a liver from anti-HBc positive donors.

MATERIALS AND METHODS

Patients

Anti-HBc screening is a general practice performed in organ donors at our hospital. Between February 1999 and February 2004, 31 HBsAg negative patients received anti-HBc positive liver. Thirty-one donors were anti-HBc positive and 16 of them were also anti-HBs positive (51.6%). Of the 31 recipients, 8 patients had a second LT or were under lamivudine treatment because they were anti-HBc and anti-HBs negative, so they were excluded from the study. The remaining 23 liver recipients were studied. Of them, 14 anti-HBc negative recipients developed protective anti-HBs levels after vaccination (40 mg/dose intramuscularly administered on days 0, 15 and 30; Engerix B, Smith-Kline Beechman, Belgium) and 9 were anti-HBc positive. Immunosuppression treatment included was of steroids and cyclosporin or tacrolimus. Characteristic and virological features of both donors and liver recipients are shown in Table 1.

Serum samples for detection of HBV markers anti-HBs and serum HBV-DNA were collected at the time of liver graft and during the post-transplant follow-up period. Liver tissues when possible were collected for analysis of HBV-DNA by PCR.

Hepatitis B virus markers

Serum samples were tested for HBsAg, anti-HBs and anti-HBc with commercially available radioimmunoassays kits (Abbott Laboratories, N Chicago, IL). Detection of viral DNA in serum was carried out by Abbott hybridization assay. Biopsy specimens were examined with immunohistochemical techniques for HBsAg and HBcAg

detection.

DNA extraction from paraffin-embedded liver tissues was performed using the MasterPure complete DNA purification kit (Epicentre, Madison, WI). Liver DNA was assayed for HBV DNA by 2 different sets of primers corresponding to the surface[13] and X genes. The sequences of the primers amplifying the X gene are as follows: HBVfe (TCTTGGACTCTCAGCAATGTCA nt 1438-1456), HBVre (GGTGAAAAAGTTGCATGGTGC nt 1583-1603), HBVfi (ACCGACCTTGAGGCATACTTCA nt 1463-1484), HBVri (CCAATTTATGCCTACAGCCTCC nt 1550-1571). PCR was started with the hot-start technique. The first round of PCR was performed with the outer primers for 30 cycles (at 95℃ for 15 s, at 55℃ for 10 s, and at 72℃ for 30 s) followed by an extension at 72 ℃ for 5 min. The second round was carried out with the inner primers for 30 cycles at 57℃ as the annealing temperature.

The selective detection of cccDNA was carried out by a nested PCR procedure as previously described with some modifications to distinguish between rcDNA genome present in virions and ccc HBV-DNA found in hepatocytes[14]. The rcDNA contains a single-stranded gap at the 5´end of the minus strand DNA. Since this region is sensitive to mung bean nuclease (MBN) leading to a disruption in the viral genome, no PCR product could be obtained using this rcDNA as a template. However, since cccDNA is a double-stranded covalently closed molecule and resistant to MBN, PCR could yield a fragment when this DNA was used as a template. MBN reaction was done as previously described[14]. The whole reaction was used as a template for the first round of nested PCR. The primers used to amplify cccDNA were HBVdr-s (TTACGCGGACTCCCCGT nt 1410-1424), 1900AS (GGTCAATGTCCATGCCCCAA nt 1769-1790), HBVfi (ACCGACCTTGAGGCATACTTCA nt 1463-1484), and HBVdr-as (GACATGAACAAGAGATGATTAGGCA nt 1706-1730). As a positive control, a PCR fragment containing the nick region of rcDNA was cloned into the pMosBlue vector (pMOSBlue blunt ended cloning kit, Amersham Pharmacia Biotech, UK). HBV DNA extracted from serum of HBsAg-positive individuals was used as a negative control. Both positive and negative controls were treated with MBN.

RESULTS

Only one patient developed *de novo* HBV infection after a follow-up of 45 ± 20.81 months (Table 1). This patient was vaccinated and developed a low anti-HBs response with anti-HBs title between 10 and 100 IU/mL. Seven months after LT, her routine biochemical tests showed abnormal level of liver enzymes (AST 97 U/L, ALT 151 U/L, GGT 136 U/L) but liver ultrasound was normal. Besides, virological analysis was found to be positive for HBsAg and serum HBV-DNA, even if her serum anti-HBs titer was 17 IU/mL. These virological tests did not reveal any other viral infections (CMV, CEV, HCV, HAV). Based on these results, *de novo* HBV infection was diagnosed and the patient was treated with lamivudine (100 mg/d). Serum HBV-DNA still remained positive, so a combined therapy of lamivudine and adefovir was administered 32 mo after LT. A second liver biopsy revealed marked steatosis with positive immunostaining for HBcAg (in nuclei and cytoplasm) and HBsAg (in cytoplasm and membrane). No tissue was obtained for the analysis of HBV-DNA.

It is known that the "a" determinant located within HBsAg is the target of immune response providing immunity against HBV infection[15]. The emergence of HBV with surface gene mutations is able to escape immune response against HBV vaccine, causing infection. In fact, HBV envelope mutants associated with the "a" determinant after HBV vaccination have been identified[16]. To know whether this might be the reason why *de novo* HBV infection occurred in this liver recipient, a serum sample taken 36 months after LT was used for HBV-DNA extraction, PCR amplification of the "a" determinant of the S gene and PCR fragment sequencing. Viral sequence revealed that this patient harbored a HBV variant with 2 point mutations at amino acid positions 127 and 145 of HBsAg. The first mutation resulted in a substitution of proline or lysine for threonine (Pro or Lys 127 Thr). The second mutation was a substitution of glycine for alanine (Gly 145 Ala). Several HBV mutants with amino acid changes in the "a" determinant have been reported in the post-transplant situation[16,17]. Among those mutations, the particular and almost invariably change is Gly 145 Arg, which can cause persistent infections[18]. Our patient also presented this change at position 145. However, the sequence analysis revealed the presence of an Ala instead of an Arg. This amino acid change has never been previously found in the literature. The change of Pro 127 Thr has been described in liver-transplanted patients [16].

A total of 18 biopsies taken at different post-LT time points from 14 recipients were obtained for the analysis of HBV-DNA in liver tissues. Open circular HBV-DNA (RC) was found in 2/14 patients (Table 1) and no cccDNA was detected in any of them. These results were reproducibly obtained in 3 repeated sets of PCR experiments. The anti-HBc negative patient with HBV-DNA in tissue (Table 1) had no detectable HBV-DNA and HBsAg in serum throughout a follow-up period of 66 mo. The anti-HBc positive recipient with viral DNA in liver (Table 1) was found to be serum HBV-DNA positive in one of his routine virological tests but this viral marker was continuously negative in the following tests. HBsAg was all negative during the whole follow-up period (66 months). No recurrent HBV infection could be considered in this patient.

DISCUSSION

Accumulating evidence suggests that HBV can be transmitted to the organ recipients from anti-HBc-positive donors through LT[19,20]. HBV transmission fluctuates among different studies between 50 % and 90 %. In our study only one patient receiving an anti-HBc positive liver developed *de novo* HBV infection as a consequence of an immune escape HBV mutant associated with the "a" determinant while in the absence of HBV mutants, none of the recipients developed HBV infection, suggesting that the presence of anti-HBs during LT, as a consequence of HBV vaccination or past HBV infection together with

anti-HBc, can protect against HBV infection transmitted by anti-HBc positive liver grafts.

Although less than 50% of the patients with cirrhosis due to HBV infection respond to HBV vaccine[21], a large number of patients can be considered as liver transplant recipients.

Anti-HBs levels >10 IU/mL due to HBV vaccine are considered protective in immunocompetent patients[22]. We consider this anti-HBs titer protective in those vaccinated patients[21].

We were aware of the short-term persistence of anti-HBs in these patients. Only 5 of them still maintained anti-HBs titers over the follow-up period while the rest of the patients lost this marker (Table 1). Since anti-HBs response is reduced in immunosuppressed patients[21,23], it is accepted that this rapid drop of anti-HBs levels is the consequence of their immunosuppressive therapy. Importantly, these results suggest that even with loss of anti-HBs during the post-LT period, HBV vaccination is effective as prophylaxis for the prevention of HBV infection in LT, which is in agreement with previous studies[24]. Another interesting finding that supports the efficacy of HBV vaccine against HBV infection is the continuous negativity for serum HBV DNA after LT during the follow-up period.

It is known that viral genome can persist in hepatocytes as a rcDNA molecule and as a cccDNA molecule, the later is required for viral replication[9]. Detection of intrahepatic cccDNA may indicate the possible ongoing viral replication[25]. Thus, its presence could explain the reactivation of HBV replication in patients receiving a liver from donors with anti-HBc. The only viral form detected in 2 recipients (1 anti-HBc negative, 1 anti-HBc positive) was rcDNA. The anti-HBc negative patient who had no history of previous HBV infection received HBV vaccine prior to LT with anti-HBs titers >10 IU/L. It is interesting to know that this patient, even maintaining HBV DNA in liver after one year of LT, did not show any virological evidence of *de novo* HBV infection during the follow-up period of 66 months (Table1). Moreover, he lost anti-HBs titers. The histopathological study at this time showed stage III fibrosis, confirming recurrence of HCV infection. The data may suggest that HBV graft infection may be infrequent. Likewise, in the anti-HBc positive recipient with viral DNA in liver tissue 23 months after LT (Table 1) and after 66 months of follow-up, no recurrent HBV infection occurred although he had positive serum HBV-DNA in one of his routine virological tests, suggesting that spontaneous seroconversion occurs in him.

However, even if the presence of anti-HBs in liver recipients seems to prevent recurrent or *de novo* HBV infection, the latest risk can still occur as seen in one of the recipients. Nevertheless, in this recipient HBV infection was not prevented by anti-HBs response because the cause was a circulating HBV mutant. The presence of circulating surface antigen-mutated HBV was proved when mutations in the "a" determinant region of the surface antigen were identified in this patient. In our study, the prevalence of HBV surface antibody escape mutants after liver transplantation was 6.6%, which is consistent with other studies[16, 26].

Our results are in agreement with an earlier study[27]. However, other reports have provided clear evidence that HBV genome is detectable in most anti-HBc positive donors[28]. The validity of our amplification method was confirmed by our control experiments using negative and positive controls. One possibility could be that DNA molecules isolated from preserved paraffin-embedded liver tissues are generally of poor quality because of the high degree of DNA degradation in these samples. However, HBV detection may be reduced beyond detectable levels but focal distribution of HBV infection cannot be excluded.

In conclusion, the presence of anti-HBs in liver recipients at the time of LT can prevent HBV recurrence or *de novo* HBV infection. Although we have described a new vaccine HBV mutant in a liver transplant recipient causing *de novo* HBV infection, the efficacy of HBV vaccine in organ recipients could not be considered as universal due to the development of immune escape HBV mutants associated with the "a" determinant which can evade the anti-HBs protection. Administration of HBV vaccine is mandatory in patients with chronic liver pathology potentially needing liver transplantation. Although these results are promising, the limited patient number may lead to an erroneous interpretation of the data. Most extensive studies including a large number of recipients need to be done.

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RAPID COMMUNICATION

Prognosis following transcatheter arterial embolization for 121 patients with unresectable hepatocellular carcinoma with or without a history of treatment

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Abstract

AIM: To retrospectively evaluate the prognosis of patients with hepatocellular carcinoma (HCC) with or without a history of therapy for HCC following transcatheter arterial embolization (TAE).

METHODS: One hundred and twenty-one patients with HCC treated with TAE from 1992 to 2004 in our hospital were enrolled in this study. Eighty-four patients had a history of treatment for HCC, while 37 did not. At the time of entry, patients with extra-hepatic metastasis, portal vein tumor thrombosis, or Child-Pugh class C were excluded. TAE was repeated when recurrence of HCC was diagnosed by elevated tumor markers, or ultrasonography or dynamic computed tomography findings.

RESULTS: Tumor size was larger and the number of tumors was fewer in patients without past treatment ($P < 0.01$). However, there were no differences in tumor node metastasis (TNM) stage or survival rate between the 2 groups. A bilobular tumor and high level of α-fetoprotein (AFP) (>100 ng/mL) were factors related to a poor prognosis in patients with a history of HCC.

CONCLUSION: The prognosis following TAE is similar between HCC patients with and without past treatment. Early diagnosis of HCC or recurrent HCC and obtaining good local control against HCC before entry to a repeated TAE course can improve prognosis.

Key words: Unresectable hepatocellular carcinoma; Prognosis; Repeated transcatheter arterial embolization

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INTRODUCTION

Liver transplantation is recognized as an effective therapy for hepatocellular carcinoma (HCC)[1]. However, a shortage of donors in Japan has led to the general use of transcatheter arterial embolization and transcatheter arterial chemoembolization (TAE) in patients with unresectable HCC without an indication of surgery and percutaneous therapy. Although disappointing results are published[2-4], the usefulness of TAE has been reconfirmed recently as some studies found that the procedure reduces the overall 2-year mortality rate and improves the survival rate of patients with unresectable HCC[5-8]. Past reports regarding the prognosis of patients with HCC are usually limited to the initial therapy, including surgery[9], percutaneous ethanol injection therapy (PEIT)[10], radiofrequency ablation (RFA)[11,12], and TAE. In previous studies of TAE, the subjects had no history of treatment for HCC. However, most patients with HCC have no indication for therapy such as surgery, PEIT and RFA due to multiple recurrences finally. No reports have evaluated prognosis and its related factors of patients with a history of HCC following a repeated TAE course. In the present study, we retrospectively evaluated the prognosis of HCC patients with or without a history of therapy for HCC following TAE.

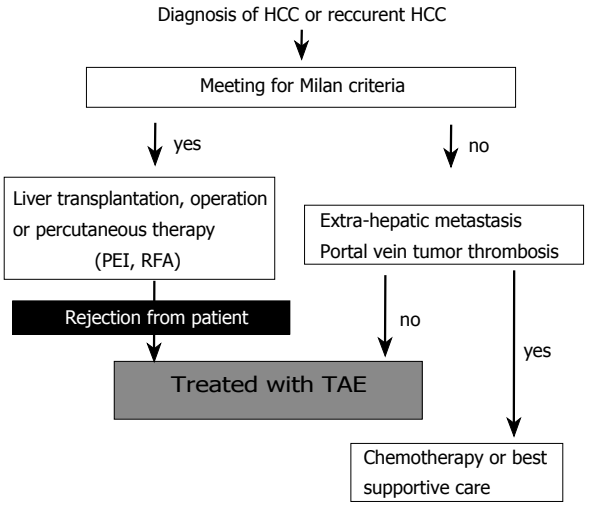

Figure 1 Strategy for treatment of HCC employed at our institution. Nearly all patients with HCC, which were outside of the Milan criteria, were recommended for a repeated TAE course.

MATERIALS AND METHODS

This was a single-center retrospective study conducted at Ehime University Hospital. One hundred and twenty-one patients with advanced HCC treated with TAE from 1992 to 2004 were enrolled in the study. After informed consent was obtained, the entry date was considered the day of the first TAE therapy after enrollment. The diagnosis of HCC was based on histological and cytologic findings or findings of dynamic computed tomography (CT). Tumor stage was established by dynamic CT, ultrasonography (US), angiography, chest CT, and bone scintigraphy examinations.

Patients with extra-hepatic metastasis, portal vein tumor thrombosis (PVTT), or Child-Pugh class C at entry were excluded from this study since the existence of PVTT and a high Child-Pugh score are poor prognostic markers and TAE can not improve these patients[5]. As a result, 37 patients with no history of treatment for HCC and 84 with such a history of surgery, PEIT, or RFA, were studied. During the 13-year study period, a total of 121 patients underwent 435 courses of TAE, with 99 (82%) outside of the Milan criteria[1]. In 37% of 121 patients, HCC was confirmed by histological examination. Others were diagnosed based on the increasing course of α-fetoprotein (AFP) and dynamic CT. All HCC nodules treated with TAE were hypervascular.

The patients treated with TAE were evaluated based on our strategy (Figure 1). TAE was repeated when HCC recurrence was diagnosed by the elevation of tumor markers, or US or dynamic CT examination findings. Experienced radiologists performed all the TAE procedures. A microcatheter was inserted into the artery feeding the tumor super-selectively after conventional hepatic angiography or angiography CT, and then a segmental or subsegmental TAE procedure[13] was performed. Before the procedure, antegrade flow in the portal vein and no obstruction of the main trunk of the portal vein were confirmed by US, dynamic CT, and portography findings via the superior mesenteric artery. Lipiodol and a gelatin sponge (Gelfoam, Upjohn, Kalamazoo, MI, USA) were used for embolization, and epirubicin hydrochloride was used together with Lipiodol in 25% of the cases. The goal of embolization was disappearance of tumor staining without complete obstruction of the hepatic artery. Patients that underwent additional chemotherapy via a subcutaneously implanted injection port, surgery, PEIT, or RFA for the purpose of reducing the size of the tumor after undergoing TAE were excluded from this study.

Table 1 Backgrounds of patients without or with history of treatment

	Patients without history of HCC ($n=37$)	Patients with history of HCC ($n=84$)	P value
Age (yr)	66.4 ± 9.9	67.0 ± 8.1	NS
Sex (male ： female)	32 ： 5	64 ： 20	NS
Frequency of positive for anti-HCV	72%	81%	NS
TNM stage (II ： III)	12 ： 25	35 ： 49	NS
Tumor size (mm)	46.4 ± 23.5	27.7 ± 16.1	$P<0.01$
Number of tumors (≤3 ： >3)	21 ： 16	23 ： 61	$P<0.01$
Monolobular ： bilobular	17 ： 20	27 ： 57	NS
Child-Pugh class (A ： B)	27 ： 10	48 ： 36	NS
Alanine transferase (IU/L)	63.8 ± 45.1	82.1 ± 64.3	$P=0.07$
AFP (≤100 ： >100 ng/mL)	23 ： 14	53 ： 31	NS
TAE with or without anti-cancer medication	13 ： 24	17 ： 67	NS
Average number of past treatments	-	2.9 ± 2.2	-
History of hepatectomy	-	18%	-
Average observation period (d)	557.6 ± 377.0	493.6 ± 390.6	NS

Anti-HCV: hepatitis C virus antibody; AFP: α-fetoprotein; TNM stage: tumor node metastasis stage.

The backgrounds of both groups at study entry are shown in Table 1. The group of patients without past treatment consisted of 32 males and 5 females, of whom 12 and 25 patients were in tumor node metastasis (TNM) stage[14,15] II and III, respectively. Furthermore, 27 were Child-Pugh class A and 10 were class B, of whom 72% were positive for the hepatitis C virus antibody (anti-HCV) and 14% for the hepatitis B surface antigen (HBs Ag).

As for the group of patients with treatment history, 64 were male and 20 female, of whom 35 and 49 were TNM stages II and III, respectively. Forty-eight patients in this group were Child-Pugh class A and 36 class B, of whom 81% were positive for anti-HCV and 16% for HBs Ag.

Determination of markers of hepatitis viruses

The presence of anti-HCV and HBs Ag was determined precisely using enzyme immunoassay kits (Imcheck-FHCV, Kokusai-Shiyaku, Kobe, Japan; AxSYM HBs Ag, Dainabot, Tokyo, Japan), according to the manufacturer's instructions.

Statistical analysis

All statistical analyses were carried out using a personal computer with StatView version 5.0 (SAS Institute, Inc., Berkeley, CA, USA). Analyses were conducted using Student's t-test, Mann-Whitney U test, Cox's proportional hazards regression model, logrank test, and the Kaplan-Meier method. $P < 0.05$ was considered statistically significant.

Table 2 Univariate analysis of patients with past treatment for HCC ($n=84$)

Factors	Number	Hazard ratio	95% CI	*P* value
Age (= and <65 ： >65)	38：46	0.99	0.95-1.02	NS
Sex (male ： female)	64：20	1.02	0.54-1.93	NS
Anti-HCV (positive ： negative)	67：17	1.31	0.64-2.67	NS
TNM stage (II ： III)	35：49	1.57	0.83-3.00	NS
Tumor size (mm)	-	1	0.98-1.02	NS
Number of tumors (≤3 ： >3)	23：61	0.88	0.47-1.67	NS
Monolobular : bilobular	27：57	2	1.04-3.86	$P<0.05$
Child-Pugh class (A ： B)	48：36	1.07	0.59-1.95	NS
AFP (≤100 ： >100 ng/mL)	53：31	1.9	1.03-3.48	$P<0.05$
History of hepatectomy (negative ： positive)	69：15	1.63	0.72-3.70	NS
Number of past treatments	-	1.05	0.93-1.19	NS

CI: confidence interval; anti-HCV: hepatitis C virus antibody; AFP: α-fetoprotein; TNM stage: tumor node metastasis stage.

Table 3 Multivariate analysis of patients with past treatment for HCC ($n=84$)

Factors	Hazard ratio	95% CI	*P* value
Existence of bilobular tumors	2.37	1.19-4.71	$P<0.05$
AFP (>100 ng/mL)	2.24	1.19-4.23	$P<0.05$

CI: confidence interval; AFP: α-fetoprotein.

RESULTS

There were no significant differences in the background findings between patients with or without past treatments for HCC, except for tumor size and the number of tumors ($P<0.01$) (Table 1). There was also no significant difference in patient distribution for TNM staging between the groups. None of the patients died due to the TAE procedure. For patients with treatment history, the average number of past treatments for HCC was 2.9±2.2 (range 1-10) and a hepatectomy was performed before entry to the repeated TAE course in 18% of these patients.

The survival rate was not significantly different between the 2 groups (Figure 2). The 1-, 2-, and 3-year survival rates were 90%, 57%, and 20% respectively in patients without past treatment, and 75%, 43%, and 25% respectively in those with past treatment. The factors related to poor prognosis in the 84 patients with past treatment for HCC were evaluated. Seventy-four of them (88%) were outside of the Milan criteria. According to univariate analysis, variables significantly associated with survival were tumor location (bilobular) and a high concentration of AFP (>100 ng/mL) ($P<0.05$). There were no relationships between the prognosis of patients with a history of treatment for HCC and other factors, including history of past hepatectomy and the number of past treatments for HCC (Table 2). Multivariate analysis showed that the existence of bilobular HCC and high concentrations of AFP (>100 ng/mL) were the factors for poor prognosis ($P<0.05$, Table 3). The survival rate of patients without both risk factors was better than that of those with both risk factors ($P<0.01$, Figure 3). In all 121 patients, the existence of bilobular HCC was related to poor prognosis ($P<0.01$), while a high concentration of AFP ($P=0.059$) and other factors including past treatments, were not related to poor prognosis.

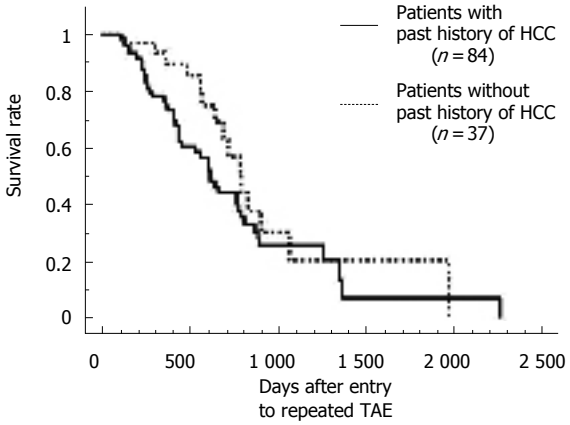

Figure 2 Survival rates of HCC patients with or without past treatment for HCC. There was no significant difference between the 2 groups. Survival rates after 1, 2, and 3 years were 90%, 57%, and 20% respectively for patients without past treatment, and 75%, 43%, and 25% respectively for patients with past treatment.

DISCUSSION

The prognosis of a patient with HCC is dependent on the hepatic reserve function and HCC staging[16,17]. A repeated TAE course is widely used for patients with unresectable HCC[18,19], though it was reported that TAE is not effective for improving the prognosis of such patients[2,3,4]. The reason for the disappointing results is that TAE is repeated within a fixed period of time although the liver reserves function and the patients have or have no recurrence of HCC. When TAE is repeated after a fixed period of time, embolization from the main trunk of the hepatic artery can lead to liver atrophy and deterioration of hepatic reserve function. Recently, the efficacy of TAE for patients with HCC has been reported, with good improvement of survival results[5-8]. Caturelli *et al*[20] reported that repeated TAE does not induce long-term deterioration of hepatic reserve function in HCC patients with Child-Pugh A and B but without PVTT.

Since repeated TAE for a fixed period without recurrence can lead to a reduction in hepatic reserve function, we think that it is important to perform TAE at the time

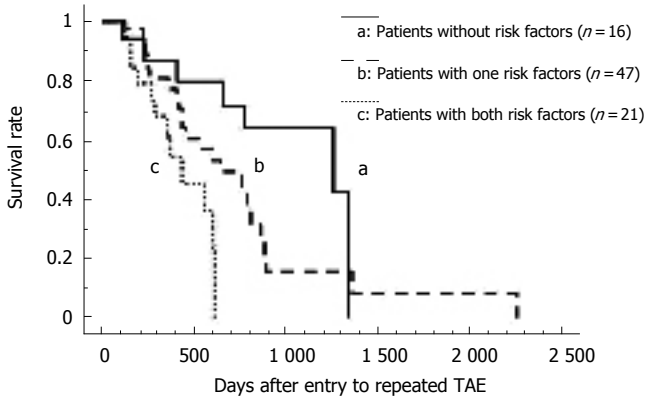

Figure 3 Survival rates of HCC patients with past treatment and with or without the 2 risk factors found in the present study. Significant differences were shown between "**a**" and "**b**" and between "**b**" and "**c**" ($P<0.01$ and $P=0.01$), while there were no significant differences between "**a**" and "**b**" ($P=0.08$). **a**: Patients without either factor [existence of bilobular tumors and high concentration of AFP (greater than 100 ng/mL; $n=16$)]; **b**: Patients with one of the factors ($n=47$); **c**: Patients with both factors ($n=21$).

when HCC recurrence is diagnosed by the elevation of tumor markers, or based on US or dynamic CT findings [21,22]. Recently, diagnostic progress of US and CT has made it easier to accurately diagnose new and recurrent HCC with low levels of AFP. Dynamic CT can offer detailed information about tumor vascularity and dynamic CT is useful to distinguish cholangiocarcinoma from HCC[23,24]. In our study metastatic liver tumor was denied from clinical course in all cases. In a large number of patients, HCC develops to an unresectable condition during the course of therapy and becomes outside of the Milan criteria. To our knowledge, the prognosis of patients with a history of HCC and factors for poor prognosis have not been reported after a repeated TAE course. In the present study, though the tumor maximum size and the number of tumors were different between the patients with or without past treatment, the survival rates of both groups after undergoing TAE did not show a significant difference, which might be due to no significant difference in TNM staging distribution between the groups.

As for the patients with past treatment, a past hepatectomy and the number of past percutaneous therapies (e.g. PEIT and RFA) did not influence the prognosis after repeated TAE in regard to maintaining liver reserve function. A high concentration of AFP and tumor location (bilobular) each had a significant influence. The elevation of AFP and existence of bilobular HCC are dependent on the malignant potential of HCC [25] and intra-hepatic metastasis, respectively.

Prognosis was not significantly different between HCC patients with or without a history of HCC following TAE. Our results show that diagnosis of HCC or recurrent HCC in the early stage and obtaining good local control against HCC before a repeated TAE course can reduce the time bias and improve prognosis.

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RAPID COMMUNICATION

Cryptogenic cirrhosis in the region where obesity is not prevalent

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Abstract

AIM: Recent studies have demonstrated that obesity is the common feature of cryptogenic cirrhosis (CC) and non-alcoholic steatohepatitis. However, there is little information on CC in the region where obesity is not prevalent.

METHODS: The clinical features, and the liver-related morbidity and mortality of CC were analyzed in Japan where the prevalence of obesity is low. Among 652 cirrhotic patients, we identified 29 patients (4.4%) with CC. Of these, 24 CC patients who were followed up for more than 6 months were compared in a case-control study with age-, sex-, and Child-Pugh score-matched controls having cirrhosis of viral etiology.

RESULTS: Obesity (BMI$\geqslant$25 kg/m^2), diabetes mellitus, and hypertriglyceridemia were more frequent, and the visceral fat area was larger in the CC patients than in the controls. The indices of insulin resistance were higher and the serum aminotransferase levels were lower in the CC patients than in the controls. Logistic regression analysis identified the elevated hemoglobin A1c, BMI $\geqslant$ 25 kg/m^2, and normal aminotransferase levels as independent predictors of CC. Kaplan-Meier analysis demonstrated lower occurrence of hepatocellular carcinoma and higher survival rate in the CC than in the controls in contrast to the similar cumulative probability of liver-related morbidity between those groups.

CONCLUSION: CC more frequently presents with the clinical features suggestive of non-alcoholic steatohepatitis compared with controls even in the region where obesity is not prevalent. The lower occurrence of hepatocellular carcinoma and higher survival rate may indicate an indolent clinical course in CC as compared with viral cirrhosis.

Key words: Cryptogenic cirrhosis; Viral cirrhosis; Non-alcoholic steatohepatitis; Hepatocellular carcinoma; Case-control study

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INTRODUCTION

Liver cirrhosis is the terminal condition of liver diseases resulting from various etiologies. Despite the recent development of diagnostic tools, no recognizable etiology can be detected in approximately 5% to 31% of cirrhotic patients who are therefore diagnosed as having cryptogenic cirrhosis (CC)[1-3]. Although several explanations such as unknown viral infections, occult alcohol abuse, or burnt out autoimmune hepatitis had been proposed as possible causes of CC, they actually induce CC only in some cases[3,4].

Obesity is an independent risk factor for chronic liver diseases, and liver fibrosis can develop in the obese patients without any known causes of liver diseases [5, 6]. Due to the recent increase of the obese population, great attentions have been paid to non-alcoholic steatohepatitis (NASH), which is characterized by obesity and insulin resistance. It has been widely recognized that NASH can progress to liver cirrhosis and hepatocellular carcinoma (HCC)[7]. Several studies have suggested that NASH may be an under-recognized cause of CC, because the prevalence of risk factors for NASH such as obesity and diabetes mellitus is definitely increased in the patients with CC[8-10]. However, these studies have been conducted

in the West where obesity is common, and there is little information on CC in the region where the dietary habits are different from those in the West and the prevalence of obesity is low. Because the key component of the association between NASH and CC is obesity[8-10], whether this association is true even in the non-obese population is an important issue.

In Japan where obesity is not prevalent, the clinical characteristics, and the liver-related morbidity and mortality of CC were investigated with a special reference to the association with NASH in this study. The aim was to clarify the clinical features of CC in the region where the prevalence of obesity is low.

MATERIALS AND METHODS

Patients

We identified 652 cirrhotic patients in whom sufficient data were available to establish the etiology of liver disease from the inpatients' registry of Nara Medical University since 1990. Liver cirrhosis was diagnosed on the basis of compatible clinical and imaging findings, and/or liver histology. The diagnosis of CC was made only after an exhaustive evaluation of the clinical history and laboratory data, from which no specific etiologies were defined. Patients with histological evidence of other defined causes of liver diseases were also excluded from the diagnosis of CC. Of 29 patients with CC, 24 patients who had been followed up for more than 6 months were classified as the CC group in our case-control study. For patients in CC group, 3 patients with cirrhosis of viral etiology [2 hepatitis C virus (HCV)-related and 1 hepatitis B virus (HBV)-related], age- (within 3 years), sex-, and Child-Pugh score-matched, were identified from the corresponding inpatients' registry in a consecutive manner. Finally, 24 patients with CC, 48 with HCV-related cirrhosis, and 24 with HBV-related cirrhosis were enrolled in the present case-control study. This study was approved by the Ethical Committee of Nara Medical University and was performed in accordance with the Declaration of Helsinki.

Methods

All patients underwent an exhaustive re-evaluation of the following clinical information: past or present evidences of diseases including diabetes mellitus, dyslipidemia, and hypertension; personal history of alcohol intake, intravenous drug use, or blood transfusion, and family history of liver diseases. The height and weight were measured, and the body mass index (BMI) was calculated as weight (kg) divided by squared height (m). The cases with ascites were evaluated after the resolution of ascites. HBV markers (surface antigen/antibody and core antibody) and HCV antibody were negative in all patients with CC. The following laboratory data were also collected at enrollment: total bilirubin, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase, albumin, γ-globulin, cholinesterase, total cholesterol, triglyceride, prothrombin activity (International normalized ratio: INR), hemoglobin A1c, α1-antitrypsin, iron storage parameters (transferrin saturation and ferritin), copper and ceruloplasmin levels, anti-nuclear antibody, anti-mitochondrial antibody, and anti-liver kidney microsomal antibody. When immune mediated liver disease could not be ruled out, liver histology was evaluated. The fasting blood levels of glucose and insulin were determined, and the homeostasis model assessment parameter of insulin resistance (HOMA-IR) and the quantitative insulin check index (QUICKI) were calculated as the indices of insulin resistance[11,12]. Liver biopsy was performed in 12 of the 24 patients with CC and was used to investigate the histological features suggestive of NASH.

Abdominal fat distribution

Computed tomograms were recorded at the umbilical level, and the visceral fat areas were measured as described previously [13]. Other tomograms were taken at the level where the liver and spleen were observed in the same slice. The Hounsfield unit of the liver and spleen was determined, and the liver/spleen ratio was calculated as an index of the hepatic fat content [14].

Assessment of the outcomes

Clinical information including the tumor markers and the imaging studies was monitored every 6 months until data analysis. In cases which lost to follow-up, up-to-date clinical information was investigated by telephone interview and/or contact with the primary care physician. The following outcomes were evaluated; (1) liver-related morbidity including variceal bleeding, ascites, jaundice, and hepatic encephalopathy, (2) occurrence of HCC, (3) mortality and cause of the death. HCC was histologically confirmed or diagnosed by elevation of α-fetoprotein and/or compatible ultrasonographic or computed tomographic findings. Death was considered to be liver-related when it happened in consequence to hepatic failure, variceal bleeding, and/or HCC.

Statistical analysis

All analyses were performed with StatView 5.0 program (SAS Institute, Cray, NC, USA). Comparisons were made using the two-tailed Student's *t* test for quantitative variables and the Chi-square test for qualitative variables. Kaplan-Meier's method and the log-rank test were used to compare the cumulative probability to liver-related morbidity and mortality. The factors associated with CC were identified using a multivariate logistic regression model. All data are expressed as mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

Etiology of liver cirrhosis

Among the 652 patients, cirrhosis was attributed to viral etiology in about 80% (HCV-related cirrhosis: 62.9%, HBV-related cirrhosis: 16.6%) and to alcohol in 9.5%. The prevalence of CC was 4.4%. The other cases resulted from various causes such as autoimmune liver diseases, hemochromatosis, Wilson's disease, Budd-Chiari syndrome, and congestion. The males were predominant in viral and alcoholic cirrhosis, whereas there was no gender difference

Table1 Characteristics of cryptogenic cirrhotic patients and matched controls

	Cryptogenic ($n=24$)	Control; all ($n=72$)	HCV-related ($n=48$)	HBV-related ($n=24$)
Age (yr)	58.2 ± 10.6	58.6 ± 8.8	58.7 ± 8.1	58.3 ± 10.2
Child-Pugh score	6.3 ± 1.4	6.5 ± 1.2	6.5 ± 1.2	6.4 ± 1.3
Follow-up (yr)	5.7 ± 4.2	5.9 ± 4.4	6.5 ± 4.2	4.7 ± 4.5
Transfusion (%)	0	26.8[b]	34.0[b]	12.5
Body mass index (kg/m^2)	25.5± 3.2	22.4±3.0[b]	22.6±3.3[b]	22.0±2.4[b]
ICGR15 (%)	31.5±16.7	27.2±12.3	28.2±12.1	25.0±12.7
Total bilirubin (mmol/L)	22±15	21±10	22±10	19±10
AST (nkat/L)	959±572	1349±772[a]	1410±573[b]	1229±1064
ALT (nkat/L)	860±640	1229±765[a]	1359±748[b]	972±747
AKP (nkat/L)	6518±2942	6638±2976	6695±2577	6529±3692
Albumin (g/L)	40±5	38±5	38±5[b]	38±6
γ-globulin (g/L)	17±3	18±5	19±4	15±5
Cholinesterase (U/L)	242.8±118.2	174.5±67.8[b]	162.1±61.7[b]	198.8±73.7
Total cholesterol (mg/dL)	170.5±43.0	156.5±35.1	151.2±31.3[b]	166.8±40.2
Triglyceride (mg/dL)	103.8±44.2	83.0±25.5[b]	84.5±26.6[b]	80.1±23.8[b]
Prothrombin time (INR)	1.15±0.15	1.19±0.14	1.21±0.14	1.16±0.14
Hemoglobin A1c (%)	6.2±1.3	5.1±0.8[b]	5.3±0.9[a]	4.8±0.7[b]
Blood glucose (mg/dL)	123.1±48.2	96.4±21.0[b]	96.9±23.0[b]	95.2±16.3[a]
Insulin (mU/L)	26.8±15.7	14.2±6.4[b]	15.9±6.6[b]	10.8±4.5[b]
HOMA-R (%)	8.6±4.8	3.6±2.5[b]	4.0±2.9[b]	2.6±1.3[b]
QUICKI	0.29±0.02	0.33±0.03[b]	0.32±0.02[b]	0.34±0.04[b]
Transferrin saturation (%)	36.3±17.5	45.2±29.7	48.5±31.1	40.0±27.8
Ferritin (mg/L)	160.0±155.3	193.5±282.9	225.0±318.1	99.3±104.8
Visceral fat area (cm^2)	102.0±39.8	56.9±35.3[b]	52.1±30.7[a]	64.3±43.9[b]
Liver/spleen ratio	1.08±0.05	1.08±0.06	1.07±0.06	1.09±0.06

[a]$P<0.05$, [b]$P<0.01$ *vs* cryptogenic cirrhosis. ICGR15:indocyanine green retention rate, INR:international normalized ratio, HOMA-R: homeostasis model assessment parameter of insulin resistance, QUICKI: quantitative insulin check index.

Table 2 Prevalence of obesity and complication in cryptogenic cirrhotic patients and matched controls

	Cryptogenic ($n=24$)	Control; all ($n=72$)	HCV-related ($n=48$)	HBV-related ($n=24$)
Body mass index: ≥25kg/m^2	54.2%	20.8%[b]	29.2%[a]	8.3%[b]
⩾30kg/m^2	16.7%	1.4%[a]	2.1%[a]	0%[a]
Visceral fat area: ⩾100cm^2	40.0%	5.3%[b]	3.7%[b]	9.1%[a]
Complication				
Type 2 diabetes mellitus	54.2%	26.4%[a]	35.4%	13.3%[b]
Hypertriglyceridemia (⩾150mg/dL)	20.8%	4.2%[a]	2.1%[b]	13.3%
Hypertension	25.0%	15.3%	18.8%	13.3%

[a]$P<0.05$, [b]$P<0.01$ *vs* Cryptogenic cirrhosis.

in CC (male/female ratio; HCV: 255/155, HBV: 82/26, alcohol: 52/10, CC: 15/14).

Characteristics of patients

The follow-up period was not significantly different between groups. Whereas one-third of the patients with viral cirrhosis were transfused, no patient with CC was transfused. BMI was significantly higher in the CC patients than in the controls of viral etiology. AST and ALT levels were lower in the CC patients than in the controls, although AST was higher than ALT in all groups. Cholinesterase, total cholesterol, and triglyceride were higher in the CC patients than in the controls. The fasting levels of blood glucose, hemoglobin A1c, insulin, and the indices of insulin resistance such as HOMA-IR and QUICKI were also higher in the CC patients than in the controls. Iron storage parameters such as transferrin saturation and ferritin were similar in all groups (Table 1). The visceral fat area was larger in the CC patients than in the controls, whereas the liver/spleen ratio was similar between groups. Obesity was more prevalent in CC patients than in controls (BMI ⩾ 30 kg/m^2: CC 16.7% *vs* controls 1.4%, $P<0.05$) (Table 2). The patients whose visceral fat area was larger than 100 cm^2 constituted 40% of the CC as compared with 5.3% of the controls ($P<0.05$). Type 2 diabetes mellitus and hypertriglyceridemia were more frequent in the CC patients than in the controls. No patient suffered from type 1 diabetes mellitus. The prevalence of hypertension was similar in all groups.

Although liver biopsy was performed in 12 of 24 patients with CC, there was no specific finding to define the etiology of liver disease. Ten of 12 biopsies, however, revealed one or more histological components suggestive of NASH such as macrovesicular steatosis,

Table 3 Factors associated with cryptogenic cirrhosis

	Odds ratio (95% CI)	*P* value
Univariate		
Body mass index (≥25 kg/m^2)	4.2 (1.51-11.48)	0.006
Visceral fat area (≥100 cm^2)	12.8 (2.16-75.32)	0.005
Diabetes mellitus	3.3 (1.26-8.60)	0.015
Dyslipidemia	6.1 (1.33-27.64)	0.020
Hypertension	1.8 (0.60-5.70)	0.285
ALT (<667 nkat/L)	3.8 (1.42-9.91)	0.008
Cholinesterase (≥192 U/L)	3.9 (1.42-10.94)	0.008
Triglyceride (≥150 mg/dL)	5.9 (1.29-26.86)	0.022
Total cholesterol (≥220 mg/dL)	3.4 (0.77-14.62)	0.108
HbA1c (≥6%)	5.5 (1.40-21.75)	0.015
HOMA-R (≥4%)	11.6 (2.46-54.45)	0.002
Ferritin (≥220 ng/dL)	0.4 (0.11-1.82)	0.255
Transferrin saturation (≥40%)	0.4 (0.11-1.82)	0.158
Multivariate		
HbA1c (≥6.0%)	7.8 (1.62-37.55)	0.010
Body mass index (≥25 kg/m^2)	6.8 (1.41-32.87)	0.017
ALT (<40 U/L)	5.0 (1.12-22.43)	0.035

CI: confidence interval, ALT: alanine transaminase, HbA1c: hemoglobin A1c, HOMA-R: homeostasis model assessment parameter of insulin resistance.

ballooning hepatocyte degeneration, neutrophilic lobular inflammation, and Mallory hyaline. Eight cases had macrovesicular steatosis in less than 30% of the hepatocytes. Ballooning hepatocyte degeneration, neutrophilic lobular inflammation, and Mallory hyaline were present in 7, 2, and 2 cases, respectively. Two cases had no inflammatory activity, 7 cases demonstrated minimal activity, and 3 cases had mild-to-moderate activity. The inflammatory infiltrates consisted predominantly of lymphocytes in fibrous bands.

Factors associated with CC

In the univariate analysis, BMI≥25 kg/m^2, visceral fat area ≥ 100cm^2, and the coincidence of diabetes mellitus and dyslipidemia, were significantly associated with CC (Table 3). Moreover, normal ALT levels (<667nkat/L), elevated levels of cholinesterase, triglyceride, and hemoglobin A1c ≥ 6.0%, and HOMA-IR ≥ 4% were significantly associated with CC. The multivariate analysis identified the elevated levels of hemoglobin A1c (OR: 7.8, 95% CI: 1.62-37.55, $P<0.05$) and BMI (OR: 6.8, 95% CI: 1.41-32.87, $P<0.05$), and normal ALT levels (OR: 5.0, 95% CI: 1.12-22.43, $P<0.05$) as independent predictors of CC.

Clinical course of CC as compared with the viral cirrhosis

During a mean follow-up of 5.7 years for the CC group ($n=24$), 2 patients experienced variceal bleeding, and 6 patients developed ascites (Figure 1). Jaundice and hepatic encephalopathy occurred in 5 patients and 6 patients, respectively. The viral group ($n=72$) was followed-up for a mean of 5.9 years, during which variceal bleeding, ascites, jaundice, and hepatic encephalopathy occurred in 12, 27, 20, and 10 cases, respectively. The cumulative probabilities of the liver-related morbidity were not significantly different between the groups. HCC occurred in 9 patients of the CC group in contrast to 53 patients of the controls (HCV: 36 patients, HBV: 17 patients) (Figure 2A). Although the cumulative probability of HCC occurrence was lower in

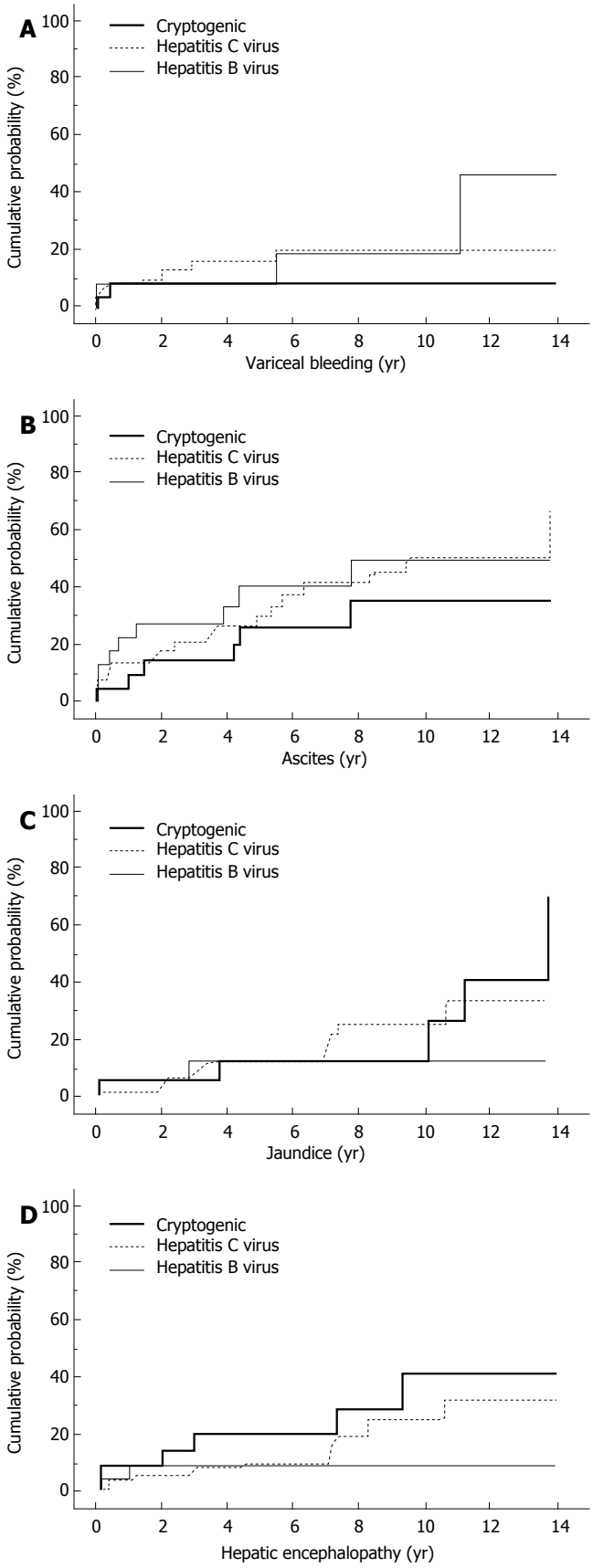

Figure 1 The cumulative probability of the liver-related symptoms for cryptogenic cirrhosis and viral cirrhosis using Kaplan-Meier's method and log-rank test.

the CC group than in the controls of viral etiology ($P<0.01$, CC *vs* HCV and CC *vs* HBV), HCC occurrence in the CC group rapidly increased 8 years after the observation. Of

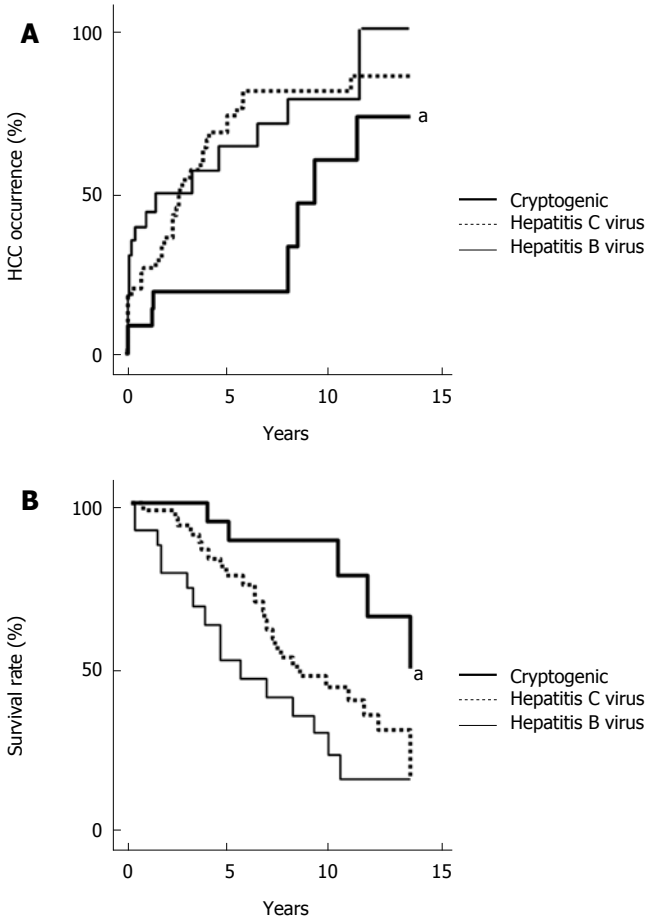

Figure 2 The cumulative probabilities of hepatocellular carcinoma and survival censoring non-liver related deaths for cryptogenic cirrhosis and viral cirrhosis. a: $P<0.01$, *vs* viral origin.

24 patients with CC, 3 died of liver failure and 2 of HCC (Figure 2B). No patient was associated with non-liver-related death. In the controls, 39 patients (24 HCV-related and 15 HBV-related) were associated with liver-related death (liver failure in 10 cases and HCC in 29 cases). The cumulative probability of survival censoring non-liver related deaths were higher in the CC group than in the controls ($P<0.05$, CC *vs* HCV, $P<0.01$, CC *vs* HBV).

DISCUSSION

Recent studies in the West have proposed that NASH may be an under-recognized cause of CC[8-10]. Although obesity is the key component of the association between CC and NASH, whether this association can be true even in the region with the low prevalence of obesity is unknown. The World Health Organization defined obesity as BMI $\geqslant$ 30[15], but in Japan, the prevalence of the population with such BMI is no more than 2%-3% in contrast to the 20%-30% in the West[16-18]. Moreover, the medical examination in 2002 ($n=6360$) in the local district where this study was performed showed that the prevalence of BMI $\geqslant$ 30 was 2.5% (data not published). We, therefore, investigated the clinical features of CC focusing on the association with NASH in Japan. This study demonstrated that in Japan, obesity, diabetes mellitus, and hypertriglyceridemia were more frequent, and insulin resistance, which is closely associated with the pathogenesis of NASH was greater in the CC group than in the controls of viral etiology. These findings are similar to those in the West, indicating that CC has the similar clinical features to NASH regardless of the prevalence of obesity.

The Japanese people as a race are known to suffer from obesity-related disorders even with a mild excess of adiposity[16,19-21]. The definition of obesity is proposed as BMI $\geqslant$ 25 in Japan, because the obesity-related disorders increase with a BMI $\geqslant$ 25[16,19]. This study demonstrated that the prevalence of BMI $\geqslant$ 30 in the CC group was 16.7% in Japan in contrast to 47% in the West. The prevalence of BMI $\geqslant$ 25 in the CC group was 54.2%, which is similar to that of BMI $\geqslant$ 30 in the West[9]. Moreover, our patients with CC, despite of the low prevalence of obesity, were accompanied with diabetes mellitus in 54.2% and hypertriglyceridemia in 20.8%, which is also similar to the reports in the West[8,9]. Although the BMI in viral cirrhosis was also lower in Japan than in the West (22.4 ± 3.0 *vs* 25.1 ± 4.2), the prevalence of diabetes mellitus was similar in these regions (26.4% *vs* 32.0%)[9]. Considering that the NASH patients in Japan are not as obese as those in the West [21], Japanese people, even with mild obesity, may suffer from NASH which may progress to CC.

Many researchers have shown that excess visceral fat is more closely related to the risk of health problems than the BMI itself[22-23]. The contribution of visceral fat area is greater in Japanese in whom the degree of whole fat accumulation is not as severe as in the West. Our present study demonstrated an increased visceral fat deposit in the CC group as compared with viral cirrhosis group. Because the increased visceral fat deposit plays a role in the pathogenesis of NASH via a production of various adipocytokines from the visceral fat tissue[23,24], the larger visceral fat area in CC further supports the association between CC and NASH. On the other hand, the hepatic fat deposit reflected by the liver/spleen ratio was similar in CC and viral cirrhosis groups. The hepatic fat deposit may decrease in the cirrhotic stage because the sinusoidal capillarization impairs the movement of gut-derived lipoproteins into the hepatocytes and the porto-systemic shunt diverts blood-borne lipids away from the liver. In fact, loss of the hepatic fat deposit has been observed in serial biopsies of NASH patients with progression to cirrhosis[7]. Because the excessive steatosis of the hepatocytes induces oxidative stress leading to the death of hepatocytes, loss of the hepatic fat deposit may be associated with lower ALT levels in CC.

This study demonstrated the lower occurrence of HCC and the higher survival rate in the CC than in viral cirrhosis, indicating that CC may take an indolent clinical course. There is a wide variation in the carcinogenic risk of CC among previously studies. Caldwell *et al*[8] showed that HCC developed in one of 71 patients with CC (1.4%). Another group reported higher prevalence of HCC in the CC group than in HCV-related cirrhosis[10]. The reason for these controversial data is unclear, but it may be attributed to the difference of the observation period. Bugianesi *et al*[25] suggested a later onset of HCC in the CC, because the patients with the CC-associated HCC were older as compared with the other cirrhosis-associated HCCs. Interestingly, this study demonstrated that although the

cumulative probability of HCC occurrence was lower in the CC than in the viral cirrhosis, HCC occurrence in the CC rapidly increased 8 years after the observation, indicating the later increase of HCC in CC. The later occurrence of HCC in CC can not be attributed to age and sex difference or degree of liver damage, because of a case-control study with age-, sex-, and Child-Pugh score matched viral cirrhosis. Therefore, HCC occurrence may be a late complication of CC.

In conclusion, the features suggestive of NASH were more frequently observed in the CC patients than in the controls of viral etiology even in Japan where obesity is not prevalent. It indicates that NASH may be a possible cause of CC regardless of the prevalence of obesity. Moreover, HCC developed less frequently and its prognosis was less severe in the CC group, indicating that CC may take an indolent clinical course. Further larger studies are necessary to understand the clinical features of CC.

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RAPID COMMUNICATION

Safety advantage of endocut mode over endoscopic sphincterotomy for choledocholithiasis

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Abstract

AIM: To evaluate whether an automatically controlled cut system (endocut mode) could reduce the complication rate of endoscopic sphincterotomy (EST) and serum hyperamylasemia after EST compared to the conventional blended cut mode.

METHODS: From January 2001 to October 2003, 134 patients with choledocholithiasis were assigned to either endocut mode group or conventional blended cut mode group at the time of sphincterotomy. The two groups were retrospectively compared for the complications after EST and serum amylase level before and 24 h after the procedure.

RESULTS: Of the 134 patients treated, 79 were assigned to conventional blended cut mode group and 55 to endocut mode group. There was no significant difference in age, sex, and serum amylase level before EST between the two groups. Complications were found in 5 patients of the endocut mode group (9%): hyperamylasemia (5 times higher than normal) in 4 and moderate pancreatitis in 1. Complications were found in 13 patients of the conventional blended cut mode group (16%): hyperamylasemia in 12 and moderate pancreatitis in 1. Serum amylase levels were elevated in both groups 24 h after EST ($P < 0.02$). The average serum amylase level 24 h after EST in the conventional blended cut mode group was significantly higher than that in the endocut mode group ($P < 0.05$).

CONCLUSION: Endocut mode offers a safety advantage over conventional blended cut mode for pancreatitis after EST by reducing hyperamylasemia.

Key words: Endocut mode; Endoscopic sphincterotomy; Choledocholithiasis

Akiho H, Sumida Y, Akahoshi K, Murata A, Ouchi J, Motomura Y, Toyomasu T, Kimura M, Kubokawa M, Matsumoto M, Endo S, Nakamura K. Safety advantage of endocut mode over endoscopic sphincterotomy for choledocholithiasis. *World J Gastroenterol* 2006; 12(13): 2086-2088

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INTRODUCTION

The main complications of endoscopic sphincterotomy (EST) are pancreatitis, hemorrhage, perforation and sepsis[1-4]. Acute pancreatitis is still the most common complication associated with the procedures. Testoni *et al*[5] have shown that pancreatitis is associated with the elevation of serum amylase level 24 h after ERCP/EST.

An automatically controlled cut system, endocut mode (ICC 200 ERBE), can reduce the danger of papillary hemorrhage but may lead to pancreatitis[6]. This study was to evaluate whether the endocut mode could reduce the complication rate of EST and serum hyperamylasemia after EST compared to the conventional blended cut mode.

MATERIALS AND METHODS

From January 2001 to October 2003, 134 patients admitted to Aso Iizuka Hospital due to choledocholithiasis were assigned to either endocut mode group or conventional blended cut mode group at the time of EST. Three gastroenterologists performed the procedure. An Olympus UES-10 electrosurgical generator (Olympus, Japan) was used for conventional cut with the blended current set at output limit 30W and the coagulation current set at output limit 15W. An Erbe ICC200 (Erbe, Germany) was used for endocut with the effect 3 current set at output limit 120W and forced coagulation current set at output limit 30W.

The two groups were retrospectively compared for the complications, pancreatitis, hemorrhage after EST and

Table 1 Age, sex, and serum amylase level before EST

	Number of patients	Yr	Male (%)	Female (%)	Amylase (IU/L)
Conventional cut group	79	73.5±1.3	54	46	102±8
Endocut group	55	70.8±1.8	56	44	101±18

Table 2 EST complications

	Hyperamylasemia (> 5 times upper normal limit)	Pancreatitis
Conventinal cut group	12	1
Endocut group	4	1

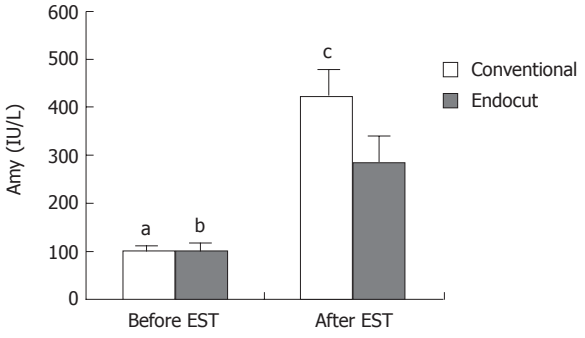

Figure 1 Average serum amylase level before and after EST. Data are expressed as mean ± SE. $^{a}P < 0.02$ *vs* conventional cut group after EST; $^{b}P < 0.02$ *vs* endocut group; after EST; $^{c}P < 0.05$ *vs* endocut group after EST.

serum amylase level before and 24 h after the procedure. Pancreatitis was defined when CT grade was higher than grade II (local pancreas swelling). A bleeding complication was defined when the patient required blood transfusion or had a drop in hematocrit level greater than 5%.

Results were presented as mean ± SE. Complications in the two groups were evaluated using χ^2 test and Fisher's exact test. Differences in serum amylase levels between the two groups were evaluated by Student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

One hundred and thirty-four patients were evaluated in this study. Seventy-nine patients underwent conventional blended cut and 55 patients underwent endocut. There were no significant differences in age and sex distribution as well as serum amylase level before EST (Table 1).

Complications were found in 5 patients of the endocut mode group (9%): hyperamylasemia in 4 (serum amylase level was 5 times higher than normal) and moderate pancreatitis in 1 patient. On the other hand, complications were found in 13 patients of the conventional blended cut mode group (16%): hyperamylasemia in 12 patients and moderate pancreatitis in 1 patient (Table 2). One patient in each group had mild bleeding not showing hematocrit decrease. No major complications such as perforation were found in both groups. There were no significant differences in the incidence of complications such as pancreatitis, hemorrhage, and hyperamylasemia between the two groups.

Serum amylase levels were elevated in both groups 24 h after EST ($P < 0.02$). The average serum amylase level 24 h after EST in the conventional blended cut mode group was significantly higher than that in the endocut mode group ($P < 0.05$, Figure 1).

DISCUSSION

Since the introduction of EST, the indications for the procedure have grown steadily. The complications of EST are similar to those of diagnostic ERCP but occur more frequently. Freeman *et al*[3] have shown that the incidence of pancreatitis after EST is 5.4% and significant risk factors for pancreatitis are sphincter of Oddi dysfunction, younger age, the number of pancreatic contrast injections, precut sphincterotomy, and difficulty of cannulation. Elta *et al*[7] reported that the type of electrocautery current affects this risk and found that the use of pure cut current is associated with a lower incidence of pancreatitis rather than blended current.

A new high-frequency current generator equipped with an automatically controlled system (endocut mode) has been currently used in EST. With endocut mode, each interval of cutting is automatically triggered by the initial electric arc sensor within the ICC 200 and is thus reproducible in duration. The automatically-fractionated cut and controlled cutting speed can prevent perforation of the major papilla[6].

In this study, we observed the most common complications such as moderate pancreatitis and mild hemorrhage in both groups, which are consistent with the other reports[3,4,6,7]. The endocut mode could reduce hemorrhage after EST[6,9-10], but leads to pancreatitis[6]. On the other hand, Ellahi *et al*[11] reported that the endocut mode does not offer a safety advantage over the conventional blended cut mode. In this study, there were no significant differences in the incidence of complications such as pancreatitis and hemorrhage between the two groups. The rate of hyperamylasemia (serum amylase level was 5 times higher than normal) was 7% in the endocut mode group and lower than 15% in the conventional blended cut mode group. But there was no significant difference in the incidence of hyperamylasemia between the two groups. In this study, the number of patients might be too small to have a statistical significance as for the incidence of complications.

A larger study is needed for the evaluation of usefulness about the endocut mode in EST, because the number of patients was less than 150 in this and other studies[6,9-11]. It was reported that the endocut mode reduces hemorrhage after EST[8]. Our results showed that the average serum amylase level 24 h after EST in the endocut mode group was significantly lower than that in the conventional blended cut mode group ($P < 0.05$), which is consistent with study of Shinozuka *et al*[9]. In this study, in comparison with a conventional blended cut mode

unit, all the examiners noted the smooth, easy EST by the endocut mode unit. Thus it might shorten the procedure time, lower the risk of major papilla's edema as well as the number of contrast injections and the serum amylase level after EST.

The endocut mode offers a safety advantage over conventional blended cut mode for pancreatitis after EST by reducing the hyperamylasemia.

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RAPID COMMUNICATION

Reduction of virus burden-induced splenectomy in patients with liver cirrhosis related to hepatitis C virus infection

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Abstract

AIM: To examine the hepatitis C virus (HCV) levels and immunological markers in cirrhotic patients after splenectomy.

METHODS: HCV RNA titers as well as cellular and humoral immune markers were determined in 20 cirrhotic patients after splenectomy and in 32 cirrhotic controls with an intact spleen.

RESULTS: Serum HCV RNA titers were lower in the splenectomized patients than in the controls ($186 \pm 225 \times 10^3$ copies/mL *vs* $541 \pm 417 \times 10^3$ copies/mL, $P < 0.01$). HCV RNA was judged to have been spontaneously eradicated in 4 splenectomized patients, but in none of the controls. Natural killer cell activity was higher in the splenectomized patients than in the controls (41.2 ± 19.3% *vs* 24.7 ± 15.3%, $P < 0.01$), and natural killer cell activity was negatively correlated to HCV RNA titers in the splenectomized patients except in those with serotype 2-related infection. The CD4/CD8 ratio was significantly lower in the splenectomized patients than in the controls.

CONCLUSION: The findings suggest that splenectomy may diminish virus burden in cirrhotic patients with HCV infection at least in part, through augmentation of natural killer cell activity.

Key words: Hepatitis C virus; Liver cirrhosis; Natural killer cell; Splenectomy

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INTRODUCTION

Persistent infection with hepatitis C virus (HCV), a parenterally transmitted RNA virus, occurs in 70%-80% of HCV-infected patients. Chronic hepatitis C progresses gradually to liver cirrhosis, a condition frequently associated with hepatocellular carcinoma (HCC)[1-3]. To prevent progression to chronic liver disease, continuous virus burden must be interrupted. However, spontaneous elimination of the virus load rarely occurs[4-8]. HCV-specific cytotoxic T cells are thought to play a principal effector role in host defense against HCV infection[9-11]. In addition, natural killer (NK) cells are believed to participate in the defense against hepatitis viruses, because the human liver contains a significantly higher number of NK cells than peripheral blood or any other organs[12]. NK cells exert various effector functions during the early phase of HCV infection, including induction of apoptosis and production of IFN-gamma and TNF-alpha[13-16]. In addition, NK cells are suggested to play a crucial role in the clearance of HCV in patients undergoing interferon therapy[17,18], but the significance of NK cells in the pathogenesis of chronic hepatitis C remains to be clarified. Although several studies have reported reduced activity of NK cells in cirrhotic patients with a history of alcohol abuse[19,20] and complication of HCC[21-23], little is known whether NK cells vary in patients with HCV-positive liver cirrhosis[24,25].

Splenectomy was a popular surgical procedure for esophageal varices associated with liver cirrhosis until endoscopic sclerotherapy has become the first line treatment[26,27]. Splenectomy may compromise the immune system because the spleen plays an important role in phagocytosis and antibody production[28,29]. On the other hand, there is some evidence that splenectomy can positively affect Kupffer cell functions in the liver, and protects against viral infection[30-33]. Ferrante *et al*[32] have insisted that a compensatory increase in the activity of NK cells in splenectomized patients might offer protection against infection and malignant disease. Pereira *et al*[31] reported that the HCV genome is detectable in spleen specimens obtained from HCV antibody seropositive patients associated with chronic schistosomiasis, suggesting that the spleen is an extrahepatic reservoir of the virus. Splenectomy may therefore influence the immune system and virus load of cirrhotic patients with HCV infection. Changes in immune

mediators and HCV burden in cirrhotic patients after splenectomy remain to be evaluated. In the present study, we retrospectively evaluated the effect of splenectomy on replication of HCV as well as cellular and humoral immunity including NK cell activity and lymphocyte subsets in cirrhotic patients with HCV infection.

MATERIALS AND METHODS

Patients

Twenty Japanese cirrhotic patients positive for anti-HCV antibody after splenectomy were enrolled in the present study (splenectomized). Splenectomy was performed in combination with surgical treatment of esophageal varices (14 cases), HCC (5 cases), or gastric cancer (1 case). The patients were referred for follow-up at our hospital. The mean duration of follow-up after splenectomy was 9.2 years. Thirty-two HCV-positive cirrhotic patients with an intact spleen served as controls and were followed up at our hospital. All controls had complications of esophageal varices, 17 of whom were treated for esophageal varices with endoscopic sclerotherapy and/or band ligation. Morphological diagnosis of post-hepatitic cirrhosis was performed in all splenectomies and 22 controls. The remaining 10 controls were diagnosed clinically based on typical clinical and laboratory findings of liver cirrhosis and characteristic liver findings at computed tomography and ultrasonography, because 6 patients showed complicated severe coagulopathy and 4 patients refused liver biopsy. No patients were diagnosed with chronic liver diseases, such as alcoholic hepatitis, autoimmune hepatitis, and chronic hepatitis B infection, or drug-induced liver disease. Patients with a history of alcohol abuse or diagnosed as HIV positive were excluded. None of our patients underwent anti-viral therapy using interferon during the period studied. Patient characteristics are shown in Table 1. Clinical features, severity of liver cirrhosis according to Child' s classification, association of HCC, HCV serotype, and liver function tests did not differ significantly between the two groups. Platelet counts were significantly higher in the splenectomized patients than in the controls. The study protocol was approved by the Ethics Committee of the Department of Internal Medicine, and informed consent was obtained from all patients.

Serum HCV RNA levels were measured to evaluate the effect of splenectomy on viral load. In addition, peripheral activity of NK cells, proportion of CD4 and CD8 subsets of T cells, and levels of serum β2-microglobulin and soluble interleukin 2 receptor (sIL2R) were examined.

Virology

The presence of HCV antibody was determined by the second and/or third generation of enzyme-linked immunosorbent assay (Ortho Diagnostic System Co., Ltd., Tokyo, Japan) and confirmed by recombinant immunoblot assay (Chiron RIBA-2 and/or RIBA-3). HCV serotype was examined by serotyping assay (SRL Laboratory Co., Tokyo, Japan) according to the method of Tsukiyama-Kohara *et al*[34]. Serotype 1 corresponded to types 1a and 1b, while serotype 2 corresponded to types 2a and 2b of the Simmonds classification[35]. Quantitative levels of HCV-RNA in serum samples were analyzed by combined reverse transcription PCR assay (Amplicor-HCV monitor; Nippon Roche, Tokyo, Japan) that could detect viral concentrations above 10^3 copies per mL[36]. If serum HCV-RNA was undetectable by this assay, we performed the Amplicor hepatitis C viral test twice, which is more sensitive and can detect as low as 10^2 copies per mL[37]. If HCV RNA was still undetectable, it was judged to indicate 'virus eradication'.

Table 1 Characteristics of cirrhotic patients with HCV infection

	Splenectomy (+)	Splenectomy (-)
Age (yr)	62.6±5.5	63.2±13.6
Gender (m:f)	8:12	13:19
HCV Serotype		
1	15	29
2	3	3
unknown[a]	2	0
Child's Classification		
A	11	18
B	6	8
C	3	6
Association of HCC	7/20	9/32
Total bilirubin (mg/dL)	1.2±0.5	1.2±0.7
Albumin (g/L)	35±6	37±4
γ-globulin (g/L)	23±0.8	20±5
AST (IU/L)	65.3±30.5	61.8±23.7
ALT (IU/L)	31.5±9.1	25.1±4.9
Platelets ($\times 10^4$ /μL)	15.2±4.5	8.1±2.7[b]
NK cell activity (%)	41.2±19.3	24.7±15.3[b]
CD4/CD8	1.2±0.4	1.6±0.6[c]
β2-MG (mg/L)	2.5±1.2	2.4±0.6[n.s]
sIL2R (U/mL)	917±445	842±190[n.s]

HCC: hepatocellular carcinoma; AST: aspartic aminotransferase; ALT: alanine aminotransferase; NK cells: natural killer cells; β2-MG: β2-microglobulin; sIL2R: soluble interleukin 2 receptor. a: undeterminable serotype; [b]$P<0.001$, [c]$P<0.05$ *vs* splenectomized patients; NS: no significant difference.

Immunological markers

NK cell activity was assessed against the k-562 cell line (Dainippon Pharmaceutical Co., Osaka, Japan) marked with ^{51}Cr using a cytotoxicity test for 3.5 h[38]. Blood samples taken from the cubital vein were collected into heparinized tubes. After centrifugation of the blood sample with a lymphocyte separation medium (Limphosepar I; Tokyo, Japan), the interface mononuclear cells were collected and suspended at a cell density of 1×10^6 /mL in RPMI-1640 medium (IBL; Gunma, Japan) and supplemented with 10% FBS (Cansera; Ontario, Canada). Peripheral blood monocytes (2×10^5 cells) were added to round-bottomed 96-well microplates containing ^{51}Cr-labeled target cells (1×10^4 cells) in 0.2 mL of RPMI-1640 medium supplemented with 10% FBS. The effector cell/target cell ratio was determined as 20. After centrifugation at 800 r/min for 5 min using an exclusive centrifuge for microplates, the cells were incubated for 3.5 h at 37℃ under 50 mL/L CO_2 in air. After incubation, the culture supernatant was harvested using PETΣ-96 (Sohken; Tokyo, Japan), and the radioactivity was determined using a gamma counter (1272 clini gamma, Wallac; Turuku, Finland). The percentage of cytotoxicity was calculated as follows: % cytotoxicity = (experimental ^{51}Cr release - spontaneous ^{51}Cr release) /(maximal ^{51}Cr release-spontaneous ^{51}Cr release) × 100.

The proportion of peripheral CD4 and CD8 subsets

Table 2 Characteristics of cirrhotic patients after splenectomy

	Age	Sex	Years After splenectomy	Serotype	HCV RNA (k copies/mL)	Child's classification	association of HCC[1]
1	75	Female	19	1	23	C	no
2	60	Female	16	Unknown[2]	Negative	A	no
3	80	Female	15	1	140	A	no
4	70	Female	15	1	130	B	no
5	63	Male	14	1	2	B	yes
6	67	Female	12	1	460	A	no
7	62	Male	12	1	Negative	A	no
8	50	Male	12	1	Negative	A	no
9	73	Female	11	Unknown[2]	Negative	A	no
10	67	Female	11	2	<1	B	no
11	50	Male	10	1	480	C	no
12	60	Female	7	1	140	B	yes
13	56	Female	7	1	160	C	yes
14	38	Male	6	1	770	A	no
15	68	Male	5	1	210	B	yes
16	73	Female	2	1	510	A	no
17	66	Female	2	2	21	A	no
18	59	Female	2	1	420	A	yes
19	58	Male	1	2	14	B	yes
20	57	Male	1	1	380	A	yes

[1] Hepatocellular carcinoma; [2] undeterminable serotype.

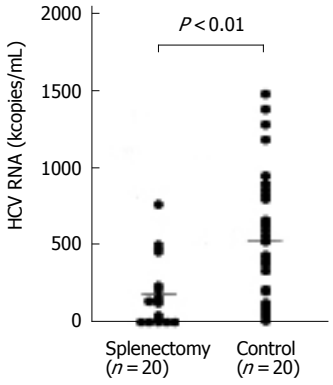

Figure 1 Serum HCV RNA titers in patients with liver cirrhosis. Splenectomized patients had significantly lower titers of HCV RNA than cirrhotic controls with an intact spleen.

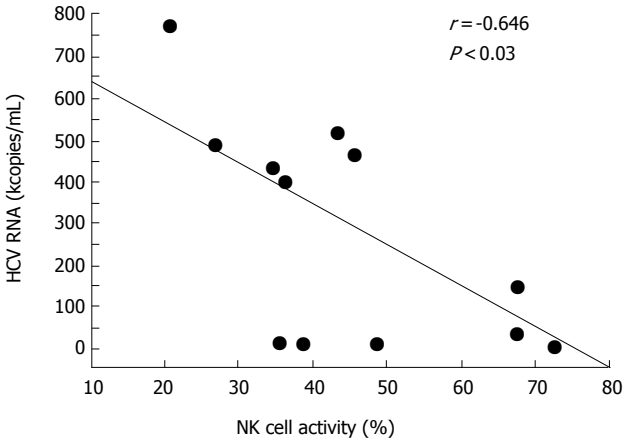

Figure 2 Relationship between HCV RNA titers and NK cell activity in non-serotype 2 splenectomized patients. There was a significantly negative correlation between HCV RNA titers and NK cell activity in non-serotype 2 splenectomized patients.

was assayed by flow cytometry. β2-microglobulin levels were measured by latex immunoaggregation assay (Eiken Co., Tokyo, Japan). sIL2R levels were determined using the cell-free IL-2R EIA kit.

Statistical analysis

Results were expressed as mean ± SD. Statistical analyses were carried out using the computer program StatView-J4.5 (Abacus Concepts, Inc. Berkeley, USA). Mean quantitative values were compared using Student's *t* test. Nonparametric data were compared using the Mann-Whitney U-test. All *p*-values were two-tailed. $P < 0.05$ was considered statistically significant.

RESULTS

HCV RNA titers (Figure 1)

Mean titers of HCV RNA were significantly lower in the splenectomized patients than in the cirrhotic controls ($186 \pm 225 \times 10^3$ copies/mL *vs* $541 \pm 417 \times 10^3$ copies/mL, $P < 0.01$). Among the serotype 1-related patients (15 splenectomized and 29 controls), the splenectomized patients showed a reduced viral load compared with the controls ($246 \pm 231 \times 10^3$ copies/mL *vs* $590 \pm 407 \times 10^3$ copies/mL, $P < 0.01$). Four (20.0%) of 20 splenectomized patients showed eradicated HCV in serum after 11 years of splenectomy in one patient, 12 years in two patients and 16 years in one patient. Two of the 4 patients had serotype 1-related HCV infection, while the serotype was undetermined in the other 2 patients (Table 2). No controls spontaneously eradicated HCV in their serum. Among the splenectomized patients with serotype 1, HCV RNA levels were lower, but not significantly lower in patients with a longer follow-up time (≥10 years) than in those with a shorter follow-up time (<9 years; $155 \pm 202 \times 10^3$ copies/mL *vs* $350 \pm 531 \times 10^3$ copies/mL, $P = 0.103$). No gender-related difference in virus load was observed in controls with serotype 1 (12 males: $649 \pm 469 \times 10^3$ copies/mL *vs* 17 females: $521 \pm 329 \times 10^3$ copies/ mL, $P = 0.43$).

Immunological markers (Table 1)

Peripheral activity of NK cells was significantly higher in the splenectomized patients than in the controls. In both splenectomized patients and controls, NK cell activity was lower in patients complicated by HCC than in those without HCC, although the difference was not significant [HCC (+): 33.6%±11.8%, HCC (-): 47.6%±18.1%, $P=0.16$]. NK cell activity in LC patients with an intact spleen was not significantly different from that in healthy subjects (data not shown).

The splenectomized patients showed a reduced proportion of CD4 cells, a similar proportion of CD8 cells and a significantly lower ratio of CD4/CD8 compared with those of the controls. Serum sIL2R and β2-microglobulin values were similar in both groups.

Relationship between HCV RNA levels and immunological markers

In the splenectomized patients without serotype 2-related infection, there was a significantly negative correlation between HCV RNA titers and NK cell activity (Figure 2). Such a relationship was not found in the controls. No relationships were found between HCV RNA levels and CD4/CD8 ratio, sIL2R, or β2-microglobulin.

DISCUSSION

This was the first retrospective study to examine the effect of splenectomy on the reduction of HCV in cirrhotic patients. We found that HCV RNA titers were significantly lower in cirrhotic patients after splenectomy than in cirrhotic controls with an intact spleen, indicating that splenectomy can reduce virus burden. The mechanisms through which splenectomy helps to reduce HCV remain to be determined, but a few possibilities have been suggested. One explanation for reduced viral load after splenectomy is that it might be immunologically based. The role of NK cells in controlling HCV replication remains obscure, but NK cells hold the potential to play a vital role in controlling HCV replication in hepatic cells using IFN-gamma-dependent mechanism[13]. Reports of the effect of splenectomy on the peripheral activity of NK cells are contradictory[27-31]. In the present study, HCV-positive cirrhotic patients splenectomy had the augmented activity of NK cells compared with those with an intact spleen. An inverse correlation between HCV RNA titers and NK cell activity was shown in the splenectomized patients, but not in the controls with an intact spleen. In addition, Ikuta *et al*[39] reported that liver mononuclear cells from splectomized mice produce a significantly larger amount of IFN-gamma than those from sham-operated mice. Taken together, increased NK cell activity post-splenectomy may reduce virus burden in HCV-positive cirrhotic patients.

The association of cirrhosis with HCC[21-23], cholestasis[40], and protein calorie malnutrition[20] is also important because these factors may influence the peripheral activity of NK cells in cirrhotic patients. Similar to previous reports, our data showed that cirrhotic patients complicated by HCC had a lower NK cell activity than those without HCC. The incidence of complication of HCC was slightly but not significantly higher in the splenectomized patients (35%) than in the controls (28%). Total bilirubin levels and severity of liver cirrhosis according to Child's classification were similar between the splenectomized patients and the controls. Accordingly, these biochemical and physiological factors did not cause increased activity of NK cells in patients after splenectomy.

Splenectomized patients with a longer follow-up time (≥10 years) had lower HCV RNA levels than those with a shorter follow-up time (9< years), although the difference was not significant. Further research should attempt to determine whether the lower HCV titers of patients with a longer follow-up time are due to the natural course of infection. To estimate the relevance of aging to virus load, we examined the changes in HCV levels over a mean period of 4.5 years in 20 patients with HCV-positive cirrhosis. The mean HCV RNA titers did not vary significantly during this period (baseline, 6.6±9.2 Meq/mL; after 4.5 years, 6.1±8.5 Meq/mL). Kato *et al*[41] reported that the amount of HCV RNA tends to increase as the duration of infection increases. These findings indicate that the virus load of splenectomized patients does not spontaneously reduce with age.

Interestingly, four splenectomized patients showed spontaneous eradication of HCV, whereas cirrhotic controls showed no such spontaneous clearance during the observation period. This is in contrast to the findings that spontaneous elimination of virus load rarely occurs in patients with persistent HCV infection and chronic liver disease[4-8]. To date, little attention has been focused on identifying the viral and host factors involved in the spontaneous disappearance of serum HCV RNA, because the time point of infection is unknown for many patients, which makes a prospective evaluation of long-term outcome of infection problematic. Evidence for factors related to the natural disappearance of HCV has been reported by Furuzaki *et al*[8]. HCV levels below 1.0 Meq/mL can contribute to natural clearance of the virus. Thus, reduced virus load caused by activated NK cells may have led to HCV eradication in four of the splenectomized patients.

The present findings suggest that splenectomy may diminish or remit virus burden in HCV-positive cirrhotic patients at least in part by increasing NK cell activity. However, simply because four HCV-eradicated patients underwent splenectomy before a method for HCV RNA assay was developed, it would be rash to conclude that splenectomy can lead to their clearance of HCV. Furthermore, influence of surgery for esophageal varices and HCC on virus load cannot be ruled out. A prospective study on the effect of splenectomy on HCV replication should be undertaken in order to clarify whether splenectomy may reduce virus burden and/or eradicate HCV in serum of HCV-positive cirrhosis.

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RAPID COMMUNICATION

Endoprosthesis implantation at the pharyngo-esophageal level: Problems, limitations and challenges

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Abstract

AIM: To present our experience with endoscopic placement of an esophageal endoprosthesis in 19 patients.

METHODS: A retrospective evaluation was made for the use of 19 stents positioned at the level of the cervical esophagus: 11 for malignant tumours (7 causing obstruction, 4 complicated by an esophago -tracheal or -cutaneous fistula), and 8 for an acquired benign tracheo-esophageal fistula due to prolonged intubation. The covered Ultraflex stent was used in all cases except two. These two patients had an esophagocutaneous fistula following laryngectomy and a Flamingo Wall stent was used.

RESULTS: Stent implantation was technically successful in all patients. Dysphagia score was improved from 3 to 2 in stenosis patients, while sealing of the fistula was achieved in all cases. The median hospital stay was 3 d for malignant tumour patients and 13.5 d for esophagocutaneous fistula patients. One Ultraflex stent and two Flamingo Wall stents were easily removed 33 d and 3 months respectively after implantation when the fistulas had totally occluded.

CONCLUSION: Endoprosthesis implantation for malignancy and/or fistula of malignant or benign origin at the level of the cervical esophagus is an easy, well tolerated, safe and effective procedure with no complications or mortality.

Key words: Cervical endoprosthesis; Pharyngo-esophageal stenosis; Dysphagia; Esophageal carcinoma; Esophagotracheal fistula

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INTRODUCTION

The insertion of an endoprosthesis is one of the most acceptable means of palliative treatment of patients with obstructing esophageal lesions and/or an existing esophagorespiratory fistula. However, the proximity to the cricopharyngeal sphincter is traditionally regarded as a relative contraindication, because of the potential problems of persistent foreign body sensation, pain, odynophagia, compression of the trachea or proximal migration of the prosthesis[1-3].

Recently, this traditional view has begun to change, as witnessed by an augmented number of case reports or small series of data presentations[1,2,4-11]. In such patients, with the inability to swallow even their own saliva at times, palliative intubation aimed at relief of dysphagia, maintenance of nutrition and avoidance of respiratory complications is the primary treatment goal, while the close relationship between the cricopharynx and the cervical lesion continues to be a challenge for every endoscopist.

Herein, we present our experience with stent placement in the cervical esophagus at the level of hypopharynx and/or the upper esophageal sphincter.

MATERIALS AND METHODS

Patients

Over the last 10 years (1995-2004), 19 patients were referred to our department to be treated endoscopically for a lesion in the cervical esophagus. There were 11 patients with a malignancy and 8 patients who spontaneously developed tracheo-esophageal fistula [TEF] after prolonged tracheal intubation for mechanical ventilatory support in the ICU.

The eleven carcinoma patients (9 males and 2 females) with a median age of 70 years (range: 62 - 82 years) suffered from: laryngeal (3 cases), hypopharyngeal (2 cases) and esophageal inlet carcinoma (2 cases), all causing severe stenosis and obstruction, two cases of high esophagotracheal fistula, one due to radical thyroidectomy and concomitant radiotherapy and the other due to an inoperable hypopharyngeal carcinoma and two cases of esophagocutaneous fistula after total laryngectomy for carcinoma, performed postirradiation (Table 1).

The seven patients with obstructing-type cancer

Table 1 Data of carcinoma patients

	Age	Gender	Disease
1	64	Male	Laryngeal carcinoma causing obstruction
2	72	Male	Laryngeal carcinoma causing obstruction
3	75	Male	Laryngeal carcinoma causing obstruction
4	65	Male	Hypopharyngeal carcinoma causing obstruction
5	68	Male	Hypopharyngeal carcinoma causing obstruction
6	70	Female	Esophageal inlet carcinoma causing obstruction
7	80	Male	Esophageal inlet carcinoma causing obstruction
8	62	Female	Esophagotracheal fistula after thyroidectomy + radiotherapy
9	64	Male	Esophagotracheal fistula after hypopharyngeal carcinoma
10	82	Male	Esophagocutaneous fistulas after total laryngectomy + radiotherapy
11	72	Male	Esophagocutaneous fistulas after total laryngectomy + radiotherapy

Table 2 Data of benign disease patients

	Age	Gender	Underlined disease
1	21	Male	Multiple trauma
2	73	Female	Multiple trauma [in septic state]
3	76	Male	Cerebral hemorrhage
4	56	Male	Cerebral hemorrhage
5	47	Female	Post-operative complications [in septic state]
6	70	Female	Post-operative complications
7	72	Male	Post-operative complications [in septic state]
8	72	Male	Post-operative complications [in septic state]

presented with a median dysphagia score of 3 (unable to swallow liquids) ranging from 2 to 4. Under diazepam-induced conscious sedation they were subjected to a Savary bougie progressive dilatation of the stenosis for one to two sessions depending on the rigidity of the tumour. Dilatation was performed over a guide wire which was advanced into the stenosis through the endoscope up to the point of 12.8 mm in diameter, to facilitate the rapid expansion of the stent.

After dilatation the endoscope was advanced to the tumour site. The total length of the stenosis and the distance of the tumor upper orifice from the incisor teeth, were carefully recorded due to their great importance for the correct placement of the stent.

The remaining 4 patients had no need for dilatation, thus immediately following insertion of the endoscope and inspection of the tumourous fistulae, the exact distance of the most proximal end of the lesion from the incisors and the total length of the lesion were recorded as above.

Eight patients had benign TEF (5 males and 3 females with a median age of 71 years, range 21-76 years) due to multiple trauma (2 cases), extended cerebral hemorrhage (2 cases) and post-operative complications (4 cases) implicating cardio-respiratory insufficiency. All patients, previously subjected to a percutaneous endoscopic gastrostomy for feeding, had an overall median intubation time of 30 d (range 15 - 80 d) and had been subjected to percutaneous tracheostomy a median of 16 d (range, 5-62 d) before diagnosis of TEF. All were characterized by poor prognosis and 4 of them were in a septic state (Table 2).

The final diagnosis of TEF was made by esophagoscopy, during which the exact characteristics of the fistula, i.e. size and its relationship with the upper esophageal sphincter as well as the distance of the most proximal end of the lesion from the incisors, were carefully recorded.

Stent placement

A self-expandable, covered -proximal release type-Ultraflex stent [Microvasive, Boston Scientific Corp., Natick, Mass] with a proximal flare of 28 mm, a body diameter of 23 mm, and a length of 120 mm or 150 mm, was used in all cases except two patients with esophagocutaneous fistulae after laryngectomy. In these cases, a Flamingo Wall stent [Microvasive Endoscopy, Boston Scientific Corp., Natick, Mass] with a proximal diameter of 24 mm, distal diameter of 16 mm, and length of 120 mm, was considered the most suitable for fistula sealing.

All stents were placed over a guide wire and no fluoroscopy was used in any case. Patients with malignant lesions were treated under diazepam-induced conscious sedation; benign TEF patients were under mechanical ventilatory support due to the underlying illness and had no need for supplementary anesthesia. The whole procedure was performed at the bedside in the ICU.

The stent delivery catheter was passed over the pre-inserted guide wire and advanced so that the proximal end of the stent was at the estimated distance from the incisor teeth. Generally, all stents were gradually deployed in such a position so that at least 2 cm of the prosthesis was over both sides of the lesion. However, our landmark was the upper esophageal sphincter. The stent was thus deployed to achieve the minimal harmful sensation with maximum security regarding stent migration as well as early overlapping by tumour overgrowth, i.e. the upper end of all stents was just within the upper esophageal sphincter or at the hypopharynx.

After stent insertion, its proper position was controlled by endoscopy and under direct vision. In some cases x-ray imaging was additionally performed (Figures 1 and 2).

RESULTS

Prosthesis implantation was technically successful in all patients and there was no procedure-related mortality. No complications occurred and no patient experienced severe pain at the site of stent placement, lasting more than 24 h and needed narcotic analgesics. Dysphagia score was improved from a median value of 3 (range, 2-4) to

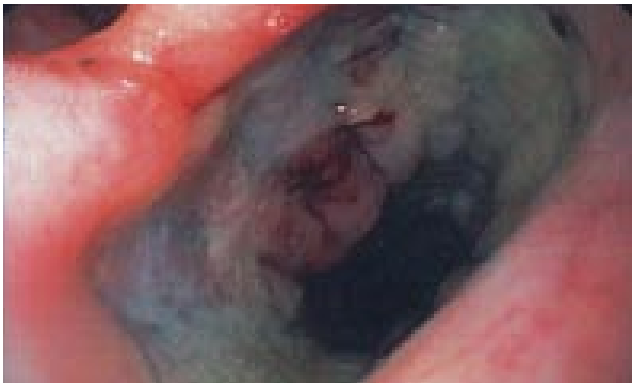

Figure 1 Endoscopic view of the soft funnel of the Ultraflex stent placed in the hypopharynx, posterior to the larynx. The stent was partially compressed in the antero-posterior direction at the level of arytenoid cartilages.

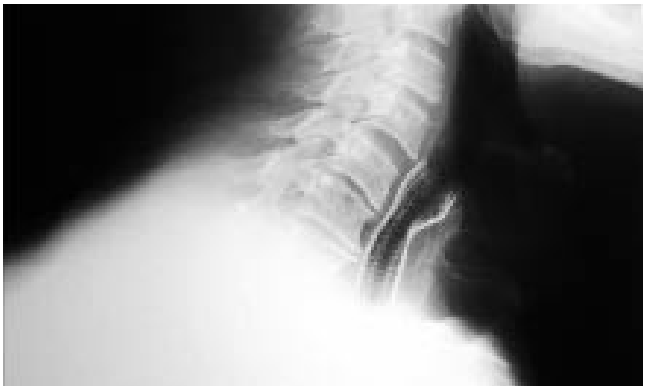

Figure 2 Plain radiograph of Ultraflex stent *in situ*, shows the fully expanded stent. Its proximal edge slightly protrudes in the hypopharynx (posterior endplate of C4).

a value of 2 (range,1-3) in esophageal stenosis patients. Fistula sealing was achieved in all cases, both benign and malignant (Table 3).

Regarding foreign body sensation, all conscious patients could well tolerate the stent placement in the first few days with no further difficulties. The two patients with esophagocutaneous fistula stented by Flamingo tubes experienced a foreign body sensation when the head/neck was bent since the proximal end of the stent was at the level of the mesopharynx, easily visible through the mouth opening (Figure 3). Both stents remained in place for three months until the fistula was totally closed, and were then removed by being grasped with retrieval forceps and pulled out without difficulty.

The median hospital stay was 3 d (range 2 - 4 d) for the 9 patients with stenosis and/or malignant esophagotracheal fistula. The other two patients with esophagocutaneous fistula remained hospitalised for 12 and 15 d, respectively due to cutaneous trauma debridement. Seven out of the eight TEF patients remained hospitalized in the ICU until their death, after 10 to 60 d, due to sepsis in 4, respiratory insufficiency in 2 and heart failure in one. The remaining patient, a 21-year-old multiple trauma victim, was weaned from a ventilator 33 d later and scheduled to be operated on for tracheoplasty. The Ultraflex stent was easily removed by simply grasping it with a pair of retrieval forceps under direct vision, just before the operation.

Table 3 Results after stent placement

Obstruction due to malignancy		**7 cases**
Stenting related mortality	0	
Improvement of dysphagia [score]	From 3 to 2	
Median hospital stay after stenting	3 d	
Malignant esophagotracheal fistula		**2 cases**
Stenting related mortality	0	
Sealing of fistula	2/2 (100%)	
Median hospital stay after stenting	3 d	
Malignant esophagocutaneous fistula		**2 cases**
Stenting related mortality	0	
Sealing of fistula	2/2 (100%)	
Median hospital stay after stenting	13.5 d	
Benign esophagotracheal fistula		**8 cases**
Stenting related mortality	0	
Sealing of fistula	8/8 (100%)	
Disease related mortality	7 cases	

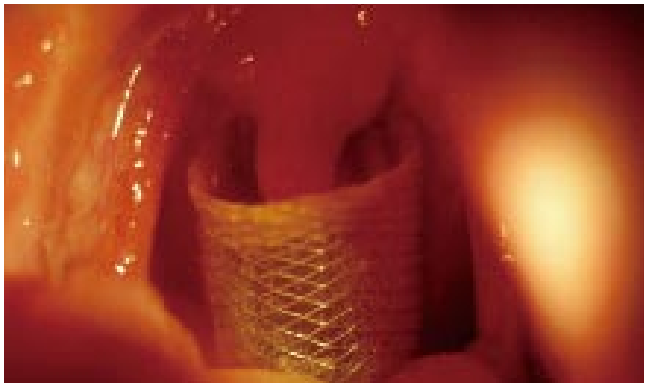

Figure 3 Direct view through the mouth opening of the upper part of the Flamingo stent placed temporarily at the cervical esophagus in the patient suffering from esophagocutaneous fistula after laryngectomy.

The 11 patients with malignancies were followed up every month. One patient with esophagocutaneus fistula died 7 months later, i.e. 4 months after fistula closure and stent removal. having developed total dysphagia due to recurrence of the disease. Unfortunately, this patient had totally refused a second stent; the second patient with esophagocutaneus fistula was alive 8 months after stenting. 5 months after fistula closure and stent removal, He is able to eat (dysphagia score 3) and no leakage has been reported; Seven patients remained alive for a median of 8 months (range 4 to18 months) and two patients were alive 10 months and 3 months after stenting. All these patients could eat semi-solid food and needed no other nutritional support.

DISCUSSION

The cervical esophagus is accepted as a segment between C6 at the pharyngoesophageal junction and the thoracic inlet at T1. It is endoscopically between 15 cm and 19cm from the incisor teeth and radiologically projects above the sternoclavicular joint[8,10]. At that level, any endoscopic procedure is more problematic even in the presence of a normal anatomical situation, since flexible endoscopy of the hypopharynx and upper esophageal sphincter is tech-

nically difficult, due to the reduced efficacy of insufflation and movements-related swallowing.

Generally, there is no report supporting placement of esophageal prosthesis for cervical lesions because of concerns about the increased risk of proximal migration of the stent into the hypopharynx and most importantly, the intolerable sensation of a foreign body[11,12]. However, there is no other acceptable means of palliating a terminal-stage tumor disease patient. This is because of the rapid decline of the patient's general condition upon the development of an esophagotracheal fistula due to aspiration pneumonia and malnutrition. On the other hand, as tumor stage is generally advanced and life expectancy is short, the major interest of any therapeutic procedure must be a rapid and successful palliation, ensuring acceptable quality of life, reducing the duration of hospital stay and cost.

There exists a general hesitation about applying stents in patients with benign diseases because of concerns regarding short-term complications and the absence of information regarding long term sequelae[3,13-15]. In our study, the patients with TEF of benign origin were all critically ill with a short term expectation of life if left untreated.

We question the risk of stent migration. In benign cases the risk is reported to be as high as 24%[15], which is probably related to a smaller mucosal surface area with less inward force anchoring the device. We experienced no migration in our cases, which might be attributed to the upper conical configuration of the stent, the external pressure induced by the cuff of the endotracheal tube, the constriction of the upper part of the stent by the upper esophageal sphincter, and mainly the absence of head-neck movements as well as swallow movements, due to the deep sedation of the patients. Moreover, the small number of patients could explain the absence of this complication in our series.

In the malignant cases, the risk of cervical stenting relates to the possibility of proximal migration, which shares the danger of sudden upper respiratory tract occlusion[3,8,10,16]. We experienced no migration in our patients, since the tumour masses were hard and protrusive and occluded the esophagus, which kept the stent in place, while in the two laryngectomy cases, it was impossible for the stent to impair breathing.

The second main concern for stenting at the level of cervical esophagus is the theoretical probability of a foreign body sensation[3,10-12]. Although such a sensation was experienced by some of our tumour-bearing patients, this was minimal and well tolerated. Most likely, by the time when a prosthesis becomes necessary, such patients especially those after laryngestomy with a Flamingo stent no longer have normal sensation, probably due to local infiltration of the nerves innervating the hypopharynx, cricopharyngeal sphincter and upper esophagus resulting in hypo/anesthesia. This propensity to infiltrate local neural structures is reflected by the frequent occurrence of unilateral or bilateral vocal cord paralysis. In addition, previous radiotherapy or surgery also impairs normal sensation. The two patients who were placed Flamingo stents for sealing the esophagocutaneous fistula after laryngectomy experienced a foreign body sensation every time they moved their heads forward. However, in these cases the stent was high at the level of the mesopharynx and easily visible even though the mouth was opened.

The procedure of stenting itself is totally uneventful. The use of local anesthesia and conscious sedation, in conjunction with a slim endoscope, can facilitate the procedure, which is time consuming only in the case of a very rigid and narrow malignant stricture requiring a guidewire to be advanced blindly through the stricture for bougie dilatation. Otherwise, the total endoscopic procedure takes only a few minutes, that is, the time needed for passing the endoscope through the lesion, making the appropriate measurements of total length of the lesion and distances of its proximal margin from upper esophageal sphincter and from incisor teeth, and advancing a guidewire through the endoscope into the stomach. The endoscope is then withdrawn and the stent is advanced 'blindly' over the guidewire, using only the numerical marks on the stent's sheath (centimeters from its proximal edge) for correct positioning. The use of general anesthesia does not facilitate the endoscopic maneuvering. However, insertion of the scope under direct inspection of the esophageal opening, by means of a laryngoscope as for tracheal intubation, is easier, but is not a reason to give general anesthesia to an otherwise conscious patient.

Thus, the eight mechanically ventilated TEF patients were under general anesthesia, while the remaining malignant lesion patients were simply given midazolam for conscious sedation. We experienced no problem with the latter group, but we could not give any favor to the former. However, this fact may be partially related to the benign nature of the disease in this group of patients.

With regard to the technical problems of proper positioning, the theoretical point of difficulty is the proximity of the mesopharynx and epiglottis. Profili *et al*[10] and Conio *et al*[17] have advised peroral administration of iodinated contrast medium for exclusive fluoroscopy guidance throughout the procedure. However, we found it was not useful and adds excessive difficulties, either because of the inability of a sedated patient to swallow or simply because of the increased time required to complete the procedure, or just because the procedure should be done on the ICU bed. We consider that the main difficulty is the accurate deployment of the proximal end of the stent close to the cricopharynx, because of the retraction of the expandable stent on deployment. The radiopaque markers used for stent placement under fluoroscopic assistance are not always reliable, because they are intended to indicate the position of a fully expanded stent. The problem was overridden when the proximally released type of Ultraflex prosthesis was used, allowing more accurate placement because we carefully measured the distances of the orifice of the upper esophageal sphincter and the proximal edge of the lesion from the incisor teeth, thus enabling us to know exactly where the proximal end of the stent should begin to deploy.

Additionally, the use of the Ultraflex stent has the advantages of being less rigid, thus reducing pain during movements of the head and neck, and has smooth edges, making it atraumatic when positioned in the hypopharynx,

despite continuous soliciting during swallowing. This prosthesis can exert a constant, gentle radial force on the esophageal wall, but withstand angulation forces better than the Song and Gianturco stents, as well as the Wallstent which is stiffer and thus less suitable for lesions in this area[12,17].

A further advantage is the rapid expansion to the full diameter, enabling all patients to ingest well-chewed food two days after intervention. The rapid expansion also results in tight fixation to the esophageal wall without any tendency to dislocate and an immediate and complete sealing. In the case of tracheoesophageal fistula, it has successful rate of 73%-100%[5,16,18-20], referring both to malignant and benign fistulae. Moreover, its funnel-shape facilitates the collection of saliva and maintains proper positioning.

For the esophagocutaneus fistula patients we preferred the Flamingo Wall stent due to a number of distinct characteristics: conical shape with proximal flaring and lange braiding angle in the upper part and small in the distal part of the stent. When swallow movements and oesophageal peristalsis propel the stent downwards, its upper end becomes trapped in the laryngoplasty area, whereas the lower end becomes stretched, thus resisting distal migration. Moreover, its polyethylene cover is on the inside of the stent, thus increasing the possibility of anchoring the metal mesh to the esophageal mucosa, since there is no stricture to hold it in place[12,21]. To the best of our knowledge, there are no comparative studies on types of stents except two which relate to the distal esophagus, suggesting that all stents offer the same degree of palliation while Ultraflex and Flamingo stents are both equally less atraumatic than the Gianturco stent[21, 22].

Stent placement in the setting of chemoradiotherapy may be associated with life-threatening complications such as esophageal perforation or bleeding. Kinsman *et al*[23] reported that the complication rate is 36.4% and the mortality rate is 23% in patients receiving radiation and/or chemotherapy, compared with 2.5% and 0% of those without prior therapy. Sumiyoshi *et al*[24] have recorded 6 sudden massive hemorrhages in a group of 22 patients. Additionally, it is likely that T4 cancers are susceptible to pressure necrosis from stent with a consequent increase in the risk of perforation into adjacent structures[24]. Fortunately, we did not experience such complications in our patients. This could be partially explained by the fact that by using the Ultraflex stent, excessive dilatation of the malignant stricture is avoided.

Finally, the quality of life becomes an overriding issue in patients with inoperable cancer, therefore the ability to swallow their saliva and maintain oral intake is important to most patients with esophageal tumours[25]. Since the most realistic goal of any palliative therapy is maximal relief of symptoms with minimal risk, the insertion of a stent with no or little risk of dilation and a minimum of post-procedure complications are the optimum. Good symptom relief of dysphagia and successful occlusion of fistulae are clearly possible by the use of a stent as shown in our malignant and benign cases and more importantly, many patients with malignant strictures or fistulae are able to swallow their saliva after stent placement.

In conclusion, the results of the present series support the thesis that the presence of a lesion within 2 cm of the cricopharyngeal muscle should no longer be considered a contraindication for the palliative or temporary use of an endoprosthesis.

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RAPID COMMUNICATION

Local regulator adrenomedullin contributes to the circulatory disturbance in cirrhotic rats

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Abstract

AIM: To investigate whether adrenomedullin, a potent vasodilator peptide, plays a role in the circulatory disturbance in cirrhosis.

METHODS: Cirrhosis was induced in rats by weekly gavage of carbon tetrachloride. Hemodynamic studies were performed *in vivo* using radioactive microspheres and *in vitro* using isolated aortic rings. The adrenomedullin concentrations were measured by radioimmunoassay.

RESULTS: Acute administration of adrenomedullin to the control rats reduced the systemic arterial pressure along with an increase of serum levels of the stable metabolite of nitric oxide (NOx), in a dose-dependent manner. Chronic infusion of adrenomedullin reduced the vascular resistance and increased the blood flow in the systemic and splanchnic circulation. Intravenous administration of anti-adrenomedullin antibody did not affect any hemodynamic parameters in the cirrhotic rats, whereas this antibody ameliorated the blunted contractile response to phenylephrine, α-adrenergic receptor agonist, in the aortic rings of the cirrhotic rats. The adrenomedullin concentrations in the aorta were higher in the cirrhotic rats than in the controls, and correlated with the mean arterial pressure in the cirrhotic rats. Moreover, adrenomedullin blunted the contractile response to phenylephrine in both of the control aorta and cirrhotic aorta, but not in the presence of NG-nitro-L-arginine methyl ester, an NO synthase inhibitor.

CONCLUSION: Adrenomedullin overproduced in the vascular wall may contribute to the circulatory disturbance in cirrhosis as a local regulator of the vascular tonus rather than a circulating hormone.

Key words: Adrenomedullin; Liver cirrhosis; Vasodilation; Circulatory disturbance; Vascular tonus; Circulating hormone

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INTRODUCTION

Arterial hypotension, high cardiac output, low vascular resistance, and hyporeactivity to vasoconstrictors are hemodynamic features in human and experimental liver cirrhosis[1-3]. These circulatory disturbances may be attributed to arterial vasodilation that results from overproduction or reduced degradation of vasodilator substances[1-3]. Several circulating vasodilator peptides including substance P, calcitonin gene-related peptide, and glucagon are increased in the cirrhotic patients[4-6]. Moreover, many studies have suggested that the vasodilator substances produced in the vascular wall such as nitric oxide (NO) and carbon monoxide, an end product of the haeme oxygenase pathway, may be coordinately associated with arterial vasodilation in cirrhosis[7-9]. Therefore, the high levels of the circulating vasodilator peptides and the increased vascular production of vasodilators can contribute to the pathogenesis of arterial vasodilation leading to the circulatory disturbance in liver cirrhosis.

Adrenomedullin (AM) is the potent hypotensive peptide discovered in the human pheochromocytoma, and is considered to cause a potent vasodilation via synthesis of NO in the vascular endothelial cells as well as an increase of the intracellular adenosine 3',5'-cyclic monophosphate in the vascular smooth muscle cells[10-11]. AM is abundant in the adrenal medulla, but is widely distributed in the human and rat organs including the vascular tissue[12,13]. Several clinical studies have

demonstrated that the circulating AM levels are increased along with progression of the liver disease and correlate with the hemodynamic parameters in cirrhosis[14-17]. Moreover, the gene expression of AM in the vascular tissue was more enhanced in the cirrhotic rats than in the controls[18]. These findings raise the possibility that AM may be involved in the circulatory disturbance in cirrhosis. However, whether the increased circulating AM and/or the enhanced vascular production of AM plays a role in the circulatory disturbance in liver cirrhosis remains to be established. In this study, the role of the circulating AM in the hemodynamic derangement in cirrhosis was investigated in the presence of exogenous AM and/or anti-AM antibody using radioactive microspheres. Moreover, the AM concentrations in the aorta were evaluated in relationship to the systemic blood pressure, and the effect of AM on the vascular tonus was investigated in the presence of exogenous AM and/or anti-AM antibody using isolated aortic rings. This study aimed to investigate whether the increased circulating AM and/or the enhanced vascular production of AM plays a role in the circulatory disturbance in liver cirrhosis.

MATERIALS AND METHODS

Animal preparation

The experiments were performed on male Sprague-Dawley rats. Liver cirrhosis was induced by weekly intragastric administration of carbon tetrachloride[19]. The rats weighing about 150 g were given phenobarbital (35 mg/dL) in the drinking water. After 2 weeks when the rats were about 250 g, the initial dose (0.04 mL) of intragastric carbon tetrachloride was begun. Body weight was monitored and the dose of intragastric carbon tetrachloride was adjusted according to the change of body weight. After 8-10 doses of carbon tetrachloride, micronodular liver cirrhosis was induced in the most rats and the half of cirrhotic rats developed ascites. The control rats were treated with phenobarbital alone. All animals received humane care and all experiments were performed according to the guidelines of the Committee for the Care and Use of Laboratory Animals in Nara Medical University.

Hemodynamic studies

Hemodynamic studies were performed by the same operator to reduce the operator-dependent variability. Ketamine (100 mg/kg, i.m.) was used as an anesthetic drug, because it closely resembled the conscious state in terms of hemodynamics[20]. The left femoral artery, right jugular vein, femoral vein, and portal vein were cannulated with PE-50 catheters. The left ventricle was catheterized with another PE-50 tube. Each catheter used was filled with heparinized saline. The cardiac output and regional blood flow were measured using radioactive microspheres[21]. A reference sample was obtained from the femoral artery for 75 seconds at a rate of 1 mL/min using continuous withdrawal pump (CFV2100; Nihon Kohden, Tokyo, Japan). Approximately 60 000 microspheres labeled with ^{57}Co (15.5 ± 0.1 μm diameter, specific activity: 610 MBq/g; New England Nuclear, Boston, MA, USA) were injected into the left ventricle 15 seconds after the start of blood withdrawal. The cardiac output was calculated as follows: The cardiac output (mL/min) = injected radioactivity (cpm) × reference blood flow (mL/min) / reference blood radioactivity (cpm). The cardiac index was expressed per 100 g of body weight. The abdominal organs were cut into small pieces and placed in counting tubes. The radioactivity of each organ was determined with a gamma counter. For calculation of the regional blood flow, the injected radioactivity was replaced by radioactivity of each organ in the previous equation. The portal venous inflow was calculated as the sum of the blood flows to the stomach, spleen, small and large intestines, pancreas, and mesentery. The vascular resistance was calculated from the ratio between the perfusion pressure and the blood flow in each vascular territory. The hemodynamics were investigated as follows: 1) Control rats were infused with either vehicle (n = 6) or human AM (0.1, 0.3, 1.0 nmol/kg/min for 10 min, n = 6, respectively) via a femoral vein catheter. Another six control rats were given human AM at a dose of 0.3 nmol/kg/min for 10 min after an intravenous injection of anti-AM antibody (500 μg/kg). The mean arterial pressure and serum levels of NO× (NO_2^- + NO_3^-), a stable metabolite of NO, were determined before and after the infusion. 2) To compare the magnitude of depressor response of the exogenous AM, cirrhotic rats were also infused either vehicle (n = 6) or human AM (0.1, 0.3, 1.0 nmol/kg/min for 10 min, n = 6, respectively) and the mean arterial pressure was determined before and after the infusion. 3) Control rats were chronically infused with either vehicle (n = 8) or human AM (1.0 μg/h, n = 8) for 14 d using a mini-osmotic pump (alzet model 2002, Alza, CA, USA). The pumps were connected to the left jugular vein cannulated with PE-60 and placed in the subcutaneous tissue. The human AM dose was determined according to a previous study[22]. On d 14 of chronic infusion, hemodynamic studies were performed using radioactive microspheres. 4) Cirrhotic rats with ascites were repeatedly injected with either anti-AM antibody (500 μg/kg, n = 8) or vehicle (n = 8) via the tail vein on the day of, and 3 and 6 d after the development of ascites. Hemodynamic studies were performed on the day after the final injection.

Isolated aortic ring studies

On the day of the experiment, the thoracic aorta was removed from control rats and cirrhotic animals with ascites and cut into 3-mm rings. The rings were suspended between two triangular stainless steel stirrups in a 20-mL jacketed organ chamber containing modified Krebs-Henseleit solution (118 mmol/L NaCl, 4.6 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, 11.1 mmol/L glucose, 27.2 mmol/L $NaHCO_3$, 0.03 mmol/L Na_2 ethyle nediaminetetraacetic acid, 1.8 mmol/L $CaCl_2$) at 37 ℃ and bubbled with 950 mL/L O_2 and 50 mL/L CO_2. The lower stirrup was anchored and the upper stirrup was attached to a force-displacement transducer (TB-652T; Nihon Kohden) to record the isometric force. All rings were

stretched to generate a resting tension of 2 g, which was optimal for contractions of the aortic rings in response to phenylephrine, an α-adrenergic receptor agonist. After 1 h of equilibration, the presence of functional endothelium was determined by the addition of acetylcholine (10 μmol/L). All rings were rinsed and allowed to equilibrate for an additional 1 h in the presence of indomethacin (10 μmol/L) to prevent the influence of the endogenous prostanoids. The aortic rings of the control rats or cirrhotic animals with ascites ($n = 8$, respectively) were incubated for 30 min with anti-AM antibody (1 mg/L) or vehicle. Another control and cirrhotic rings were incubated with either human AM (100 nmol/L) or vehicle in the presence or absence of NG-nitro-L-arginine methyl ester (L-NAME) (30 μmol/L) ($n = 8$, respectively). Then, the cumulative dose-response curves to phenylephrine (1 nmol/L to 10 μmol/L) were evaluated. On completion, the rings were dried and weighed. The force of contraction was expressed as mg of contraction per mg of dried tissue. From each dose-response curve, the maximum response (Rmax) and phenylephrine concentration required for 50% of the maximum response (EC_{50}) were calculated with a nonlinear regression method using computerized curve-fitting software (StatView 5.0 program, Abacus Concept Inc., Berkeley, CA, USA), and were used to compare the contractility and reactivity of phenylephrine-induced contraction, respectively.

Measurements

The AM concentrations in the aorta were measured in the control rats and the cirrhotic animals with/without ascites ($n = 10$, respectively). The aorta was homogenized for 1 min in 10 volumes of 1 mol/L acetic acid and immediately heated at 100 ℃ for 10 min. The homogenates were centrifuged at 15 000 g for 10 minutes at 4 ℃. The supernatants were frozen until analyzed. The AM concentrations were measured by radioimmunoassay[18]. The protein concentrations were determined by Bradford's method[23]. Serum NOx levels were measured as previously described[24].

Chemicals

The human AM and anti-AM antibody were kindly supplied from Diagnostic Science Division, Shionogi & Co., Ltd. (Settsu, Japan). The anti-AM antibody which specifically binds to the C-terminal structure of AM and neutralize the effect of AM, belongs to immunoglobulin G1 subclass and equally cross-reacts with the rat AM [1-50], but not with calcitonin gene-related peptide or amylin[25]. The mouse IgG, KCl, acetylcholine, L-NAME, indomethacin, and phenylephrine were purchased from Sigma Chemical (St. Louis, MO, USA).

Statistic analysis

All analyses were performed with StatView 5.0 program (Abacus Concept Inc.). Comparisons were made using the two-tailed Student's t test for quantitative variables. All data are expressed as mean ± SE. $P < 0.05$ was considered statistically significant.

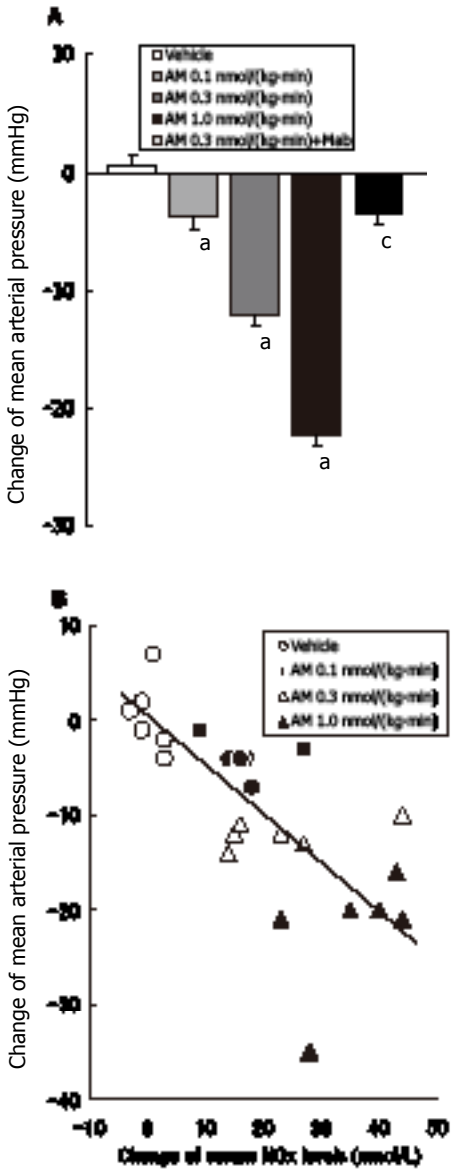

Figure 1 Changes of the mean arterial pressure and serum NOx levels by acute administration of exogenous adrenomedullin. **A**: Arterial pressure; $^{a}P < 0.05$ *vs* vehicle; $^{c}P < 0.05$ *vs* AM 0.3 nmol/(kg·min); **B**: serum NOx levels. mean ± SE. $r = -0.72$, $P < 0.05$, $n = 6$.

RESULTS

Acute administration of exogenous AM

Acute administration of human AM reduced the systemic arterial pressure in the control rats in a dose-dependent manner (Figure 1A, the changes of the mean arterial pressure, vehicle: 0.5 ± 1.6 kPa, AM 0.1 nmol/(kg·min): -3.8 ± 0.8 kPa, $P < 0.05$ *vs* vehicle, AM 0.3 nmol/(kg·min): -12.0 ± 0.6 kPa, $P < 0.05$ *vs* vehicle, AM 1.0 nmol/(kg·min): -22.2 ± 2.7 kPa, $P < 0.05$ *vs* vehicle, respectively). The changes of the mean arterial pressure by AM infusion (0.3 nmol /(kg·min)) were abolished by the pre-treatment with anti-AM antibody (-3.5 ± 0.6 kPa, $P < 0.05$). Moreover, AM infusion increased serum NOx levels in a dose-dependent manner, and the changes of serum NOx levels

Table 1 Hemodynamic effects of chronic administration of adrenomdullin in control rats

	Vehicle ($n=8$)	AM ($n=8$)	P value
Mean arterial pressure (mmHg)	132±3	122±4	<0.05
Cardiac index (mL/min·100g bw)	26.0±2.1	34.1±2.9	<0.05
Systemic vascular resistance (mmHg·min·100g bw/mL)	5.3±0.4	3.8±0.4	<0.05
Portal pressure (mmHg)	6.9±0.5	7.0±0.5	NS
Portal venous inflow (mL/min·100g bw)	3.5±0.4	5.0±0.6	<0.05
Portal venous system resistance (mmHg·min·100g bw/mL)	2.1±0.3	1.6±0.3	NS
Splanchnic arterial resistance (mmHg·min·100g bw/mL)	38.5±4.1	25.7±4.0	<0.05

Control rats were chronically infused with either vehicle or human adrenomedullin (AM) (1.0 mg/h) for 14 d using mini-osmotic pump. On day 14 of chronic infusion, hemodynamic study was performed using radioactive microspheres. Values are presented as mean±SE of 8 separate experiments.

Table 2 Effects of anti-adrenomedullin antibody on hemodynamics and aortic ring contraction of cirrhotic rats.

	Vehicle	Anti-AM antibody
Hemodynamic effects		
Mean arterial pressure (mmHg)	117 ± 3	121 ± 4
Cardiac index (mL/min·100g bw)	39.5 ± 2.9	38.2 ± 3.8
Systemic vascular resistance (mmHg·min·100g bw/mL)	3.1±0.4	3.4±0.4
Portal pressure (mmHg)	13.0 ± 0.4	12.8 ± 0.5
Portal venous inflow (mL/min·100g bw)	6.8 ± 0.8	5.6 ± 1.0
Portal venous system resistance (mmHg·min·100g bw/mL)	2.2 ± 0.3	2.6 ± 0.4
Aortic ring contraction		
Rmax (mg/mg tissue)	998 ± 96	1499 ± 137a
EC50 (nmol/L)	85.7 ± 11.8	53.8 ± 6.2

Cirrhotic rats with ascites were repeatedly injected either vehicle or anti-adrenomedullin (anti-AM) antibody [500 mg/(kg·times)] via tail vein on the day of occurrence of ascites, 3 and 6 d after. Hemodynamic study was performed on the next day of the final injection. Values are presented as mean±SE of 8 separate experiments. The contraction of aortic rings to phenylephrine was evaluated in the presence of vehicle or anti-AM antibody. Values are presented as mean±SE of 8 rings. Rmax: maximal contraction to phenylephrine, EC_{50}: phenylephrine concentration required for 50% of Rmax, $^aP<0.05$ *vs* vehicle.

by AM infusion negatively correlated with the changes of the mean arterial pressure (Figure 1B, $r=-0.72$, $P<0.05$). Exogenous AM reduced the systemic arterial pressure in the cirrhotic rats, as well (vehicle: -0.5±1.8 kPa, AM 0.1 nmol/(kg·min): 0.3±1.9 kPa, AM 0.3 nmol/(kg·min): -4.0±0.6 kPa, AM 1.0 nmol/(kg·min): -13.2±1.7 kPa, $P<0.05$ *vs* vehicle, respectively), but the magnitude of depressor response in the systemic arterial pressure was lower in the cirrhotic rats than in the controls.

Chronic administration of exogenous AM

In agreement with the results of acute administration, chronic administration of exogenous AM caused systemic hypotension as compared with vehicle infusion (Table 1). Chronic infusion of AM increased the cardiac index and reduced the systemic vascular resistance as compared with vehicle infusion. Moreover, chronic AM infusion increased the portal venous inflow and reduced the splanchnic arterial resistance as compared with vehicle infusion. The portal pressure and portal venous system resistance were unchanged by chronic AM infusion.

Effects of anti-AM antibody on hemodynamics and vascular tonus in cirrhotic rats

To evaluate whether the circulating endogenous AM is associated with the circulatory disturbance in cirrhosis, the effects of anti-AM antibody on the hemodynamics were investigated in cirrhotic rats with ascites (Table 2A). Despite the repeated administration of anti-AM antibody that neutralizes the circulating AM, the systemic and splanchnic circulations of the cirrhotic rats were both unchanged. To evaluate whether the endogenous AM in the vascular tissue plays a role in the vascular tonus in the cirrhotic rats, the effects of anti-AM antibody on the phenylephrine-induced contractile response of the control and cirrhotic aortas were evaluated. In the cirrhotic aorta, the anti-AM antibody enhanced the contractility of the phenylephrine-induced contraction without affecting the reactivity as compared with vehicle-treatment (Table 2B). On the other hand, this antibody did affect the contractile response of the control aortas as compared with vehicle.

AM concentrations in the aorta

The AM concentrations in the aorta were higher in the cirrhotic rats than in the controls (Figure 2A, 21.9±2.3 *vs* 12.9±1.2 fmol/mg, $P<0.05$). The cirrhotic rats with

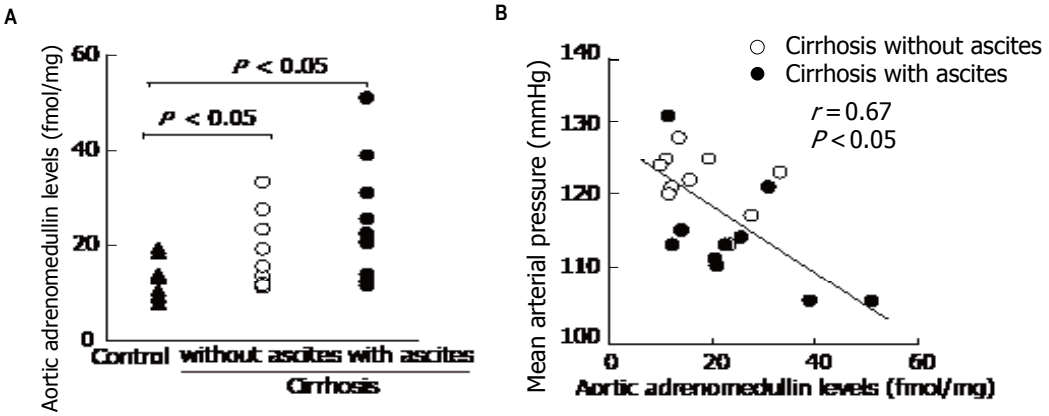

Figure 2 Adrenomedullin concentrations in the aorta in the control and cirrhotic rats (**A**) and their relation with the mean arterial pressure (**B**). ▲ : Control rats, ○ : Cirrhotic rats without ascites, ●: Cirrhotic rats with ascites. mean ± SE. $n = 10$.

Table 3 Contractile response of control and cirrhotic aortic rings to adrenomedullin and/or N^G-nitro-L-arginine methyl ester

	Control		Liver cirrhosis	
	Rmax (mg/mg tissue)	EC_{50} (nmol/L)	Rmax (mg/mg tissue)	EC_{50} (nmol/L)
Vehicle	1860±152	68.8±9.4	998±96[a]	85.7±11.8
AM	1330±118[a]	82.7±12.3	698±94[c]	91.0±12.8
L-NAME	2362±182[a]	61.8±7.1	2274±148[c]	72.1±6.7
L-NAME+AM	2242±91[a]	55.7±9.5	2092±219[c]	53.8±6.2

The contraction of aortic rings to phenylephrine was evaluated in the presence of vehicle or anti-AM antibody. Values are presented as mean±SE of 8 rings. Rmax: maximal contraction to phenylephrine, EC^{50}: phenylephrine concentration required for 50% of Rmax, [a]$P < 0.05$ *vs* vehicle-treated control aorta, [c]$P < 0.05$ *vs* vehicle-treated cirrhotic aorta. AM, adrenomedullin; L-NAME, N^G-nitro-L-arginine methyl ester.

ascites showed the highest aorta AM concentration (24.8 ± 4.0 fmol/mg) which was approximately two-fold increased as compared with that in the controls. Moreover, the AM concentrations in the aorta negatively correlated with the mean arterial pressure in the cirrhotic rats ($r = -0.67$, $P < 0.05$, Figure 2B).

Interaction of AM with NO in phenylephrine-induced contraction of the aortic rings

To investigate the interaction between AM and NO on the vascular tonus, the effect of AM on phenylephrine-induced contraction of the aortic rings was examined in the presence or absence of L-NAME (Table 3). AM-treatment reduced the contractility of the aorta as compared with vehicle-treatment in both of the control and cirrhotic aortas (Rmax: control; 1330 ± 118 *vs* 1860 ± 152 mg/mg tissue, $P < 0.05$, cirrhosis; 698 ± 94 *vs* 998 ± 96 mg/mg tissue, $P < 0.05$), whereas the reactivity showed no difference between AM-treatment and vehicle-treatment (EC_{50}: control; 82.7 ± 12.3 *vs* 68.8 ± 9.4 nmol/L, cirrhosis; 91.0 ± 12.8 *vs* 85.7 ± 11.8 nmol/L). L-NAME potentiated the contractility of both the control and cirrhotic aorta as compared with vehicle without affecting the reactivity (Rmax: control; 2362 ± 182 mg/mg tissue, $P < 0.05$, cirrhosis; 2274 ± 148 mg/mg tissue, $P < 0.05$, EC_{50}: control; 61.8 ± 7.1 nmol/L, cirrhosis; 72.1 ± 6.7 nmol/L). When the aortic rings were pre-treated with L-NAME, AM had no effects on the contractile response of both the control and cirrhotic rings (Rmax: control; 2242 ± 91 mg/mg tissue, cirrhosis; 2092 ± 219 mg/mg tissue, EC_{50}: control; 55.7 ± 9.5 nmol/L, cirrhosis; 53.8 ± 6.2 nmol/L).

DISCUSSION

Arterial vasodilation leading to a low systemic vascular resistance is the most outstanding hemodynamic alteration in the human and murine liver cirrhosis[1-3]. This vasodilation has been suggested to be a major pathogenic mechanism for hyperdynamic circulation characterized by arterial hypotension, hypervolemia, and high cardiac output[1-3]. Although the precise mechanism of the vasodilation in liver cirrhosis remains unknown, the overproduction or reduced degradation of endogenous vasodilators may play a major role. AM, a potent vasodilator peptide, is overproduced by various stimuli including endotoxin, cytokines, vasoactive substances and/or shear stress[26,27], which are enhanced in liver cirrhosis[28,29]. Moreover, the circulating AM levels and the gene expression of AM in the vascular tissues are both elevated in the human and/or murine liver cirrhosis[14-18]. These findings indicate that AM may be implicated in the circulatory disturbance in liver cirrhosis. The aim of this study was to investigate whether the increased circulating AM and/or the enhanced vascular production of AM plays a role in the circulatory disturbance in liver cirrhosis.

Acute administration of exogenous AM to control rats reduced the systemic arterial pressure along with an increase of serum NOx levels in a dose dependent manner, and

the changes of serum NOx levels correlated with the changes of the mean arterial pressure. Moreover, chronic infusion of AM (1.0 μg/h for 14 d) which keeps plasma human AM concentrations within the physiological limit[22] reduced the vascular resistance and increased the blood flow in both of the systemic and splanchnic circulations. These results, together with the existence of AM receptors in the vascular endothelial cells[11], suggest that AM may cause arterial vasodilation via NO synthesis in the vascular wall and lead to a hemodynamic alteration resembling liver cirrhosis.

Whether the circulating endogenous AM is associated with the circulatory disturbance in liver cirrhosis remains to be established. In this study, we used the anti-AM antibody to abolish the effect of the circulating endogenous AM. This antibody possesses an extremely potent neutralizing potency against AM[25], which may allow us to elucidate the role of the endogenous AM in the circulatory disturbance in cirrhosis. Five hundred μg/kg of the anti-AM antibody abolished hypotension following AM infusion at the dose of 0.3 nmol/kg/min for 10 min. Our previous study showed that plasma concentrations of AM in the cirrhotic rats with ascites were 19.2 ± 5.4 fmol/mL[18]. Therefore, the dose of anti-AM used in this study corresponds to that to neutralize the 200-fold of the circulating AM levels of the cirrhotic rats, indicating the enough dose to neutralize the effect of the circulating AM. However, this neutralizing antibody did not affect any hemodynamic parameters in the cirrhotic rats. Moreover, most of the circulating AM is reportedly occupied by glycine-extended AM, an inactive intermediate form of AM, and reflects the process of AM production in the tissue[30]. Therefore circulating endogenous AM may not play a significant role in the circulatory disturbance of liver cirrhosis.

There are no significant difference among the AM levels in plasma samples obtained from the hepatic vein, renal vein, pulmonary artery and femoral artery in cirrhotic patients[15]. The lack of significant arterio-venous difference in the AM levels in various vascular territories suggests that the increased plasma AM levels in cirrhotic patients do not result from an increased production in a specific organ. In this study, the AM concentrations in the aorta were more enhanced in cirrhotic rats than in the controls, and correlated with the mean arterial pressure in the cirrhotic rats. It is, therefore, possible that the endogenous AM contributes to the circulatory disturbance in cirrhosis as a paracrine and/or autocrine regulator of the vascular tonus rather than a circulating hormone. To investigate the role of the endogenous AM in the vascular tonus of cirrhotic rats, we performed isolated aortic ring studies. The cirrhotic aorta showed a blunted contractile response to phenylephrine as compared with the controls. The anti-AM antibody ameliorated the blunted contractile response in the cirrhotic aorta, but did not affect the contraction of the control aorta. This finding, together with the increased AM concentrations in the cirrhotic aorta, indicates that the endogenous AM in the aorta may play a role in the vascular tonus in liver cirrhosis.

It is widely recognized that NO plays a major role in the blunted vascular tonus in cirrhosis[31,32]. Moreover, AM and NO stimulate the synthesis and secretion of each other[11,33]. We, therefore, investigated the interaction between AM and NO in the contractile response of the cirrhotic aorta. In both of the control and cirrhotic aortas, AM reduced the contractile response, but not in the presence of NO synthase inhibitor. Prostanoids are unlikely to explain the blunted contractile response caused by AM, because sufficient indomethacin was used to inhibit cyclooxgenase. These findings, together with an increase in serum NOx levels by the exogenous AM infusion, indicate that AM overproduced in the vascular wall of cirrhotic rats may regulate the vascular tonus via NO synthesis in a paracrine and/or autocrine manner. In liver cirrhosis, an increase in hepatic vascular resistance is the initial phenomenon leading to portal hypertension. This is primarily due to the structural distortion of the hepatic microcirculation caused by cirrhosis, but is also associated with a deficient NO production in the liver, which results in an increased hepatic vascular resistance in contrast to an increased portal inflow via a vasodilation in pre-hepatic (splanchnic) vessels[34]. Considering the interaction between AM and NO, AM may be associated with portal hypertension through an imbalance of the NO production in the hepatic and pre-hepatic vessels.

Interestingly, the magnitude of hemodynamic alteration caused by AM infusion to the control rats was less than that in the cirrhotic animals with ascites, despite the similar hypotension in both groups. It has been considered that the circulatory disturbance in cirrhosis is initially caused by arterial vasodilation and thereafter promoted by an increase in the blood volume resulting from the impaired water and sodium excretion by activation of the endogenous vasoconstrictive systems[1-3]. Because the chronic infusion of AM suppresses the renin activity and aldosterone concentration[22,35], the lack of activation of the vasoconstrictor system may result in a less hemodynamic alteration in the AM-infused rats as compared with the cirrhotic animals. This finding provides further support for the concept that activation of the vasoconstrictive system as well as arterial vasodilation is essential for the circulatory disturbance in liver cirrhosis.

In conclusion, the AM concentrations in the aorta were elevated and negatively correlated with the systemic arterial pressure in the cirrhotic rats. Anti-AM antibody ameliorated the blunted contractile response of the cirrhotic aorta, whereas neutralization of circulating AM by anti-AM antibody did not affect the hemodynamic parameters in cirrhosis. These findings indicate that increased AM production in vascular tissue may contribute to the circulatory disturbance in cirrhosis, acting as a local regulator of the vascular tonus rather than a circulating hormone.

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RAPID COMMUNICATION

Circadian variation in expression of G_1 phase cyclins D_1 and E and cyclin-dependent kinase inhibitors p16 and p21 in human bowel mucosa

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Abstract

AIM: To evaluate whether the cellular proliferation rate in the large bowel epithelial cells is characterized by circadian rhythm.

METHODS: Between January 2003 and December 2004, twenty patients who were diagnosed as suffering from primary, resectable, non-metastatic adenocarcinoma of the lower rectum, infiltrating the sphincter mechanism, underwent abdominoperineal resection, total mesorectal excision and permanent left iliac colostomy. In formalin-fixed and paraffin-embedded biopsy specimens obtained from the colostomy mucosa every six hours (00:00, 06:00, 12:00, 18:00 and 24:00), we studied the expression of G_1 phase cyclins (D_1 and E) as well as the expression of the G_1 phase cyclin-dependent kinase (CDK) inhibitors p16 and p21 as indicators of cell cycle progression in colonic epithelial cells using immunohistochemical methods.

RESULTS: The expression of both cyclins showed a similar circadian fashion obtaining their lowest and highest values at 00:00 and 18:00, respectively ($P < 0.001$). A circadian rhythm in the expression of CDK inhibitor proteins p16 and p21 was also observed, with the lowest levels obtained at 12:00 and 18:00 ($P < 0.001$), respectively. When the complexes cyclins D_1-p21 and E-p21 were examined, the expression of the cyclins was adversely correlated to the p21 expression throughout the day. When the complexes the cyclins D_1-p16 and E-p16 were examined, high levels of p16 expression were correlated to low levels of cyclin expression at 00:00, 06:00 and 24:00. Meanwhile, the highest expression levels of both cyclins were correlated to high levels of p16 expression at 18:00.

CONCLUSION: Colonic epithelial cells seem to enter the G_1 phase of the cell cycle during afternoon (between 12:00 and 18:00) with the highest rates obtained at 18:00. From a clinical point of view, the present results suggest that G_1-phase specific anticancer therapies in afternoon might maximize their anti-tumor effect while minimizing toxicity.

Key words: G1 phase proteins; CDK inhibitors; Cell proliferation; Circadian rhythm

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INTRODUCTION

Eukaryotic cell division occurs in four phases of the cell cycle. The cells are prepared for DNA replication in G_1-phase. DNA is replicated during the S phase, a gap (G_2) period which allows preparation for mitosis before chromosome segregation and cytokinesis in M phase (mitosis). During development, differentiation, or growth factor withdrawal, cells can enter an inactive period (G_0-phase) before returning to G_1-phase[1].

Cyclins represent important regulators of cell cycle process. There are at least 15 distinct cyclin genes in the human genome that fall into three categories, and each category regulates specific passage through the cell cycle[2,3]. Passage through G_1 to S phase is regulated by cyclins C,

regulated by cyclins A and B$_{1-2}$[4-7]. Their levels in cell cytoplasm increase or decrease depending on the stage of cell cycle[2, 3, 8, 9].Elevated nuclear expression of cyclins favour the progression from one phase to the next while their low expression decelerates this progression[2-4, 7].

Cell cycle progression is mediated by the activation of a family of protein kinases, known as cyclin-dependent kinases (CDKs). CDKs constitute a large family of proteins, which act in a variety of key regulatory pathways, including control over the cell cycle and gene transcription[2, 9]. They are also divided in G$_1$-phase CDK (CDK4), S-phase CDK (CDK2) and M-phase CDK (CDK1)[8, 9]. Their levels in the cells remain fairly stable, but each must bind to the appropriate cyclin (whose levels fluctuate) in order to be activated[3, 8]. Cyclin D$_1$ (an early G$_1$-phase cyclin) mainly binds to and activates CDK4 and CDK6[8, 10] while cyclin E (a late G$_1$-phase cyclin) binds to and activates CDK2[8, 10].

CDK inhibitors inhibit the passage through the various phases of the cell cycle[11, 12]. The CDK inhibitors p16 and p21 protein families mediate regulation of cyclin/CDK activity[11, 12]. p16 family contains four members and inhibits CDK4 and CDK6, forming binary complexes with CDK4 *in vitro*[11, 12]. p21 family contains three members and interacts with both cyclin D and E complexes in G$_1$-phase and preferentially inhibits CDK2 activity[10].

In mammals, physiological (e.g. cardiac rhythm) or biochemical (e.g. hormone levels) processes vary in a regular and predictable periodic manner, with respect to the time of the day, which is called endogenous circadian rhythm[13, 14]. At the cellular level, each cell goes through the cell cycle in an orderly and controlled fashion, where the multiple steps associated with each phase should be successfully completed before progressing to the next phase[15]. At the tissue level, experimental[10, 16, 17] and clinical[18-26] studies suggest that a greater proportion of cycling cells in a specific organ, enters S-phase and mitosis at specific times of the day.

The present study was designed to evaluate whether the cellular proliferation activity in the large bowel epithelial cells shows variation over the 24 hours of a day and if this variation is characterized by circadian rhythm. As indicators for cellular proliferation activity we examined the quantitative expression of cyclins D$_1$ and E, as well as the inhibitor proteins p16 and p21 in biopsy specimens taken from the normal bowel mucosa of a permanent colostomy at six-hour intervals.

MATERIALS AND METHODS

Patients

Between January 2003 and December 2004, eighty-six patients suffering from colon and rectal cancer were surgically treated in our department. Among them, there were twenty patients (twelve men and eight women, median age 67 years, interquartile range 55-78 years) who were diagnosed as suffering from adenocarcinoma of the lower rectum infiltrating the sphincter mechanism. In all patients, the preoperative and intraoperative staging work-up revealed primary, resectable, non-metastatic lower rectal cancer. In order to achieve an oncological procedure, all patients underwent abdominoperineal resection, total mesorectal excision and permanent left iliac colostomy. All patients were operated on electively and neither neoadjuvant nor adjuvant chemo-radiotherapy was administered during the period of the study.

Biopsies

Biopsy specimens of bowel mucosa were obtained from the site of colostomy of these twenty patients undergone an abdominoperineal resection. Biopsies were collected at the time when the bowel retained its normal function after the operation and the colostomy was fully functional. Patients received the usual in-hospital low fibre diet. In order to avoid disturbance of cell proliferation, no enemas, bowel preparations and paraffin oil were used[18, 25]. All patients followed their usual sleep schedule (sleeping between 22:00 and 07:00) without receiving any sedatives[18, 19, 22, 25]. Their sleep pattern was interrupted only at the time when the colostomy specimens were taken. Specimens of 2-4 mm^2 were collected every 6 hours from the colostomy mucosa, approximately 3 cm lower to the colostomy orifice, using biopsy miniforceps. The examinations were performed at 00:00, 06:00, 12:00, 18:00 and 24:00. The biopsies were fixed in 10% buffered formalin and embedded in paraffin wax using conventional techniques.

Immunohistochemistry

Immunostainings for cyclins and CDK inhibitors were performed using mouse monoclonal antibody for cyclin D$_1$, rabbit polyclonal antibody for cyclin E, mouse monoclonal antibody for p16 protein and mouse monoclonal antibody for p21protein (Santa Cruz Biochemicals, Santa Cruz, California, USA) with a Vectastain Elite ABC-peroxidase kit (Vector Laboratories, Peterborough, United Kingdom) and a liquid DAB substrate-chromogen system (DAKO, Glostrup, Denmark) according to the manufacturer's instructions. An additional step of antigen retrieval (citrate buffer at pH 6.1 and microwave heating) was performed before antibody incubation for p21. The sections were counterstained with hematoxylin (Merck, Darmstadt, Germany). The percentage of positively stained cells in immunohistochemistry experiments was obtained by counting epithelial cells in each case by two independent observers.

Statistical analysis

All graphics were constructed using Microsoft Excel for Windows XT Professional. Statistical differences between the groups were determined by the Student's *t*-test. $P < 0.05$ was considered statistically significant. All statistical calculations were performed using the STATA statistical package (StataQuest Version 4.0, College Station, Texas, USA, 1995).

RESULTS

The median and interquartile range (IR) values of the percentage of the positively stained cells in immunohistochemistry for every single protein examined throughout the day of the present study, are presented in Table 1. The observed differences in the expression of the studied proteins between 00:00 and 24:00, could be explained by the fact that more bowel mucosa epithelial cells entered the

Table 1 Percentage of stained cells in immunohistochemistry for cyclin D1, cyclin E, protein p16 and protein p21 expression in bowel mucosa specimens

TIME	Cyclin D1 (Median + IR)	Cyclin E (Median + IR)	p16 (Median + IR)	p21 (Median + IR)
00:00	6 (4 - 8)	4 (2.75 - 5)	13 (11 - 15.25)	14 (10.75 - 16.25)
06:00	7 (5 - 8)	5.5 (5 - 7)	10 (8 - 11.25)	10 (8 - 11)
12:00	8 (6 - 9)	8.5 (7.75 - 10)	8 (6.75 - 8.25)	8 (6.75 - 8)
18:00	13 (11 - 15)	13.5 (11.75 - 16)	13.5 (9 - 15.25)	6 (5 - 7.25)
24:00	8 (6.75 - 10)	8 (6 - 9.25)	15 (12.75 - 16)	14 (11 - 15)

IR: Interguartile range.

proliferative activity as time passed.

The interindividual values for cyclin D_1 expression varied between 3% and 19%. Its expression gradually increased between 00:00 and 18:00 and gradually decreased between 18:00 and 24:00, obtaining the lowest and highest values at 00:00 and 18:00, respectively. The differences between the highest values of cyclin D_1 expression at 18:00, as compared to the values of the remaining examined periods, were highly statistically significant ($P < 0.001$).

The inter-individual values for cyclin E expression varied between 1% and 17%. Its expression gradually increased between 00:00 and 18:00 and then gradually decreased, obtaining its lowest and highest values at 00:00 and 18:00, respectively. Similarly to cyclin D_1, the differences between the values of cyclin E expression at 18:00, as compared to the values of the remaining examined periods, were highly statistically significant ($P < 0.001$).

The inter-individual values for the inhibitor protein p16 expression varied between 4% and 19%. Its expression gradually decreased between 00:00 and 12:00 and gradually increased between 12:00 and 24:00, obtaining its lowest and highest values at 12:00 and 24:00, respectively. The differences between the low values of p16 expression at 12:00, as compared to the higher values of the remaining examined periods, were highly statistically significant ($P < 0.001$).

The inter-individual values for the inhibitor protein p21 expression varied between 6% and 14%. Its expression gradually decreased between 00:00 and 18:00 and gradually increased between 18:00 and 24:00, obtaining its lowest values at 18:00 and its highest values at 00:00 and 24:00. The differences between the lowest values of p21 expression at 18:00, as compared to the values at 00:00, 06:00 and 24:00, were highly statistically significant ($P < 0.001$).

The present study concluded that expression of both cyclins showed circadian rhythm in a similar fashion. The higher levels of both proteins were obtained between 12:00 and 24:00 (highest at 18.00), while the lower levels were observed between 00:00 and 12:00 (lowest at 00:00).

A circadian rhythm in the expression of inhibitor proteins p16 and p21 was also observed. The lower levels of p16 expression were obtained between 06:00 and 12:00 (lowest at 12:00), while the lower levels of p21 expression were obtained between 12:00 and 18:00 (lowest at 18:00).

When the complexes of cyclins D_1 - p21 (Figure 1A) and E - p21 (Figure 1B) were examined, the present the expression of both cyclins was adversely correlated to the p21 expression throughout the day. The highest levels of cyclins were correlated to the lowest levels of p21.

When the complexes of cyclins D_1 - p16 (Figure 1C) and E - p16 (Figure 1D) were examined, high levels of p16 expression were correlated to low levels of cyclin expression at 00:00, 06:00 and 24:00. Meanwhile, the highest expression levels of both cyclins were correlated to high expression levels of p16 at 18:00.

DISCUSSION

How the circadian variation in proliferation at the tissue level relates to the control of the cell cycle, is a subject of continuous study[22]. The predictable association between certain cell cycle proteins and defined events during the cell cycle may be used to study the timing of cell cycle phases in normal tissues[22]. In malignant tissue, the expression level of cyclins and their inhibitors may have important prognostic and therapeutic implications, especially in the cell cycle phase-dependent toxicity of anticancer agents[20, 21].

Bucchi *et al*[18] are the first to investigate the presence or absence of rhythm in normal bowel mucosa proliferation. In their study, rectal biopsies were obtained every 2 or 3 h for 24 h using standard biopsy forceps during flexible sigmoidoscopy from 16 volunteers under fasting and fed conditions. Incorporation of [^{3}H] thymidine was measured in each specimen. Both fed and fasting subjects showed circadian variation in DNA synthesis in rectal mucosa that peaked at 07:00. Although thymidine incorporation fell during fasting, the circadian rhythm remained intact.

Marra *et al*[19] investigated the proliferation rhythm within the rectal crypt epithelium using the ^{3}H-thymidine autoradiographic method and calculated the ratio (labeling index) of the S-phase cells to total cells in the crypt. By taking biopsies every 4 h using standard biopsy forceps via rectoscopy from 23 normally fed subjects, they found a circadian rhythm in the labeling index with a peak at 01:28 in the morning. The base of the crypt and the upper 40% (which contains mainly differentiated cells) did not show circadian variation in the labeling index.

Brandi *et al*[25] studied the circadian variations of rectal cell proliferation in five patients by taking biopsies via proctoscopy from apparently normal mucosa 10 cm from the anal verge every 6h in a 24h period. Labeling index was evaluated as the percentage of labeled cells with respect to the whole cell population in the crypt. The results of the study suggest that rectal cell proliferation fluctuates during the day with the lower rates noted between 22:00 and 02:00.

By measuring the ratio of the S-phase cells to the whole cell population with the [^{3}H] thymidine technique, all previously mentioned studies[18, 19, 25] have demonstrated a rhythmicity in the rectal mucosa proliferation. Due to potential limitations of the [^{3}H] thymidine technique [27], most recent studies on the cellular proliferation rhythm in different tissues, are focused on the immunohistochemically quantitative expression of phase specific proteins. In the present study we examined the quantitative expression of the G_1-phase specific cyclins D_1 and E and the CDK

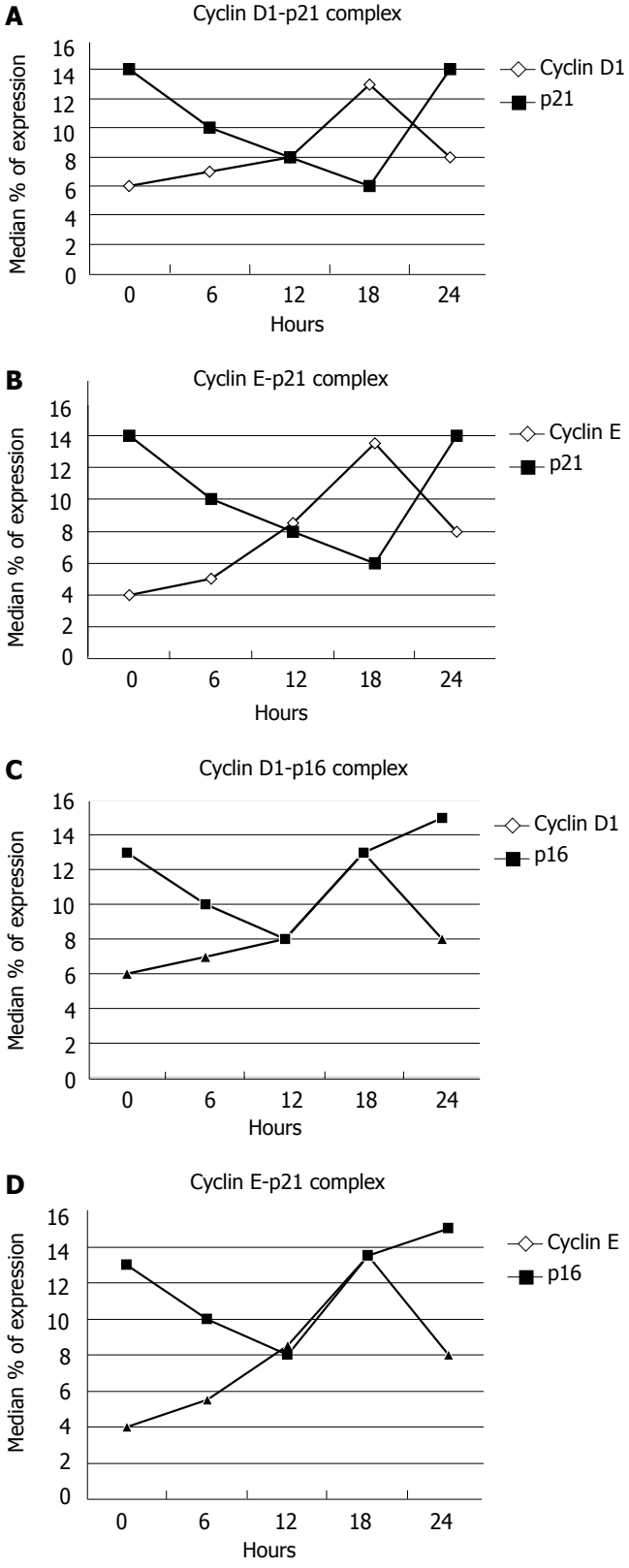

Figure 1 Graphical presentation of simultaneous quantitative expression of cyclin D1 and p21 (**A**), cyclin E and p21 (**B**), cyclin D1 and p16 (**C**), and cyclin E and p16 (**D**) in colostomy specimens sampled every 6 hours for 24 hours.

inhibitors p16 and p21 in normal large bowel mucosa.

Quantitative cyclin D_1 expression has been observed in normal bowel mucosa[28], dysplastic and non-dysplastic adenomatous polyps of the small and large bowel[18, 23, 29]. Its over-expression is related to adenocarcinoma of the colon and rectum (independently of the differentiation or Dukes' stage)[23], pancreatic[30], esophageal[30], endometrial[30], head and neck cancers[31].

Normal cells maintain strict control of cyclin E activity, while its deregulation plays a fundamental role in carcinogenesis. Cyclin E activities mainly consist of passage of cells through the restriction point "R" for cells entering the division from the resting state to G_1-phase[32]. Cyclin E functions not only as a S-phase entry regulator, but also plays a direct role in the initiation of DNA replication, by inducing S-phase specific genes[33, 34]. It is expressed higher than in many human tumors[35, 36] and the accumulation of cyclin E is considered a marker for the transition from adenoma to adenocarcinoma[37, 38].

p16 is a tumor suppressor gene and regulates cell proliferation by inhibiting CDK4 and CDK6 activities. Transient expression of p16 leads to inhibition of DNA synthesis by hypophosphorylating the retinoblastoma (Rb) gene protein[39-42]. The p16/Rb tumor suppressor pathway is frequently defective in many human tumors either by inactivating p16 or Rb or by over-expressing cyclin D_1 or CDK4. In cases of colorectal cancer, only a low frequency of p16 mutation has been found[39, 43]. Dai *et al*[44] hold that p16 expression begins in the earliest detectable stages of human colonic neoplasia and exerts a continuous constraint of tumor growth. A recent experimental study[45] showed that interaction of p16 expression and CDK4 may become a new prognostic marker in colorectal cancer. Tada *et al* [46] studied the possible role of p16 in the development of colonic neoplasms and found that p16 is overexpressed in 98% of adenocarcinomas and that colorectal cancer with reduced p16 expression is more aggressive in lymphatic infiltration.

The p21 protein family can interact with both cyclin and CDK subunits. Members of the p21 family interact with cyclins D and E during the G_1-phase of the cell cycle, preferentially inhibiting CDK2 activity and promoting assembly of cyclin D/CDK 4 complex *in vivo* and *in vitro*[47]. p21 constitutes the first molecule which is considered as a wild-type activated factor (WAF1) due to its upregulation by the tumor suppressor protein p53 and also as a cell-derived inhibitor of DNA synthesis[48]. Expression of p21 has been detected *in vivo* by immunohistochemistry in cells of upper crypts and lower villus. The above mentioned areas are associated with enterocyte differentiation[48] which is associated with a withdrawal from the cell cycle and the transcriptional activation of p21 either dependently or independently of the tumor suppressor p53[48-50].

The results of the present study demonstrated that the expression of both cyclins showed a similar circadian fashion, with the higher levels obtained between 12:00 and 24:00 (highest at 18.00) and the lower levels between 00:00 and 12:00 (lowest at 00:00). These findings partly support the theory for coordinating and cascading activity between them during the G_1 phase of the cell cycle [10]. As cyclin D_1 represents an early G_1 phase cyclin, while cyclin E constitutes a late G_1 phase cyclin, simultaneous increase and decrease of their expression during the same periods of time, require further investigation. A future study focusing on their expression at shorter intervals may disclose more accurately their fluctuation during the day. Circadian rhythm was also observed in the expression of

both inhibitor proteins, with the lowest values obtained at 12:00 and 18:00 for p16 and p21 expression, respectively. By comparing cyclins D_1-p21 and E-p21 complexes, the lowest expression levels of p21 and the highest expression levels of both cyclins were observed at 18:00. When the complexes of cyclins D_1-p16 and E-p16 were examined, the inhibitory action of p16 protein, successfully arrested the cell cycle during the night and early in the morning. Why high expression levels of p16 correlate to the highest expression levels of both cyclins at 18:00 remains unclear.

In conclusion, the expression of all examined parameters (which are involved in the progression from the G_1- to S-phase of the cell cycle) is characterized by circadian rhythm. Colonic epithelial cells seem to enter the G_1 phase of the cell cycle during afternoon between 12:00 and 18:00, because during that period the higher expression levels of cyclins D_1 and E correlate to the lower expression levels of the expression of CDK inhibitor proteins p16 and p21. From a clinical point of view, the present results suggest that G_1-phase specific anticancer therapies in afternoon might maximize their anti-tumor effect while minimizing toxicity[2, 3, 51-53]. Further studies on the accurate circadian rhythm in anatomically intact human colonic epithelium and malignant tissues are required.

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RAPID COMMUNICATION

Portal vein thrombosis: Prevalence, patient characteristics and lifetime risk: A population study based on 23 796 consecutive autopsies

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Abstract

AIM: To assess the lifetime cumulative incidence of portal venous thrombosis (PVT) in the general population.

METHODS: Between 1970 and 1982, 23 796 autopsies, representing 84% of all in-hospital deaths in the Malmö city population, were performed, using a standardised protocol including examination of the portal vein. PVT patients were characterised and the PVT prevalence at autopsy, an expression of life-time cumulative incidence, assessed in high-risk disease categories and expressed in terms of odds ratios and 95% CI.

RESULTS: The population prevalence of PVT was 1.0%. Of the 254 patients with PVT 28% had cirrhosis, 23% primary and 44% secondary hepatobiliary malignancy, 10% major abdominal infectious or inflammatory disease and 3% had a myeloproliferative disorder. Patients with both cirrhosis and hepatic carcinoma had the highest PVT risk, OR 17.1 (95% CI 11.1-26.4). In 14% no cause was found; only a minority of them had developed portal-hypertension-related complications.

CONCLUSION: In this population-based study, PVT was found to be more common than indicated by previous clinical series. The markedly excess risk in cirrhosis and hepatic carcinoma should warrant an increased awareness in these patients for whom prospective studies of directed intervention might be considered.

Key words: Epidemiology; Venous thrombosis; Portal hypertension; Cirrhosis; Gastrointestinal cancer

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INTRODUCTION

Extrahepatic portal vein thrombosis (PVT) may be caused by a variety of conditions including cirrhosis, cancer and abdominal infectious and inflammatory processes. In the wake of thrombus organisation and vessel involution follows development of tortuous collaterals, so called cavernous transformation[1]. Symptom development is often insidious and related to the progression of portal hypertension. The concept of PVT as a rare disease is mainly based on clinical series and case reports[2-7]. Estimates of frequency and of distribution of etiological fractions vary widely between studies[2, 4,5,6, 8,9,10,11]. To some extent this may be explained by lack of precision in these small series, but a contribution of case selection and ascertainment bias should also be considered.

For several decades, the city of Malmö in southern Sweden has been a centre for epidemiological research, including clinical and autopsy-based studies of cardiovascular diseases[12,13,14,15]. Between 1970 and 1982, close to 24 000 autopsies, comprising 84% of all in-hospital deaths in the city, were performed. All procedures followed a standardised protocol including examination of the portal vein and its major tributaries. In consequence with the course in PVT, the prevalence at autopsy can be regarded as a proxy for the cumulative incidence during lifetime to develop PVT. The size of the cohort together with the high autopsy rate thus provides a unique opportunity to yield a robust estimate of risk in the general population. Further aims have been to evaluate likely causes to PVT and clinical characteristics related to portal hypertension, and to estimate the absolute risk of PVT in high-risk disease categories as characterised by autopsy findings.

MATERIALS AND METHODS

Study cohort

Malmö is a city with a single referral centre for post-

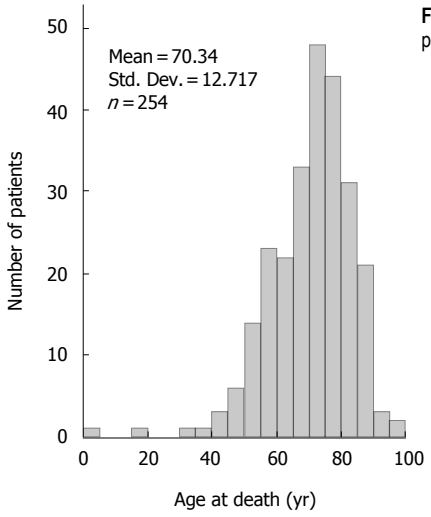

Figure 1 Age distribution of patients at death.

mortem examinations, the Department of Pathology at Malmö University Hospital. Between January 1, 1970 and December 31, 1982, when the city population declined from 264 000 to 230 000 inhabitants, 35 784 deaths occurred in the Malmö population. Among the 28 196 deaths occurring among in-patients at the three hospitals, which served the Malmö population during this period, a total of 23 796 clinical autopsies were performed, corresponding to an autopsy rate of 84%.

All autopsies were performed using a standardised protocol and carried out or supervised by senior pathologists. All findings were classified and coded according to the Standardised Nomenclature of Pathology (SNOP), as defined by the College of American Pathologists in 1965. The death certificates were issued by the pathologist. Based on the clinical picture and on autopsy findings, an underlying cause of death and up to six contributing causes were determined and classified using the ICD-8 code.

Classification of PVT patients in relation to potential causes

All cases with SNOP code 48-80-3700/3703, denoting *portal vein thrombosis* (*PVT*), were identified and validated individually. The following disease categories in the literature regarded as major causes of PVT were identified[5]:

-*cirrhosis,* where classification was based on SNOP codes 4850 - 4857 and/or cirrhosis as underlying or contributing death cause (ICD 8 code 571.00 - 571.99). PVT patients with cirrhosis were further classified according to presence or absence of *primary hepatic cancer* (hepatocellular carcinoma or intrahepatic cholangiocarcinoma);

-*primary hepatobiliary cancer,* comprising primary hepatic cancer (with or without cirrhosis), extrahepatic cholangiocarcinoma and gall bladder carcinoma;

-*secondary malignancy of the hepatobiliary region* (metastatic malignancy to the liver, biliary tract, gall bladder, duodenum or pancreas), further classified according to primary tumour location as expressed by SNOP code;

-*myeloproliferative disorders*: non-specific myeloproliferative disorder (SNOP code 7690); agnogenic myeloid metaplasia (7691); myelofibrosis (7692); histiocytic medullary reticulosis (7698); megablastic erythropoesis (7706); neutrophilic (7711) and eosinophilic (7713) leukocytosis; leukaemoid reaction (7714); erythrocytosis (7715); reticulocytosis (7716); lymphocytosis (7718); thrombocytosis (7719); erythrophagocytosis (7763); erythroid (7771), granulocytic (7772), reticular cell (7777) and megakaryocytic (7778) hyperplasia; polycythaemia vera rubra (7779); and chronic myeloic leukaemia (9867);

-*major abdominal infections and inflammations,* including liver abscess, abdominal abscess, purulent peritonitis, acute necrotising pancreatitis and ulcerative colitis.

In consequence with the possibility PVT being the result of more than one factor, a patient might be included in more than one of these categories. PVT patients in whom none of the above causes could be established were identified and analysed separately.

Classification of risk groups for PVT

PVT prevalence and risk, expressed as odds ratio, were assessed in relation to the following disease categories: *cirrhosis* (with and without primary hepatic cancer), *primary hepatobiliary cancer* (with and without cirrhosis), *secondary hepatic malignancy* (secondary malignancy of the hepatobiliary region not being readily definable in terms of SNOP codes) and *myeloproliferative disorders*. Presence of secondary hepatic malignancy was based on SNOP code 56-8006. Among these, patients with metastatic disease from pancreatic, gastric and colorectal carcinoma were further identified and similarly analysed. For the other disease categories, patients were classified using the definitions stated in the previous section.

Definition of clinical complications in PVT patients

In patients with PVT, the prevalence at autopsy of the following complications typically related to portal hypertension was ascertained and analysed in relation to disease categories: ascites, oesophageal varices and gastrointestinal bleeding.

Statistical analysis

Distributions of age at death were expressed in terms of means and variance and groups defined by presence or absence of potential causal factor compared with one-way analysis of variance (ANOVA). Proportions were compared using two-sided Fisher's exact test for univariate analyses. The PVT risks in relation to respective disease category were also expressed in terms of odds ratios with computation of 95% confidence intervals.

RESULTS

PVT prevalence and patient demographics

PVT was found at autopsy in 254 (1.1 %) of the 23 796 patients; in 125 (1.0 %) out of 12 157 men and 129 (1.1 %) of the 11 639 women ($P = 0.57$). The age distribution is depicted in Figure 1. With the exception of one patient, who was less than 1 year old, the age ranged between 17 and 96 years. Mean (95 % CI) age was 68 (66-70) years in

Table 1 Prevalence in patients with portal vein thrombosis (PVT) of conditions known to be major causes: Age- and gender distribution within categories

Patient category	*n* (%) of PVT patients	*n* (%) within risk category	Age (yr) Median	Mean (95% CI)	Female gender (%)
Cirrhosis [1]	72 (28)		70	68.5 (65.8 - 71.3)	28 (39)
with primary hepatic cancer		26 (36)			
without primary hepatic cancer		46 (64)			
Primary hepatobiliary cancer [1]	59 (23)		72	72.1 (69.4 - 74.8)	26 (44)
hepatic carcinoma [2]		38 (64)			
extrahepatic biliary / gall bladder carcinoma		21 (36)			
Secondary malignancy of the hepatobiliary region [1]	111 (44)		70	69.0 (66.7 - 71.2)	59 (52)
from pancreatic carcinoma		47 (42)			
gastric carcinoma		20 (18)			
colorectal carcinoma		11 (10)			
lung cancer		7 (6)			
malignant lymphoma		5 (4)			
mammary adenocarcinoma		4 (4)			
other primary cancer [3]		17 (15)[1]			
Myeloproliferative disorders [1]	7 (3)		72	65.4 (44.2 - 86.6)	5 (71)
Major abdominal infection / inflammation [1,4]	25 (10)		73	71.9 (66.5 - 77.3)	15 (60)
No cause identified	36 (14)		73	70.8 (65.9 - 75.7)	21 (57)
All patients with PVT	254 (100)		72	69.9 (68.3 - 71.4)	129 (51)

[1] categories not mutually exclusive. [2] hepatocellular carcinoma (26), intrahepatic cholangiocarcinoma (6), mixed (4), anaplastic (1) and unspecified (1) carcinoma. [3] malignant melanoma (3), female genital tract cancer (3), uroepithelial cancer (2), urinary bladder carcinoma (2), prostatic carcinoma (2), oesophageal cancer (1), duodenal cancer (1), renal cancer (1), adrenal cancer (1), sarcoma (1). [4] liver abscess (13), peritonitis (8), acute necrotising pancreatitis (4), abdominal abscess (2), ulcerative colitis (1) (more than one alternative possible).

Table 2 Prevalence and relative risk (odds ratio) of PVT in relation to disease condition with major risk of PVT

Patient category	*n* (%)	PVT (%)	O.R. (95% C.I.)[1]	*P* value	O.R. (95% C.I.)	*P* value
Cirrhosis	1193 (5.0)	72 (6.0)	7.9 (6.0 - 10.5)	< 0.001		
with primary hepatic cancer	182	26 (14.3)	17.1 (11.1 - 26.4)	< 0.001	3.5 (2.1 - 5.8)	< 0.001
without primary hepatic cancer	1011	46 (4.5)	5.2 (3.7 - 7.2)	< 0.001	1[2]	
Primary hepatobiliary cancer	698 (2.9)	59 (8.5)	10.8 (8.0 - 14.7)	< 0.001		
hepatic carcinoma	392	38 (9.7)	11.5 (8.0 - 16.5)	< 0.001		
with cirrhosis	182	26 (14.3)	17.1 (11.1 - 26.4)	< 0.001	2.8 (1.3 - 5.6)	0.004
without cirrhosis	210	12 (5.7)	5.8 (3.2 - 10.6)	< 0.001	1[2]	
extrahepatic biliary / gall bladder carcinoma	313	21 (6.7)	7.2 (4.5 - 11.4)	< 0.001		
Secondary hepatic malignancy						
from all tumours	3446 (14.5)	113 (3.3)	4.9 (3.8 - 6.2)	< 0.001		
from pancreatic carcinoma	312	36 (11.5)	13.9 (9.6 - 20.2)	< 0.001	5.2 (3.4 - 7.8)[3]	< 0.001[3]
gastric carcinoma	316	18 (5.7)	5.9 (3.6 - 9.7)	< 0.001	1.9 (1.2 - 3.2)[3]	0.019[3]
colorectal carcinoma	637	13 (2.0)	2.0 (1.1 - 3.5)	0.028	0.5 (0.3 - 1.0)[3]	0.063[3]
Myeloproliferative disorders	231 (1.0)	7 (3.0)	3.0 (1.4 - 6.3)	0.012		
All patients	23796 (100)	254 (1.0)				

[1] *vs.* all patients with absence of respective condition. [2] reference category. [3] *vs.* patients with liver metastatic disease from other cancer forms.

men and 71 (69 - 74) years in women. Median age at death was 72 years; 70 years in men and 74 years in women.

Distribution of potential causative factors in PVT patients

Seventy-two (28 %) of the PVT patients had cirrhosis, and one third of those had also primary hepatic cancer (Table 1). In all 59 PVT patients (23 %) had primary hepatobiliary cancer and 111 (44 %) had secondary malignancy of the hepatobiliary region. Major abdominal infectious or inflammatory disease was present in 25 (10 %), 7 (3 %) had myeloproliferative disorders, whereas in 36 (14 %) of the patients with PVT, none of the above causal factors could be identified. The age distribution in these categories was similar (Table 1). Sixty-one per cent of the PVT patients with cirrhosis were men ($P = 0.018$), otherwise there were no gender differences.

PVT prevalence and risk in relation to presence of disease

Cirrhosis was present in 5 % (1193 / 23796) of the cohort and related to a 7.9 times increased odds for PVT (95 % CI: 6.0-10.5) (Table 2). Patients who also had primary hepatic cancer had a 3.5 times higher odds for PVT (95 % CI 2.1-5.8) than cancer-free cirrhosis patients, with a PVT prevalence of more than 14 %. Secondary hepatic malignancy was associated with 4.9 times increased odds (95 %

Table 3 Portal hypertension-related complications in categories of PVT based on likely etiology

Patient category	*n*	*n*	Complication					
			Ascites		Oesophageal varices		Gastrointestinal bleeding	
			n (%)	*P* value[2]	*n* (%)	*P* value[2]	*n* (%)	*P* value[2]
Cirrhosis[1]	72		45 (62)	<0.001	42 (58)	<0.001	34 (47)	<0.001
with primary hepatic cancer		26	21 (81)		13 (50)		7 (27)	
without primary hepatic cancer		46	24 (52)		29 (63)		27 (59)	
Primary hepatobiliary cancer[1]	59		34 (58)	0.016	15 (25)	0.18	15 (25)	0.72
without cirrhosis		33	13 (39)		2 (6)		8 (24)	
Secondary hepatobiliary malignancy[1]	111		46 (41)	0.61	4 (4)	<0.001	15 (14)	0.002
Myeloproliferative disorders[1]	7		4 (57)	0.47	2 (29)	0.62	2 (29)	0.67
Major abdominal infection / inflammation[1]	25		12 (48)	0.67	1 (4)	0.06	5 (20)	0.81
No cause identified	36		6 (17)	<0.001	2 (6)	0.036	3 (8)	0.02
All patients with PVT	254		110 (43)		48 (19)		59 (25)	

[1] Categories not mutually exclusive; [2] in comparison with all PVT patients not belonging to category.

CI 3.8 - 6.2). Within this group, pancreatic cancer patients had a 6.7 times higher odds for PVT (95 % CI: 3.4 - 13.1) than patients with liver metastatic disease from colorectal cancer.

Complications related to portal hypertension

Among the patients with PVT, the highest rates of complications related to portal hypertension were found in the subgroup with concomitant cirrhosis: 62 % had developed ascites, 58 % had oesophageal varices and 47% terminal gastrointestinal bleeding (Table 3). Corresponding figures in all PVT patients were 43 %, 19 % and 25 % ($P<0.001$ for all three comparisons), whereas of the 36 patients where no cause of PVT could be identified at autopsy only a minority had these signs of portal hypertension: ascites was only present in 6 (17 %; $P<0.001$), oesophageal varices in 2 (6 %; $P=0.036$) and only 3 (8 %; $P=0.020$) had developed gastrointestinal bleeding.

DISCUSSION

Our finding of an overall risk in the general population of 1 % to develop PVT during lifetime does not corroborate the concept of PVT being a rare disease. The figure might even be an underestimation, since it cannot be ruled out that in some cases of partial thrombosis earlier in life a spontaneous resolution may have occurred. It should therefore be relevant to view these patients with renewed interest.

Among the major causes of PVT in adults, cirrhosis is generally named the most common, followed by neoplasia[5-6]. In this study the order of magnitude was reversed, with cancer in two thirds of the patients. By nature of being an autopsy study it is a selection of the sickest patients, and in some of these the thrombosis may be a late event in advanced cancer disease. Discrepancies in proportion of cirrhosis patients compared with previously published case series might also reflect patient selection and ascertainment bias resulting from an increased diagnostic activity in high-risk categories, but one should also consider the impact of disease duration. An intriguing finding was the heterogeneity of risk within the group of metastatic malignancy, with a significantly higher risk with pancreatic cancer in comparison with other major gastrointestinal adenocarcinomas.

When assessing the risk for a patient with a certain condition to develop an infrequent complication like PVT, knowledge from case series on the distribution of causes to that complication may be helpful and is sometimes the only available information. It may be misguiding, though. In this study, secondary malignancies accounted for a twice as large fraction of PVT cases as primary hepatobiliary cancer, but the actual risk of PVT in patients with secondary malignancies was less than half of that associated with primary hepatic cancer. In patients with both cirrhosis and hepatic cancer the risks appeared to be additive resulting in a prevalence of 14%. Whether increased diagnostic and therapeutic activities would be effective to reduce this rate is to be considered for further studies. A recent study, though, indicates that even hepatocellular cancer patients, who have developed portal vein tumour thrombosis, may benefit from an active treatment if timely diagnosed with respect to hepatic function[16].

In one sixth of the PVT patients no explanation could be found despite the thorough examination. This group differed from the other patients by a low frequency of complications typical to portal hypertension. A cross-sectional study like the present has obvious limitations for assessment of previous diseases, and this group may harbour some cases of juvenile PVT, though recent longitudinal studies indicate that PVT is an infrequent complication to umbilical vein sepsis, and in the western world the risk of this complication to umbilical vein catheterisation has declined considerably over time[5,17-18]. Similarly, it cannot be ruled out that the thrombosis in the odd case might have been caused by surgical trauma not evident from the recent patient history. With increasing possibilities to detect various trombophilic disorders the proportion of non-explainable, so called idiopathic PVT has gradually become smaller[5,10,19-22]. The present study does not allow an investigation of markers of the haemostatic system, but it is not unreasonable that the

PVT in some of these cases may be an expression of a hypercoagulability state.

Autopsy rates have declined considerably also in Malmö, following changes in legislation, and it is unlikely that a similar study will ever be performed again. With regards to the setting in time, some limitations should be discussed. Apart from aspects of the design previously viewed, the potential effects on the epidemiology of an increasing use of antithrombotic therapies should be considered. The study does not allow an assessment of actual medications, but even today the majority of these conditions would not warrant primary or secondary anticoagulant prophylaxis according to guidelines[23].

From this first population-based study we conclude that PVT is more common than previously indicated by clinical series. The greatly increased risk in patients with cirrhosis and hepatic carcinoma should warrant an increased awareness in these patients. While anticoagulation therapy might be considered for certain patient categories, this remains to be evaluated in prospective studies.

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RAPID COMMUNICATION

Effects of hypobaric hypoxia on adenine nucleotide pools, adenine nucleotide transporter activity and protein expression in rat liver

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Abstract

AIM: To explore the effect of hypobaric hypoxia on mitochondrial energy metabolism in rat liver.

METHODS: Adult male Wistar rats were exposed to a hypobaric chamber simulating 5000 m high altitude for 23 h every day for 0 (H0), 1 (H1), 5 (H5), 15 (H15) and 30 d (H30) respectively. Rats were sacrificed by decapitation and liver was removed. Liver mitochondria were isolated by differential centrifugation program. The size of adenine nucleotide pool (ATP, ADP, and AMP) in tissue and mitochondria was separated and measured by high performance liquid chromatography (HPLC). The adenine nucleotide transporter (ANT) activity was determined by isotopic technique. The ANT total protein level was determined by Western blot.

RESULTS: Compared with H0 group, intra-mitochondrial ATP content decreased in all hypoxia groups. However, the H5 group reached the lowest point (70.6%) ($P < 0.01$) when compared to the control group. Intra-mitochondrial ADP and AMP level showed similar change in all hypoxia groups and were significantly lower than that in H0 group. In addition, extra-mitochondrial ATP and ADP content decreased significantly in all hypoxia groups. Furthermore, extra-mitochondrial AMP in groups H5, H15 and H30 was significantly lower than that in H0 group, whereas H1 group had no marked change compared to the control situation. The activity of ANT in hypoxia groups decreased significantly, which was the lowest in H5 group (55.7%) ($P < 0.01$) when compared to H0 group. ANT activity in H30 group was higher than in H15 group, but still lower than that in H0 group. ANT protein level in H5, H15, H30 groups, compared with H0 group decreased significantly, which in H5 group was the lowest, being 27.1% of that in H0 group ($P < 0.01$). ANT protein level in H30 group was higher than in H15 group, but still lower than in H0 group.

CONCLUSION: Hypobaric hypoxia decreases the mitochondrial ATP content in rat liver, while mitochondrial ATP level recovers during long-term hypoxia exposure. The lower level of extra-mitochondrial ATP may be related to the decrease of ANT activity during hypoxia exposure.

Key words: Adenine nucleotide pool; Hypoxia; Liver; Mitochondria

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INTRODUCTION

The liver is the largest metabolic organ in the body. It performs a number of important and complex biological functions that are essential for survival. It also plays important roles in metabolism of carbohydrates, proteins, lipids, drugs, as well as in bile formation and secretion. Energy metabolism is closely related to these normal functions of liver. Mitochondria are the "energy factory" of cells. The adenine nucleotide transporter (ANT) is the most integral protein in inner mitochondrial membrane and consists of two identical subunits of 32 KD[1]. It catalyzes the transporter of cytosolic ADP and mitochondrial ATP in the process of phosphorylation[1]. Consequently, ANT is an important link between the cytosolic energy consumption and mitochondrial energy process yield[1,2]. Hypoxia could influence mitochondrial oxygenation respiration function[3,4] and F0-F1 ATPase activity of rat brain[4]. However, little is known about the relationship between the effects of hypobaric hypoxia on mitochondrial energy metabolism changes and ANT function in rat liver exposed to hypoxia. We therefore used

HPLC, isotopic assay and Western blot to examine the inner- and extra-mitochondria adenine nucleotide pool, ANT activity and its total protein level of rat liver exposed to hypobaric hypoxia.

MATERIALS AND METHODS

Chemicals

[2,8-^{3}H] ADP was obtained from Perkin-Elmer. Atractyloside (ATR), adenosine-5'-diphosphoric acid (ADP), albumin bovine serum (BSA), nitroblue tetrazolium chloride (NBT), 5-bromo-4-chloro-3-indolye-phosphate (BCIP) and mouse anti-goat IgG-alkaline phosphatase (IgG-AP) were supplied by Sigma. Goat anti-human ANT polyclonal antibody was purchased from Santa Cruz.

Animals and treatments

Adult male Wistar rats (150-200 g) were used in the experiments. The rats were exposed to a hypobaric chamber simulating 5000 m high altitude for 23 h every day for 0 (H0), 1 (H1), 5 (H5), 15 (H15) and 30 d (H30) respectively. Animals were fed with laboratory chow and tap water *ad libitum*. Rats were sacrificed by decapitation at seal level (H0 group) and hypobaric chamber simulating 4000 m high altitude (H1, H5, H15, H30 groups). After decapitation the liver tissues were immediately excised, and part of it was frozen in liquid nitrogen, and then transferred to -70℃ for storage until use. The remaining liver tissues were rapidly placed into ice-cold isolation medium [0.25 mol/L sucrose, 10 mmol/L 4-(2-hydroxymethyl)-1-piperazine ethane sulfonic acid (HEPES), 1 mmol/L ethylene diamine tetraacetic acid (EDTA), pH 7.4][5]. The tissues were chopped finely with scissors while being washed three times with ice-cold isolation medium and then manually homogenized by 15 up and down strokes with Teflon glass pestle. The liver mitochondria were isolated by centrifugation as established in our laboratory[3,4]. The protein content in the mitochondria suspension was assayed by Lowry's method using BSA as the standard.

Assay of mitochondria adenine nucleotide pool

Adenine nucleotides were separated and quantitated by HPLC[4,6]. Briefly, 500 mmol/L ice-cold $HClO_4$ was added to 200 μL liver mitochondria suspension and after 5 min incubation at 4 ℃, the liquid was centrifuged (12 000 r/min, 20 min) at 4 ℃ and the supernatant was saved and neutralized with 1 mol/L K_2CO_3 to pH 6.5-7.0. The liquid was centrifuged (12000 r/min, 10 min) at 4 ℃ again. The samples were maintained frozen at -70 ℃ until defrosted and analysed by HPLC equipped with high pressure pumps (Waters, USA), fit with 4.6 mm× 250 mm hypersil 18 (5 μm pore size, from Sigma). The samples were applied in a total volume of 20 μL. The results were shown as nmol adenine nucleotides/mg mitochondrial protein.

Assay of liver tissue adenine nucleotide pool[5,6]

The liver tissues stored at -70 ℃ were rapidly transferred into ice-cold 0.5 mol/L $HClO_4$, chopped finely with scissors, and then manually homogenized by 10 up and down strokes with Teflon glass pestle. The homogenate was placed at 4℃ for 5 min, centrifuged (12 000 r/min, 20 min) at 4 ℃. The supernatant was saved and neutralized with 1 mol/L K_2CO_3 to pH 6.5-7.0. The liquid was centrifuged (12 000 r/min, 10 min) at 4 ℃ again and maintained frozen at -70 ℃ until supernatant was analyzed by HPLC as described above. The results were expressed as nmol/mg tissue.

^{3}H- ADP label and liquid scintillation

The activity of ANT was determined at 4℃ by isotopic technique[1]. ^{3}H- ADP label was stopped by atractyloside, a specific inhibitor of ANT. Fifty microliter mitochondrial suspension solution was diluted by 150 μL ice-cold isolation medium. Twenty microliter ^{3}H- ADP (specific activity 33.9 Ci/mmol ADP) 0.3 μmol/L were added. After 10 s incubation at 4 ℃, the reaction was inhibited by 50 μL 3.2 nmol/L ATR, then centrifuged (12 000 r/min, 20 min) at 4 ℃. The precipitation was dissolved with 1 ml ice-cold isolation medium, centrifuged (12 000 r/min, 20 min) at 4℃ again for 20 min and the process was repeated three times. The pellet was digested at 70 ℃ with 7 mol/L $HClO_4$ 200 μL, 8.8 mol/L H_2O_2 400 μL for 40 min. The 200 μL sample was dissolved in 1 ml scintillator (5 g PPO was dissolved by 700 mL dimethylbenzene and 300 mL anhydro-ethanol) and measured in liquid scintillation counter. ANT activity was calculated from the radioactivities of H^3-ADP [count per min (cpm)]. Nonspecific binding of H^3-ADP to mitochondria was determined by incubation of mitochondrial samples with 50 μL 3.2 nmol/L ATR prior to addition of 0.3 μmol/L ^{3}H- ADP. The results were expressed as pmol ADP/min per milligram mitochondrial protein.

Western blot

The rat liver mitochondrial ANT protein level was determined by Western blot[1]. The mitochondria sample was solubilized in sample buffer and supplemented with 5 μL β-mercaptoethanol. Following that, it was heated for 10 min at 100 ℃ and supplemented with 5 μL β-mercaptoethanol again. Electrophoresis was performed on a 12% polyacrylamide slab gel with Tris/glycine running buffer. The staking gel contained 5% polyacrylamide. The lanes were loaded with 30 μL aliquots of solubilized mitochondria (10 μg). After separation the protein bands were stained with Commassie blue for 4 h or transferred to polyvinylidene difluoride (PVDF) sheet for 90 min. The PVDF sheet was washed twice with Tris-buffered saline (TBS) containing 0.1% Tween-20 (0.1% TBST), pH 7.4 at room temperature. ANT polyclonal antibody at a 1:1000 dilution was used. Incubation was performed in 0.1% TBST, pH 7.4 for at least 12 h at 4℃. The sheet was washed six times with 0.1% TBST at room temperature. Mouse anti-goat IgG coupled with alkaline phosphatase at a 1:1000 dilution was used as secondary antibody. Incubation was performed in 0.1% TBST, pH 7.4 for 1 h at room temperature. The sheet was washed six times with 0.1% TBST again. Antigen was visualized by luminescence (NBT/BCIP). Signals were quantified with Smartview (Furi

Table 1 Changes of total adenine nucleotide pool in liver tissue during hypoxia exposure (nmol/mg tissue, mean ± SD)

Group	*n*	AMP	ADP	ATP
H0	6	9.32±1.63	9.02±0.87	4.00±0.26
H1	6	7.14±1.41	5.53±0.71[b]	2.40±0.29[b]
H5	6	4.90±0.74[b]	4.57±0.48[b]	2.41±0.17[b]
H15	6	6.08±1.57[a]	5.40±1.31[b]	3.11±0.25[b]
H30	6	6.58±1.19[a]	6.21±1.36[b]	3.27±0.30[b]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* H0 group.

Table 2 Changes of intra-mitochondrial adenine nucleotide pool of rat liver during hypoxia exposure (nmol/mg protein, mean ± SD)

Group	*n*	AMP	ADP	ATP
H0	6	6.84±1.57	11.34±1.97	5.47±0.54
H1	6	3.68±0.42[b]	4.52±1.07[b]	3.86±0.20[b]
H5	6	2.72±1.09[b]	4.76±1.56[b]	4.03±0.25[b]
H15	6	3.94±0.68[b]	5.07±0.10[b]	4.50±0.35[b]
H30	6	3.77±0.67[b]	8.70±2.11[b]	4.72±0.60[a]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* H0 group.

Science and Technology Co., Ltd., Shanghai, China).

Statistical analysis

Results were expressed as mean ± SD. Significant difference was determined by one-way ANOVA followed by LSD test between different groups. Statistical analyses were performed using SPSS 12.0 software.

RESULTS

Changes of adenine nucleotide pool in rat liver tissue during hypoxia exposure

Compared with H0 group, liver tissue ATP and ADP content decreased significantly in all hypoxia groups ($P<0.01$). Tissue AMP in groups H5, H15, and H30 was significantly lower than in H0 group ($P<0.05$ and $P<0.01$), while H1 group had no marked change compared to H0 group (Table 1).

Changes of intra-mitochondrial adenine nucleotide pool of rat liver during hypoxia exposure

Compared with H0 group, intra-mitochondrial ATP content decreased in all hypoxia groups, which in H1 group was 70.6% of that in H0 group ($P<0.01$) reaching the lowest point. Intra-mitochondrial ADP and AMP levels showed the same change and were significantly lower in H0 group than that in all hypoxia groups ($P<0.01$) (Table 2).

Changes of adenine nucleotide pool in extra-mitochondria of rat liver during hypoxia exposure

Compared with H0 group, extra-mitochondrial ATP and ADP content decreased significantly in all hypoxia groups ($P<0.05$ and $P<0.01$). Extra-mitochondrial AMP in groups H5, H15, and H30 was significantly lower than

Table 3 Changes of adenine nucleotide pool in extra-mitochondria of rat liver during hypoxia exposure (nmol/mg tissue, mean ± SD)

Group	*n*	AMP	ADP	ATP
H0	6	8.64±1.58	8.08±0.93	3.45±0.22
H1	6	6.77±1.40	5.07±0.74[b]	2.01±0.27[b]
H5	6	4.63±0.82[b]	4.09±0.60[b]	2.01±0.15[b]
H15	6	5.68±1.60[a]	4.90±1.34[b]	2.67±0.27[b]
H30	6	6.21±2.01[a]	5.37±1.53[b]	2.80±0.33[a]

[c]

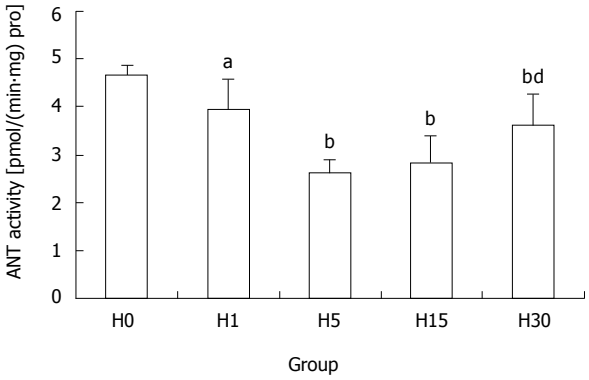

Figure 1 Change of mitochondrial ANT activity of rat liver during hypoxia exposure (mean±SD). [a]$P < 0.05$, [b]$P < 0.01$ *vs* H0 group; [d]$P < 0.01$ *vs* H15 group.

that in H0 group ($P<0.01$), while in H1 group it had no marked change compared with H0 group (Table 3).

Change of mitochondrial ANT activity of rat liver during hypoxia exposure

Compared with H0 group, the activity of ANT decreased significantly in all hypoxia groups, which in H5 group was 55.7% of that in H0 group ($P<0.01$), being the lowest. Activity in H30 group was higher than that in H15 group ($P<0.01$), but was still lower than in H0 group ($P<0.01$) (Figure 1).

Effect of hypoxia on mitochondrial ANT protein expression

ANT protein expression in H5, H15, and H30 groups, compared with H0 group, decreased significantly, which in H5 group was the lowest point (27.1%) ($P<0.01$). The expression in H30 group was higher than that in H15 group ($P<0.01$), but was still lower than that in H0 group ($P<0.01$) (Figures 2 and 3).

DISCUSSION

ATP is the direct energy for cell usage. Mitochondrial ATP level is influenced by two factors. First, mitochondrial ATP is produced by oxidative phosphorylation. Mitochondria oxidative respiration and phosphorylation states are the main factors that affect the ATP level. Second, mitochondrial ATP provides energy for cytoplasm as well as for its own demand such as the synthesis of mitochondrial DNA, RNA and proteins, etc. Our previous work showed that during hypoxia exposure, mitochondrial

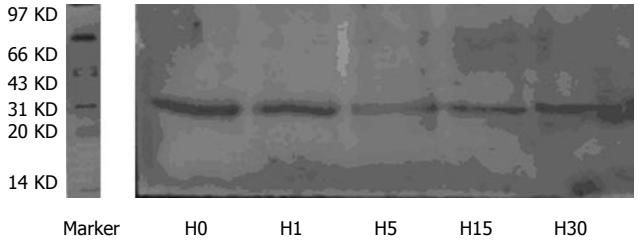

Figure 2 Western blot results of mitochondrial ANT protein in rat liver exposed to hypobaric hypoxia.

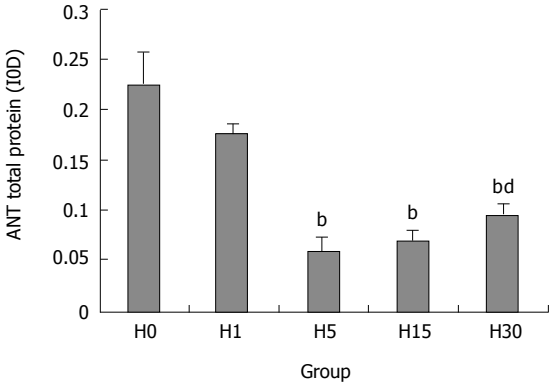

Figure 3 The effect of hypoxia on mitochondrial ANT total protein level (mean± SD). $^{b}P < 0.01$ *vs* H0 group; $^{d}P < 0.01$ *vs* H15 group.

ATP content and F_0-F_1 ATPase activity of rat brain decreased significantly compared with control [4,7]. This indicates that hypoxia could influence ATP production and then the mitochondrial ATP level. However, there were no reports about the hypobaric hypoxia effect on rat liver mitochondrial adenine nucleotide pool.

The current results showed that ATP level in rat liver mitochondria reduced during hypoxia exposure, which in H1 group was the lowest point, 70.6% of control ($P<0.01$). The decrease of mitochondrial ATP content may be related to the following factors. First, mitochondrial oxidative respiratory function was inhibited. Our previous study showed that hypobaric hypoxia inhibited the oxidative respiratory function of mitochondria in rat brain[3,4,6]. Our results (data not shown) also revealed that hypoxia significantly decreased the mitochondrial three state oxygen consumption and respiratory control rate in rat liver, while significantly increased the rat liver mitochondrial four state oxygen consumption. Second, hypoxia decreased the mitochondrial membrane potential (MMP). Our results (data not shown) demonstrated that hypoxia inhibited the MMP of rat hepatocytes. However, MMP is the motive power of mitochondrial ATP synthesis. Thirdly, hypoxia lowered the mitochondrial F_0-F_1 ATPase activity[4,8]. Fourthly, the lower mitochondrial ATP content may be also related to the reduction of intra-mitochondrial ADP concentration[1], intra-mitochondrial Ca^{2+} content[9] and extra-mitochondrial adenine nucleotide pool[10, 11] .

ANT is the most integral protein in inner mitochondrial membrane and consists of two identical subunits of 32 KD[1]. It is a key energy link between the mitochondria and cytoplasm since it catalyses the transmembrane exchange between ATP synthesized by the F1-Fo ATP synthase inside mitochondria and ADP generated by the metabolism in cytoplasm[1,6]. The ADP/ATP exchange follows the Michaelis-Menten kinetics and ANT activity is moderate, 1500-2000 molecules per min[12]. Schonfeld et al reported that matrix adenine nucleotides and the ANT protein content are associated with the changes of the ANT activity in rat heart mitochondria[1]. Rulfs et al also reported that matrix adenine nucleotide concentration influenced the ANT activity in rabbit liver mitochondria[13]. However, little is known about the effect of hypobaric hypoxia on mitochondrial ANT activity in rat liver.

Our study showed that the activity of ANT after hypoxia exposure decreased significantly. The ANT activity was the lowest point, 55.7% of control after hypoxia exposure for 5 d ($P<0.01$), while after hypoxia exposure for 30 d it was higher than after 15 d exposure ($P<0.01$), but was still lower than control. This indicates that hypoxia could inhibit the mitochondrial ANT activity in rat liver. The decrease of ANT activity may be related to the following factors. First, the ANT protein level and content was the main factor. Our results showed that ANT protein expression decreased significantly in H5, H15, H30 groups, which in H5 group was 27.1% of that in H0 group. Second, MMP also influenced the ANT activity[14]. Passarella *et al* reported that helium neon laser increased the rate of ADP/ATP exchange through increasing the MMP in rat liver[15]. In the presence of an MMP of about 100 mV positive inside, the rates of the [^{14}C] ATP_{out}/ADP_{in} exchanges were stimulated[3]. All these indicate that MMP is one of the most important factors that affect the ANT activity. The mechanism that decrease of MMP reduced ANT activity is not clear. Thirdly, the change of ANT conformation also influenced its activity. ANT has two conformational states, cytosolic conformation (c-confromation) and matrix conformation (m-conformation)[16,17]. ANT is not a pore, which opens or closes simply as a response to stimuli. Conformational changes have to occur to release nucleotides to the matrix (and the reverse) without creating leakage in the membrane. Fourthly, it was reported that the size of mitochondrial adenine nucleotide pool influenced the ANT activity. Previous studies showed that the postnatal increase in the matrix adenine nucleotides concentration contributed to the increase of ANT activity in rat liver[13] and heart[1].

The decrease of extra-mitochondrial ATP level influences the mitochondrial carrier family including ANT synthesis and transport. Extra-mitochondrial ATP level is mainly determined by the ANT activity. However, the lower ANT protein level has an identical role in influencing ANT activity during hypoxia. So the ANT activity-ANT protein level- ATP content form the vicious cycle and aggravate the dysfunction of cell energy metabolism during hypoxia exposure.

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CASE REPORT

Liver cell adenoma: A case report with clonal analysis and literature review

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Abstract

We report a case of liver cell adenoma (LCA) in a 33-year-old female patient with special respect to its clonality status, pathogenic factors and differential diagnosis. The case was examined by histopathology, immunohistochemistry and a clonality assay based on X-chromosomal inactivation mosaicism in female somatic tissues and polymorphism at androgen receptor focus. The clinicopathological features of the reported cases from China and other countries were compared. The lesion was spherical, sizing 2 cm in its maximal dimension. Histologically, it was composed of cells arranged in cords, most of which were two-cell-thick and separated by sinusoids. Focal fatty change and excessive glycogen storage were observed. The tumor cells were round or polygonal in shape, resembling the surrounding parenchymal cells. Mitosis was not found. No portal tract, central vein or ductule was found within the lesion. The tumor tissue showed a positive reaction for cytokeratin (CK) 18, but not for CK19, vimentin, estrogen and progesterone receptors. Monoclonality was demonstrated for the lesion, confirming the diagnosis of an LCA. Clonality analysis is helpful for its distinction from focal nodular hyperplasia.

Key words: Liver Cell adenoma; Clonality; Literature review

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INTRODUCTION

Liver cell adenoma (LCA) is a rare benign liver tumor. It is considered a hepatocellular neoplasm, while some authors hold that LCA originated from liver progenitor cell[1]. A female predominance was noticed for the development of LCA based on the reports from Western countries, with a majority of cases associated with a long-term use of oral contraceptives or other steroids. Other diseases should be excluded before establishing the diagnosis of an LCA. It is indeed a difficult task to distinguish LCA from focal nodular hyperplasia (FNH) when the morphologic features are not prominent and a central scar is absent. Then some molecular approaches, including clonality analysis, may be helpful. In this article, a case of LCA in a female Chinese patient is presented, with its clonality status demonstrated.

CASE REPORT

A hepatic mass was found in the right lobe of a 33-year-old woman during her routine medical check-up. She was then admitted to Tangdu Hospital in Xi'an in January 28, 2003. She had never used oral contraceptives, and she had no history of alcohol abuse or hepatitis. No record of HCC or any hereditary disease was found among her family members. The parameters of routine clinical biochemistry, including values of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), γ-glutamyltransferase (γ-GTP) and concentrations of α-fetoprotein (AFP) and plasma proteins, were all within normal ranges. The laboratory tests failed to show any positive signal in her serum for hepatitis B surface antigen (HBsAg) or anti-hepatitis C virus (HCV) antibody.

Ultrasonography revealed a solid mass in the posterior part of the right lobe of liver. Computed tomography (CT) scanning showed reduction of density for the lesion sizing 2.0 cm in diameter (Figure 1), indicating malignant potential. Laparotomy was then performed on February 1, 2003. Size of the liver appeared normal, with a mass found in the right lobe. Partial hepatectomy was then performed. Appearance, color and texture of the surrounding liver

were normal, without any indication of cirrhosis, pronounced fibrosis or cholestasis.

Histological and immunohistochemical procedures

The sample was fixed in 40 g/L formaldehyde solution, embedded in paraffin. Sections of 4 μm in thickness were prepared and stained by hematoxylin and eosin (HE), Masson trichrome methods and periodic acid-Schiff (PAS) reaction. Immunostaining was carried out using a streptavidin-labeled peroxidase (S-P) kit (KIT9730) as described previously[2]. The primary antibodies used in this study included those against cytokeratin (CK) 18, CK19, vimentin, CD34, estrogen receptor (ER), progesterone receptor (PR), AFP, S-100 protein, HBsAg, hepatitis B core antigen (HBcAg), as well as an anti-HCV antibody. All of the reagents for immunostaining were supplied by Maxim Biotechnology Corporation Limited, Fuzhou, China.

Clonal analysis

Sections of 10 μm in thickness were prepared, deparaffinized, rehydrated and HE stained. Neoplastic tissues were dissected using a syringe needle from 4 different tumor areas, sizing 0.5 cm × 0.5 cm for each. Normal liver tissue was also isolated from the surrounding parenchyma at 3 different sites of the same size and analyzed in a parallel way as reference samples. Genomic DNA was extracted using a QIAamp kit (Qiagen, Mannheim, Germany). Polymorphism was examined at the androgen receptor (AR) and phosphoglycerokinase (PGK) loci as described previously[3,4], with the former gene proved polymorphic at the CAG short-tandem repeat (STR) located in exon 1. Loss of X-chromosomal inactivation mosaicism was demonstrated by pretreatment of DNA with methylation-sensitive restriction enzyme *Hba* I and amplification via nested PCR. The CAG STR-polymorphic alleles were resolved on a 100 g/L denaturing polyacrylamide gel at 120 V for 4 h. A reduction of at least 50% in density of either band, as compared to that obtained using the sample not treated with *Hba* I, is regarded as loss of X-chromosomal inactivation mosaicism [5].

RESULTS

A partial resection liver specimen, sizing 4.0 cm × 3.0 cm × 2.0 cm, presented with a spherical mass of 2.0 cm in its maximal dimention. The mass was beneath the hepatic capsule, yellow brown in color and soft in its texture, without any necrotic focus or fibrotic scar in its cut surface. There was a clear border, but not a fibrotic septum between the lesion and surrounding liver tissue that appeared red brown and apparently normal. Microscopically, the lesion was composed of cells arranged in two-cell-thick cords, with the cell cords separated by sinusoids (Figure 2A). Focal fatty change and excessive glycogen storage were present (Figure 2A and 2B). The tumor cells were round or polygonal, apparently resembling the surrounding liver parenchyma cells in size and shape. Mitosis was not found. There was no portal tract or hepatic venule in the tumor. Ductule or scattered ductular cells (the so-called "oval cells"[6,7] or "liver progenitor cells"[1]) were absent, and immunostaining for the ductular cell markers, including CK19 and S-100 protein[7,8], failed to show any positive cell within the lesion. The tumor cells were positive for CK18 (Figure 2C), but negative for AFP, vimentin and p53 protein. They did not show any positive signal for ER or PR. Both neoplastic and the adjacent parenchymal tissues were negative for HBsAg, HBcAg and HCV antigen.

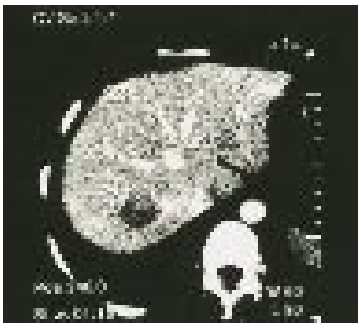

Figure 1 CT scanning showing a lesion with reduction of density in the posterior part of the right lobe of liver.

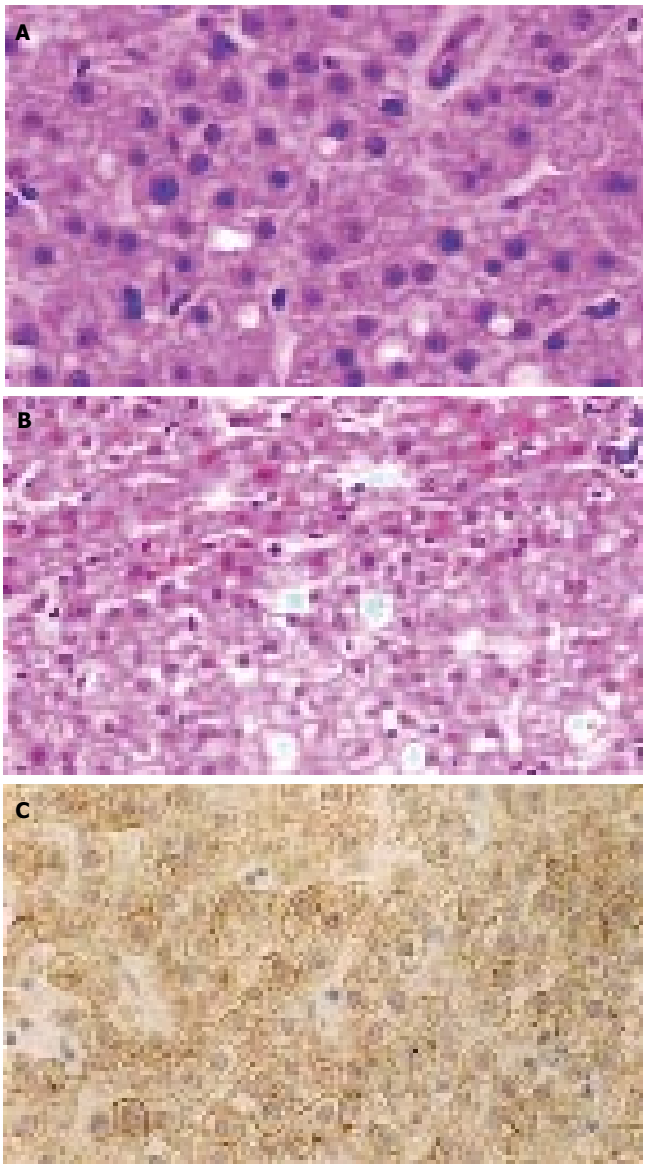

Figure 2 Liver cell adenoma (× 400). **A:** Similar to normal hepatocytes, arranged in two-cell-thick cords separated by hepatic sinusoids (HE); **B:** Showing the border between LCA (lower) and surrounding liver parenchyma (upper) (HE); **C:** CK18 immunoreactivity (S-P).

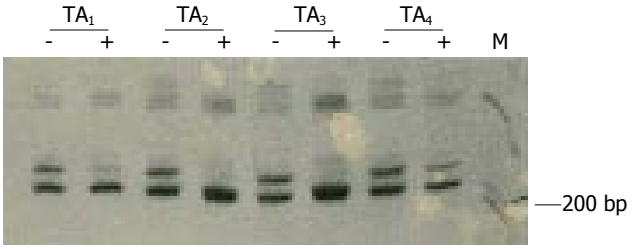

Figure 3 Clonal analysis on tissues from 4 tumor areas (TAs). The pretreatment with *Hha* I results in loss of the upper band for TA_2 and TA_3, and intensity reduction by factors of 71.8% and 57.1% for TA_1 and TA_4, respectively. +, with H*ha* I pretreatment; -, without *Hha* I treatment.

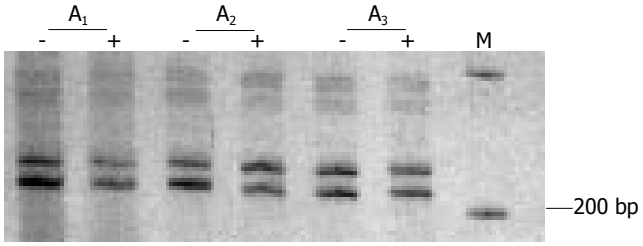

Figure 4 Data of clonality test of three areas of liver parenchymal tissues (A_1-A_3). Neither of the bands on gel shows marked intensity change for the samples pretreated with Hha I (+) compared to those not treated (-).

Clonality status of the lesion was determined by an assay based on the CAG-STR polymorphism at exon 1 of AR gene. Pretreatment with *Hha* I resulted in pronounced reduction or loss of the upper band for all of the tissue samples from 4 different areas of the lesion (Figure 3), demonstrating loss of the X-chromosomal inactivation mosaicism. The adjacent liver parenchyma, however, did not show the change (Figure 4). The data proved the monoclonal, neoplastic nature of the lesion, confirming the diagnosis of LCA. The patient has survived for 30 mo after the operation without any indication of recurrence.

DISCUSSION

LCA is a rare benign hepatic neoplasm, accounting for less than 2% of all hepatic tumors[9]. A pronounced female predominance was noted mainly by authors from Western countries. It often occurs in women of 20 to 40 years during their child-bearing period[10,11], being closely associated with long-term use of steroids, mainly oral contraceptives[12-14]. In fact, it was rarely reported before the introduction of oral contraceptives in 1960s. Edmondson *et al*[15] found only two cases in 48 900 necropsies performed in Los Angeles General Hospital during the period from 1918 to 1954. In 1973, Baum *et al*[16] pointed out the possible link between the use of oral contraceptives and LCA development. Data from several reports has confirmed the etiologic association. Leese *et al*[17] reported 24 cases of HA, 16 (66.7%) of them with a history of using oral contraceptives. Tumor regression was observed in some cases after withdrawal of the hormones[18,19], and then the tumor remained silent or grew slowly for many years, or even progressed to HCC[20], albeit infrequently. Complete remission was observed in an LCA patient, who had used oral contraceptives for 8 years, after the hormone withdrawal for 9 months[21].

Through literature review, the clinicopathological data of 127 cases of LCA reported from Chinese patients were collected and compared to those of 130 patients from Western countries, with the male/female ratios being 1.8/1 and 1/2.9, respectively. The ages of the Chinese patients ranged from 2 to 73 years, with their mean and average values estimated to be 31.0 and 35.8 years, respectively. For patients from Western countries, the ages ranged from 11 months to 82 years, with their mean and average values 30.0 and 31.6 years, respectively. Only 3.9% (5/127) of Chinese patients had a history of using oral contraceptives, the percentage being much lower than that for the patients from Western countries (54/130, 41.5%). Difference is evident, therefore, between the LCAs occurring in China and in Western countries in their etiologic associations, indicating possibly different pathogenic pathways. This, at least partly, provides an explanation for the distinct gender distribution pattern in Chinese patients.

Other factors are also linked to LCA development, including glycogen storage diseases [22] and administration of danazol[23], phenobarbital[24] and androgenic/anabolic steroids[9,25-33]. Among the 130 cases from Western countries, 4 (3.1%) were found to have glycogen-storage diseases, but only one (0.8%), in the Chinese group, was with the association. For some LCA cases, there seemed no identifiable pathogenic factor. The majority of the cases from the Chinese group fell into this category. Among the 127 Chinese patients, 13 (10.2%) were seropositive for HBsAg, and 8 of them were with chronic hepatitis[34-37]. Among the patients from Western countries, however, only one (0.8%) was shown to be an HBsAg-carrier. In consideration of the high prevalence (up to 10%[38]) of HBsAg-carrier state in China, the data does not provide support for the role of persistent HBV infection in development of the solitary LCA.

Most of LCAs grow slowly and are asymptomatic or cause only mild symptoms, while rupture and hemorrhage may occur in some tumors (15%-33%[39]), and malignant transformation was also observed in a minority of the cases (5/39, 12.8%[20]). Ultrasonography, CT scanning and magnetic resonance imaging (MRI) are useful for detecting hepatic occupation and determining its location and size. Imaging approaches may be helpful for identifying LCA from other hepatic lesions, and a peritumorous halo, demonstrated by CT scanning, was considered indicative of LCA[40,41]. These features, however, are not specific[40], and the accurate preoperative diagnosis of LCA, particularly the distinction from HCC, is frequently problematic. This is even more serious as a surgical consideration in China where incidence of HCC is overwhelmingly high compared to that of LCA. For the pathologists, well differentiated HCC and FNH are among the hepatic lesions that should be excluded before making a diagnosis of LCA.

HCC is diagnosed usually at ages between 40 and 60 years, with a pronounced male predominance. In China, including Hong Kong and Taiwan, about 80% of HCCs were found in patients with chronic hepatitis B and cirrhosis or advanced liver fibrosis. An elevated level of circulating AFP is indicative of HCC, but this change may

not be evident in patients with an early-stage, often well differentiated, HCC. Liver-cell plates more than three cells thick, acinar structures, increased nuclear/cytoplasmic ratio, prominent nucleoli, mitoses, increased cytoplasmic basophilia, loss of the reticulin fibers, absence of Kupffer cells, presence of vascular invasion or immunoreactivity for AFP, all indicate HCC. However, none of these features can be relied upon with certainty, as some are also seen in the hepatic lesions with high-grade SCC, including the premalignant nodules of altered hepatocytes[2], adenomatous hyperplasia[42-46] and LCA. The most helpful parameters, in our consideration, are thickness of the liver-cell plates (more than three cells), cell density (an increase of two folds compared to the surrounding liver parenchyma) and vascular invasion[38]. It should be noted that male, cirrhosis, and chronic HBV infection strongly indicate that a hepatic neoplasm is malignant. Conversely, female and a history of oral contraceptive use support the diagnosis of LCA. Exceptional difficulties may be encountered in some of the LCAs associated with long-term use of anabolic steroids or metabolic disorders. Some of them may show pronounced architectural disturbance and cellular atypia, which make their distinction impossible from well-differentiated HCCs based on histological grounds alone. Malignant transformation is proposed for such cases[13,20,47,48], but they often show more favorable clinical courses, or even regression after withdrawal of the steroid[21]. Such lesions may represent the borderline hepatocellular neoplasm, and their behaviors should be determined by careful postoperative observations.

FNH occurs most commonly in young women, having similar etiological associations and clinical manifestations to LCA[49-51]. It is a localized lesion, frequently solitary, within an otherwise normal or nearly normal liver. The lesion is similar to cirrhosis by its histology, and a central stellate fibrous region containing large vessels can often be found. Its development has been attributed to the vascular malformation[52,53]. Usually, FNH is readily distinguished from LCA by its central scar, multinodularity and presence of proliferating bile ductules in the fibrous septa. It may become a diagnostic problem, however, when the central fibrous region is not evident. Data from different laboratories have demonstrated the polyclonal cell composition and indicated non-neoplastic nature for the lesion[54,55]. In contrast, an LCA was shown to be monoclonal[56], and our data confirm the conclusion that LCA is a neoplastic lesion. The clonality assays, therefore, are helpful for the differential diagnosis between LCA and some FNH lesions without an identifiable central scar.

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CASE REPORT

Adult intussusception caused by cystic lymphangioma of the colon: A rare case report

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Abstract

We experienced a case of intussusception caused by cystic lymphangioma of the colon in a 32 years old female who was admitted to our hospital for the chief complaint of bloody stool. In the colonoscopic examination, cystic mass with stalk which had smooth mucosal surface was noted at the descending colon. Abdominal ultrasonography and computed tomography revealed left colon intussusception with a multilocular cystic tumor as a leading point. Emergent operation was performed. On the histopathologic examination, the cystically dilated spaces lined by endothelium and septated by fibrous septa were present. The pathological diagnosis was cystic lymphangioma of the colon. Although intussusception due to lymphangioma in an adult are rare, it should be taken into consideration that it is possible diagnosis.

Key words: Cystic lymphangioma of the colon; Adult intussusception

Kim TO, Lee JH, Kim GH, Heo J, Kang DH, Song GA, Cho M. Adult intussusception caused by cystic lymphangioma of the colon: A rare case report. *World J Gastroenterol* 2006; 12(13):2130-3132

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INTRODUCTION

Lymphangioma is a benign tumor consisting of several expanded lymphatics, each lymphatics is surrounded by benign endothelial cells and it could be developed in any parts of the body. However, it is developed primarily in the neck, the axillary area, the scapula and other areas where lymphatic organs are abundant[1,2]. Lymphangioma is hardly developed in the digestive organs and the development of the colon is very rare. It was reported for the first time by Chisholm and Hillkowitz in 1932 and recently, with the increased application of colonoscopy and endoscopic ultrasonography, these cases are on the rise[4,5]. We report a case who was diagnosed as intussusception due to cystic lymphangioma by abdominal ultrasound, computed tomography and colonoscopy.

CASE REPORT

A 32-year-old female was admitted to our hospital due to bloody stool for 3 d. In her past, family and social history, specific findings were not detected. At the time of visit, blood pressure of 100/60 mmHg, pulse rate of 76 per min and body temperature of 36.7 ℃ was recorded. In physical examination, the acute ill appearance was detected and she were conscious. The conjunctiva was pale and the head-neck lymph node was not palpated. In abdominal examination, the liver or the spleen was not palpated and the bowel sound was normal. But, left lower quadrant pain was detected, however, the rebound tenderness was not detected. In laboratory findings, in peripheral blood test, the number of leukocyte was 16 140/mm^3, hemoglobin was 128 g/L, and platelet was 317 000/mm^3. In biochemical test was all normal. CEA, CA 125 and CA 19-9 showed normal limits.

In colonoscopic examination, a cystic mass with a stalk of the descending colon was detected (Figure 1). The mass was covered with the normal mucosa with smooth surface and palpated with the tissue biopsy forceps, it was cystic that changed readily. On the next day, the patient reported severe abdominal pain and thus abdominal ultrasonography and abdominal computed tomography were performed. In abdominal ultrasonography, a cystic mass divided by several septa was detected and the situation was that intussusception by this mass (Figure 2). In abdominal computed tompgraphy, a mass was detected and in the proximal area, intussusception was detected (Figure 3). The patient underwent emergent surgery and the partial resection of the descending colon was performed. The resected specimen was a mass 2.5 cm× 3.5 cm× 5.0 cm in size and similarly to ultrasonographic finding. The specimen was a bag shape, its inside was divided into several fibrous septa and filled with clear serous liquid (Figure 4). In histological finding, the cyst was located below the mucosal mem-

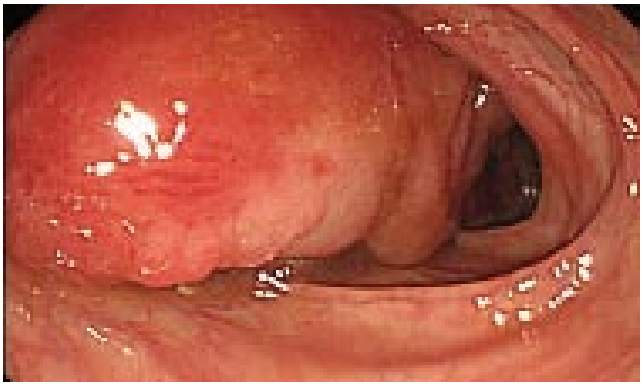

Figure 1 Colonoscopic examination shows a 2.5 cm x 2.5 cm x 5.0 cm sized round, smooth surfaced mass covered by normal mucosa in the descending colon. This mass is pedunclated cystic nature, and compressible pillow sign positive.

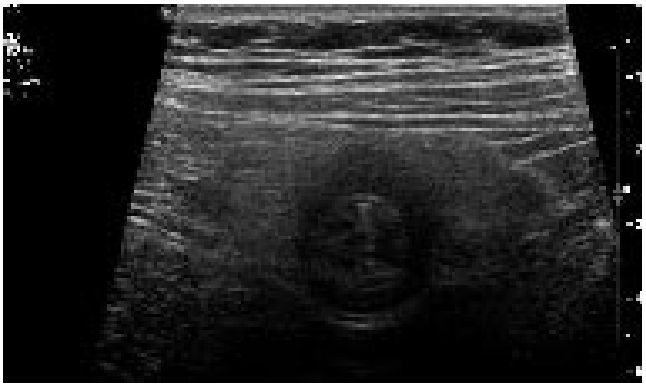

Figure 2 Ultrasonography of the mass shows a multilocular cystic lesion with septa.

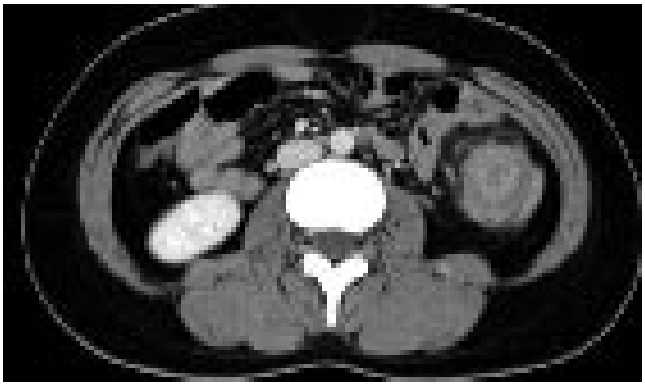

Figure 3 Computed tomography shows the cystic lesion in the descending colon, occupying the whole lumen, and also shows multicentric target sign and sausage-shaped inhomogeneous soft-tissue mass.

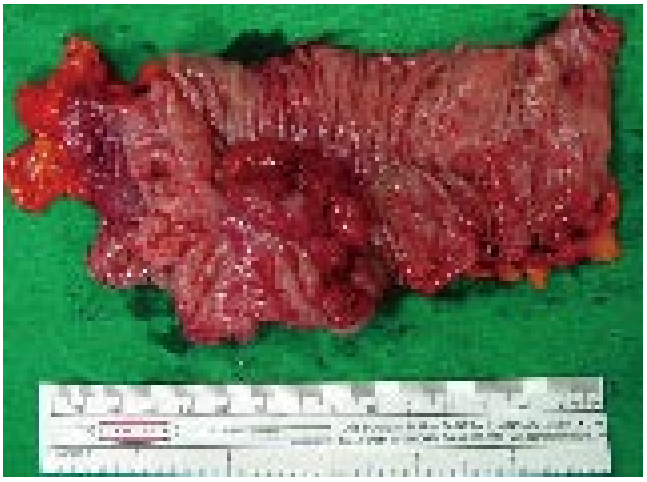

Figure 4 Macroscopic finding of the resected specimen reveals 2.5 cm x 3.5 cm x 5.0 cm sized multiseptated submucosal cystic tumor, which changed shape easily. The tumor shows fluctuation and serous clear fluid was aspirated by needle puncture.

brane, the inside was surrounded by normal epithelial cells and divided by several fibrous septa (Figure 5). Based on the above findings, she was diagnosed as intussusception caused by cystic lymphangioma.

DISCUSSION

Intussusception in adults is developed relatively infrequently. They are equivalent to 1% of colonic obstructive patients and they are equivalent to 5 - 10% of total intussusception[1,2]. Different from in children, they occur secondarily by definite lesions. For the diagnosis of such intussusception in adults, computed tomography has been reported to be the most useful radiological method[1]. This case is a patient that intussusception is caused by the cystic lymphangioma in the descending colon. Lymphangioma within the abdominal cavity is rare, and it occupies less than 5% of the entire lymphangioma[3]. Most reported cases are the cases detected accidentally during colonoscopy or barium enema and definitely diagnosed histologically by the resection of the lesion[5, 7]. Pathophysiologically, according to its shape, it is classified into 3 types: (1) simple capillary, (2) cavernous and (3) cystic. As in our case, cystic cases are most frequent. Recently, with the increase of the application of colonoscopy, the reported cases of the lymphangioma of the colon are increased [4,5]. The findings suggesting lymphangioma are the characteristics that are the round and soft surface, a wide base area, pink color and semi-transparent. In response to the peristaltic movement or the position of patient, its shape changes and it has the characteristic that the diameter and the location change upon pressing by forceps. In our case also, it was stalked cystic and the smooth surface of mass was covered with the normal mucosa of large intestine, and palpated with the biopsy forceps, it showed the pattern of readily changing its shape. The disease should be differentiated from other mass lesions, such as lipoma, cavernous angioma, leimyoma and enteric duplication cyst. However, endoscopic findings are very diverse, subjective and it is not of help to the diagnosis. So, the confirmation of its structure by endoscopic ultrasonography is important for the differential diagnosis[6]. In ultrasonography, it is detected as a mass limited to the submucosal area, separated well from adjacent tissues, and divided by several septa and for the cases showing such findings, it could be definitely diagnosed without further tests, and for the asymptomatic cases, the follow-up observation without treatment is sufficient[7]. Occasionally, nonspecific symptoms, in other

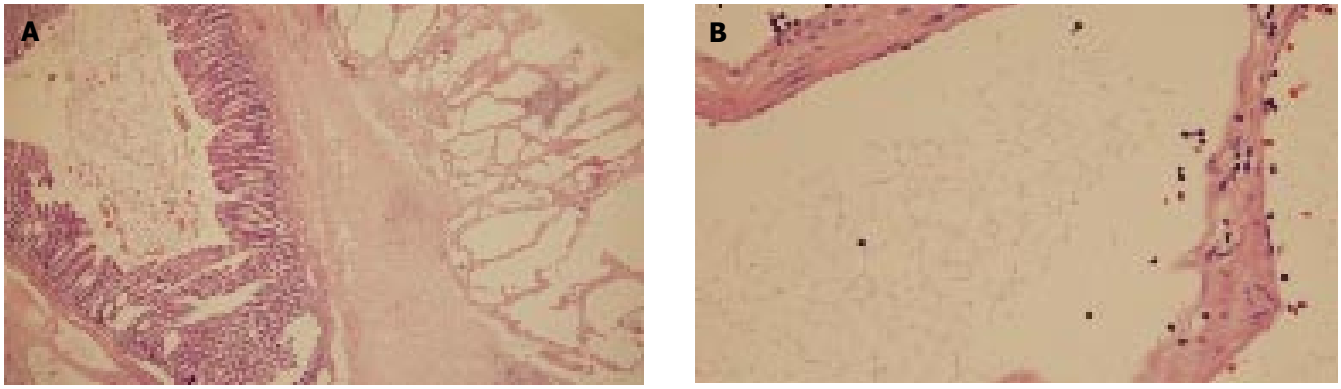

Figure 5 (**A**) Cystically dilated spaces are covered with single layer of endothelial cells and separated by fibrous septa are present in the submucosa. The overlying colonic mucosa is normal (H & E stain, x100). (**B**) Atypical cell are not noted, and endothelium is lined by benign cuboidal cells (H-E, original magnification, x400).

words, hemorrhage or abdominal pain may be reported, it may induce protein loss enteropathy, and intussusception may be developed by the mass[8]. Our case is also the example that the patient was admitted for bloody stool and developed intussusception on the next day. In such cases, if symptomatic or with complications, it should be removed by surgery or endoscopic resection. The indications of endoscopic polyp resection are staked masses or partially staked masses less than 2 cm in size[6]. However, in the cases of masses with a large diameter, surgical resection is required. It has been reported that generally, in the patient with intussusception, there are a large volume of hemorrhage, or protein loss enteropathy, a large mass in size, and asymptomatic patients showed a mass in a small size[9]. We experienced a case of intussusception in a 32-year-old female patient admitted for bloody stool, and it is an important causality although rare, therefore, the case is reported here together with a review of the literature.

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CASE REPORT

Spontaneous rupture of splenic hamartoma in a patient with hepatitis C virus-related cirrhosis and portal hypertension: A case report and review of the literature

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Abstract

Spontaneous rupture is a rare complication of splenic hamartoma. A review of the literature revealed only four such cases. To the best of our knowledge, this is the first report of spontaneous rupture of splenic hamartoma associated with liver cirrhosis and portal hypertension. A 53-year-old woman, who was followed up for aortic dissection and hepatitis C virus (HCV)-related liver cirrhosis, was referred with sudden left chest and shoulder pain. An abdominal ultrasound showed intraabdominal bleeding, and computed tomography indicated rupture of a splenic tumor. Emergent splenectomy was carried out. The postoperative course was uneventful, and the patient was discharged on the 13th postoperative day. Pathology revealed the tumor to be a ruptured splenic hamartoma. The non-tumorous splenic parenchyma revealed congestive changes. We consider that the presence of liver cirrhosis and portal hypertension are risk factors for spontaneous rupture of the splenic hamartoma.

Key words: Splenic hamartoma; Spontaneous rupture; Hepatitis C virus; Cirrhosis; Portal hypertension

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INTRODUCTION

Splenic hamartoma is a rare benign tumor, and is usually asymptomatic. Its spontaneous rupture is an uncommon, but life-threatening, complication. A review of the literature revealed only four such cases [1-4]. Furthermore, there has been little discussion of underlying disease and no previous reports of a ruptured splenic hamartoma in a patient with chronic liver disease. Here we report a case of spontaneous rupture of splenic hamartoma associated with hepatitis C virus (HCV)-related cirrhosis and portal hypertension.

CASE REPORT

A 53-year-old woman was referred to our emergency care center with sudden left chest and shoulder pain without a history of trauma. She was being followed up for aortic dissection (DeBakey typeⅢb) and HCV-related liver cirrhosis. The aortic dissection had been stable for seven years and the pseudo-lumen was also patent. Portal hypertension and moderate splenomegaly was associated with the liver cirrhosis. During the clinical examination, the patient lost consciousness and her blood pressure dropped below 60 mmHg, though rapid infusion of saline prompted a swift recovery of consciousness and blood pressure. Initially, ischemic heart disease was suspected, but a chest X-ray and electrocardiogram showed no abnormal findings. Laboratory data were: hemoglobin, 8.2 g/dL; platelets, $5.9 \times 10^4/m^3$; total bilirubin, 1.2 mg/dL; asparate aminotransferase, 63 IU/L; alanine aminotransferase, 53 IU/L, albumin, 2.8 g/dL; prothrombin time, 74% to control. An abdominal ultrasound revealed fluid collection suggestive of intraabdominal bleeding. Computed tomography indicated rupture of a splenic tumor, which had been not detected by the previous screening for the aortic dissection (Figure 1). The patient was transferred to our intensive care unit, where conservative treatment was started, beginning with a blood transfusion. Interventional treatment was not initially selected because of the aortic dissection. However, her blood pressure dropped again, necessitating an emergency operation.

At laparotomy, there was a massive hemorrhage with recovered blood weighing 2 880 g. Active bleeding continued from the ruptured spleen, and a splenectomy was performed. The spleen weighed 520 g and measured 15 × 10

Table 1 Literature review of spontaneous rupture of splenic hamartoma

Author	Year	Age/gender	Spleen weight/size	No. of tumors	Tumor size	Underlying disease
Morgenstern *et al* [1]	1984	73 yr/F	117 g/11×7×4.5 cm	1	2.5 cm	no
Ferguson *et al* [2]	1993	48 yr/F	459 g/15.5×8.0×4.5 cm	1	5.4 cm	no
Yoshizawa *et al* [3]	1999	5 mo/F	230 g/6×9×6 cm	1	5.0 cm	no
Ballardini *et al* [4]	2004	60 yr/M	NA	1	NA	lung cancer (chemotherapy)
Present case	2005	53 yr/F	520 g/15×10×4 cm	4	4.5 cm	liver cirrhosis, portal hypertension

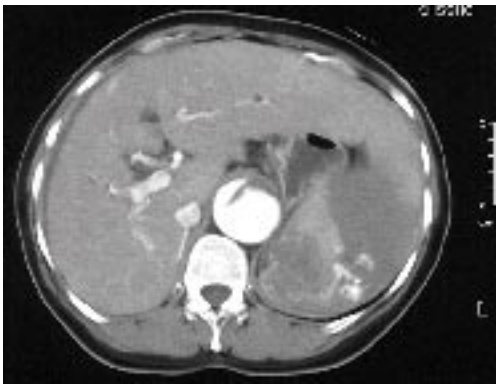

Figure 1 Enhanced computed tomography showed intraabdominal fluid collection near the spleen, and contrast material outflow from the splenic tumor.

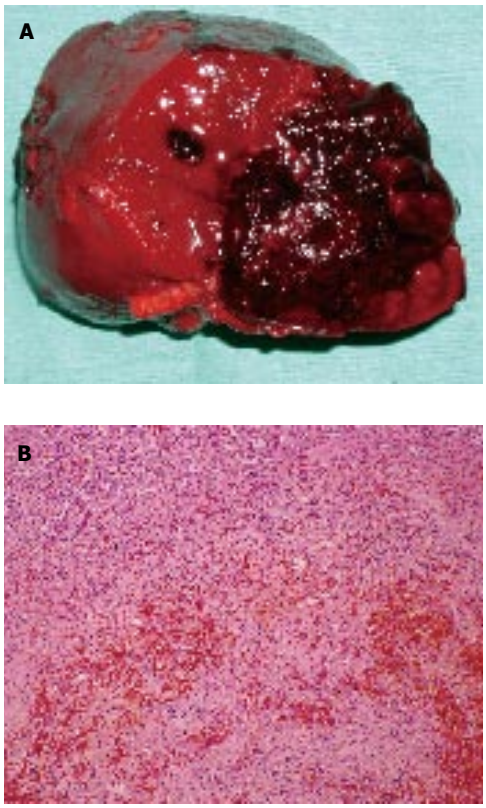

Figure 2 **A:** Macroscopic findings for the ruptured splenic hamartoma. The hamartoma is well demarcated from the surrounding parenchyma and significant hemorrhage is shown; **B:** Microscopy showed a red pulp with no lymphatic structures, or trabeculae of the spleen. (hematoxylin-eosin, original magnification, ×200).

cm with subcapsular hemorrhage. Macroscopically, there were four nodules on the cut surface of the specimen. The nodule near the upper pole was the largest, 4.5 × 3.0 cm in diameter, and ruptured, causing intraabdominal hemorrhage (Figure 2A). Microscopically, the nodules were not encapsulated and composed of sinusoidal spaces and cords of Billroth as in normal red pulp without lymphatic follicles or fibrous trabeculae (Figure 2B). The vessel spaces linked by plump endothelial cells were filled with a mixed population of erythrocytes and lymphoreticular cells. Pathology revealed the tumor to be a ruptured splenic hamartoma, of the red pulp type. The non-tumorous splenic parenchyma showed proliferation of splenic sinuses and cords with extramedullary hematopoiesis, indicating congestive changes. These findings corresponded to the splenomegaly caused by portal hypertension. The postoperative course was uneventful, and the patient was discharged on the 13th postoperative day.

DISCUSSION

Splenic hamartoma is a rare benign tumor composed of all the normal constituents of splenic parenchyma arranged in a disorganized fashion. The incidence has been reported as 0.024-0.13% from autopsy cases [5, 6]. Clinical symptoms include mass effect due to splenomegaly, pancytopenia, hematological disorders, and spontaneous rupture [5-7]. The average spleen weight and size of all reported hamartomas were about 600 g and 5.1 cm, respectively [6, 7].

Spontaneous rupture of splenic hamartoma is rare. The cause of rupture has not been specified in any of the small number of cases reported to date (Table 1). The cases discussed in these reports showed mild to moderate splenomegaly, however, the weight of the spleen (117-459 g) was below the average, and the size of the hamartomas (2.5 cm-5.4 cm in diameter) did not far exceed the average. There were also no underlying diseases, except for one case, in which the patient had lung cancer. These cases indicate that the weight of the spleen, the size of the hamartoma, or any underlying diseases, is not closely related to rupture of the hamartoma. In contrast to these cases, our case was characterized by moderate splenomegaly in association with hepatitis C virus-related cirrhosis. The patient had no other risks of spontaneous

splenic rupture, such as a history of trauma.

Whether or not hepatitis C virus-related cirrhosis contributed to the rupture of the splenic hamartoma in our case is unclear. Spontaneous rupture of the spleen is a rare complication of liver cirrhosis [8-14]. In these reported cases, portal hypertension associated with liver cirrhosis has been suspected of playing a role in splenic rupture. In our case, portal hypertension and splenomegaly due to cirrhosis was apparent, and microscopy revealed congestive changes in the non-tumorous splenic parenchyma. Since the hamartoma nodules are not encapsulated and composed of sinusoidal spaces and cords of Billroth as in normal red pulp, it is quite likely that the hamartoma itself was affected by portal hypertension. Although an association of splenic hamartoma with liver cirrhosis and portal hypertension has been demonstrated in several studies [1, 15-17], to the best of our knowledge, this is the first report of spontaneous rupture of splenic hamartoma associated with liver cirrhosis and portal hypertension.

Recent progress in imaging enables us to detect splenic tumors, and elective surgery is sometimes conducted [18-20]. Operative indications for splenic hamartoma include the possibility of malignant disease and the risk of spontaneous rupture. If splenic hamartoma is suspected by image findings, elective splenectomy should be taken into consideration to make a definitive diagnosis and to prevent rupture [21]. We consider that the presence of liver cirrhosis and portal hypertension are risk factors for spontaneous rupture of the splenic hamartoma.

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CASE REPORT

A case of primary biliary cirrhosis complicated by Behçet's disease and palmoplantar pustulosis

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Abstract

A 46-year-old woman was diagnosed with palmoplantar pustulosis (PPP) at the Department of Dermatology, Fukushima Medical University Hospital in 2000, and was treated with ointment. However, because liver dysfunction developed in 2003, she was referred to our department, where primary biliary cirrhosis (PBC) was also diagnosed on the basis of clinical findings. One year later, at the age of 49, she developed manifestations of Behçet's disease (BD), including erythema nodosum in the lower extremities. Because she had a history of uveitis, recurrent oral ulceration was present, and the HLA typing was positive for B51, BD was additionally diagnosed. Liver function normalized within three months of the start of treatment with ursodesoxycholic acid (UDCA). This is the first case of PBC associated with BD and PPP.

Key words: Primary biliary cirrhosis; Behçet's disease; Palmoplantar pustulosis

Iwadate H, Ohira H, Saito H, Takahashi A, Rai T, Takiguchi J, Sasajima T, Kobayashi H, Watanabe H, Sato Y. A case of primary biliary cirrhosis complicated by Behçet's disease and palmoplantar pustulosis. *World J Gastroenterol* 2006;12(13):2136-2138

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INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease associated with a variety of other conditions, including Sjögren's syndrome, Hashimoto's thyroiditis, scleroderma or other components of CREST syndrome, and inflammatory arthritis [1,2], but its association with Behçet's disease (BD) is very rare, and so is its association with palmoplantar pustulosis (PPP) [3]. We report the first case of PBC associated with BD and PPP, and discuss the relationship among these three diseases in terms of the environmental factors, such as infectious agents, described in the literature.

CASE REPORT

A 37-year-old woman suffered from uveitis in 1991. In 2000, she was diagnosed with palmoplantar pustulosis (PPP) at the Department of Dermatology, Fukushima Medical University Hospital, and was treated with ointment. However, liver dysfunction developed, and she was referred to our department in March 2003 at the age of 49 years.

At our first medical examination, she complained of no symptoms involving the oral mucosa or extremities except the lesion of pustulosis. Blood pressure was normal, and there was no history of diabetes mellitus or hyperlipidemia. Laboratory data showed liver dysfunction with elevated levels of AST (149 IU/L), ALT (340 IU/L), ALP (428 IU/L; normal, 125-335 IU/l), GGT (228 IU/L; normal, 6-30 IU/l), T-Bil (0.7 mg/dL) and IgM (130 mg/dL; normal, 52-270 mg/dL). The titer of anti-mitochondrial autoantibodies (AMA) was 1:20, and the index of AMA-M2 antibody was 32 (normal, <7). Results of hepatitis B and C serologic tests were negative. After one month, subarachnoid hemorrhage occurred owing to the bursting of a cerebral artery aneurysm. An emergency brain operation was performed, revealing four cerebral artery aneurysms including the ruptured one (Figure 1), which were clipped successfully. Her hospital course was otherwise uneventful, and her slight cerebral signs resolved within days of the operation, and she was discharged from the hospital, neurologically intact. Liver biopsy was, therefore, postponed. PBC associated with PPP was diagnosed on the basis of clinical findings based on 'Criteria for Diagnosis of PBC in Japan' by the Study Group for Autoimmune Hepatitis, a subdivision of the Research Group for Intractable Hepatitis, sponsored by the Ministry of Health and Welfare of Japan [4]. Liver function normalized within three months of the start of treatment with ursodesoxycholic acid (UDCA) (600 mg/d).

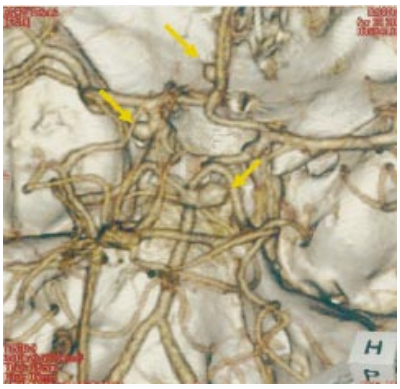

Figure 1 Computed tomographic angiography shows aneurysms of the left. internal carotid-posterior communicating artery, left. anterior communicating artery, and right. posterior cerebral artery (arrows).

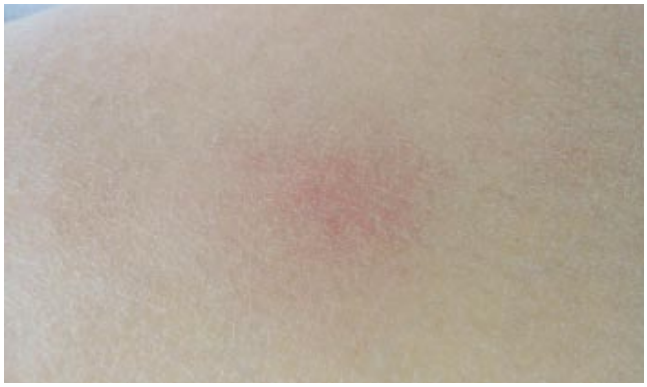

Figure 2 Typical eruption of erythema nodosum shows erythematous nodules on the anterior aspect of the leg.

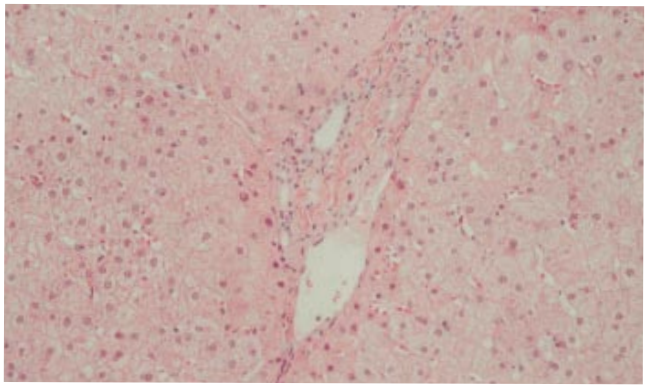

Figure 3 Liver biopsy specimen shows slightly enlarged portal tracts with no evidence of chronic nonsuppurative destructive cholangitis. Hematoxylin and eosin, original magnification ×100.

Erythema appeared in the lower extremities in July 2004 (Figure 2). Oral aphthous ulcers were sometimes noted, too. Lymphadenopathy was not recognized. No abnormal findings were noted in the chest or abdomen. Laboratory data at the time were as follows: increased level of CRP, 3.1mg/dL (normal, <0.3) and ESR, 73mm/h (normal, 3-15); WBC, 9 400/mm^3, (normal, 2800-8800). Liver function and renal function were normal. HLA typing was positive for A2, A30 (19), B54 (22), B51 (5), Cw1 and DR9. Because HLA B51 was positive, a skin biopsy of the erythema was performed. Histological examination showed erythema nodosum characterized by a neutrophilic inflammatory infiltrate involving the septa of the subcutaneous tissue. Incomplete type Behçet's disease (BD) was diagnosed on the basis of recurrent oral aphthous ulcers, erythema nodosum, and past history of uveitis according to the diagnostic criteria from the Behçet's Disease Research Committee of Japan (1987 revision)[4]. A non-steroidal anti-inflammatory drug was administered orally as palliative treatment, and erythema nodosum resolved.

Liver biopsy was performed in January 2005, but histological examination did not show typical chronic nonsuppurative destructive cholangitis (CNSDC) (Figure 3) because liver function was improved by 2-year UDCA treatment.

DISCUSSION

BD is a well-known multisystem inflammatory disorder of unknown etiology that is characterized by oral and genital ulcers, uveitis, and a variety of other manifestations, such as erythema nodosum, polyarthritis, thrombophebitis, and ulceration of the intestinal mucosa [6]. Involvement of the liver is less common; however, systemic amyloidosis and Budd-Chiari syndrome have been documented [7, 8]. Susceptibility to BD is associated with the HLA B51 allele.

There have been only a few cases of PBC associated with BD [9]. In the present case, the clinical manifestations of BD were oral aphtha, erythema nodosum and uveitis, and there was no genital ulcer or intestinal involvement. However, it is possible that the intracranial aneurysms were related to BD. Cerebral artery aneurysms in patients with BD are uncommon, but there are 14 cases reported in the literature [10]. These cases are quite similar to ours in which the aneurysms are multiple. Furthermore, in our case there was no risk factor that would produce a cerebral vessel event: hypertension, hyperlipidemia, or diabetes mellitus. Some BD case reports describe the efficacy of steroid therapy for multiple nonruptured cerebral artery aneurysms [11-13]. Kerr *et al*, however, report a patient with BD who developed multiple aneurysms and subarachnoid hemorrahge in rapid succession under steroid therapy [14]. In our patient, follow-up brain computed tomographic angiography was performed after surgical treatment, but no aneurysms were detected.

PBC is an autoimmune liver disease characterized by the occurrence of AMA. Clinically, PBC is associated with a large variety of other diseases, such as arthropathy, CREST syndrome, and autoimmune thyroiditis [15]. To our knowledge, however, no report has ever described the simultaneous development of three autoimmunological diseases, PBC, BD, and PPP.

This is the first case report of PBC complicated by BD and PPP, which is a localized type of psoriasis. Although psoriasis is characterized by proliferation of the epidermis, the immune system plays a prominent role in its development [16]. Interaction between keratinocytes and T cells is involved in the pathogenesis, just as interaction between biliary epithelial cells and T cells is in the pathogenesis of PBC. In BD, lymphocytic infiltration is observed around the arteries. Moreover, some workers suggested that some microbial antigen is closely associated with the development of these three diseases [17-19].

Memory T cells specific to these bacterial antigens may be activated by antigens on the arteries, skin or liver. We believe that patients with PBC associated with BD and PPP hold the key to clarifying the nature of these diseases.

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CASE REPORT

Chest wall metastasis from unknown primary site of hepatocellular carcinoma

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Abstract

Previous reports of a solitary metastatic hepatocellular carcinoma have been rare. Because this tumor has a different treatment modality and prognosis, an accurate differential diagnosis is essential. Here we report a rare case of a solitary chest wall metastasis from unknown primary site of hepatocellular carcinoma. It involves a 51-year-old man who was admitted to our hospital because of a palpable left upper chest wall mass. The mass was resected and pathologic examination confirmed a diagnosis of metastatic hepatocellular carcinoma. Despite our investigation, no evidence was found that indicated the primary origin of the hepatocellular carcinoma. Four months later, the patient was admitted again because of spinal cord compression at the third and fourth thoracic vertebrae. Emergent decompressive laminectomy was performed and microscopic features revealed the same pathology as the initial chest wall mass resected 4 months earlier. After one year, a follow-up abdominal computed tomography (CT) still revealed no evidence of primary hepatocellular carcinoma.

Key words: Chest wall; Hepatocellular carcinoma; Metastasis

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INTRODUCTION

The most common causes of a chest wall mass in adults are infection or tumor,[1] and the majority of the malignant masses are hematological in origin. Malignant chest wall masses arising from a primary focus in the lung (metastatic lung cancer) or the liver (metastatic hepatocellular carcinoma) are rare.[2] Primary hepatocellular carcinoma often metastasizes out of the liver. Metastasis typically occurs in patients with advanced stages of the disease, and the most common site is the lungs, followed by the abdominal wall, lymph nodes, bone, adrenal gland, pancreas, kidney and spleen.[3,4] Katyal *et al* [5] categorized the intrahepatic stage of the tumor according to TNM staging, and reported that extrahepatic metastasis of small hepatocellular carcinomas (confined to stage one) was rare. References indicates that hepatocellular carcinomas found outside the liver without a primary focus are usually due to carcinogenesis of ectopic liver. Cases of hepatoid adenocarcinoma have previously been reported, but a solitary extrahepatic hepatocellular carcinoma that has metastasized to the chest wall has rarely been reported. Because these types of tumors have different treatment modalities and prognoses, an accurate differential diagnosis is essential. Here, the authors have diagnosed a solitary metastatic hepatocellular carcinoma of the chest wall, with an unknown primary focus, and have been closely following the patient for one year since the diagnosis.

CASE REPORT

A 51-year-old male visited the hospital because of a left upper chest wall mass that developed during the prior 8 months. Ten years ago he was diagnosed with chronic viral hepatitis B but was lost to follow-up in the intervening period. The patient had not received any treatment since the mass was first discovered, and the mass progressively grew in size until the patient sought treatment at the hospital. The patient was a heavy drinker and smoker, with a drinking history of two bottles of beer per week for 30 years, and a smoking history of 15 pack years. There was no significant family history. The patient's initial vital signs were stable and he appeared relatively well, and was alert and responsive. On physical examination, a hard, palpable mass about 10 cm × 12 cm in size was noted on the left upper anterior chest wall. Palmar erythema and spider angioma on the anterior chest wall were noted, but there were no other specific complaints. Initial laboratory findings were

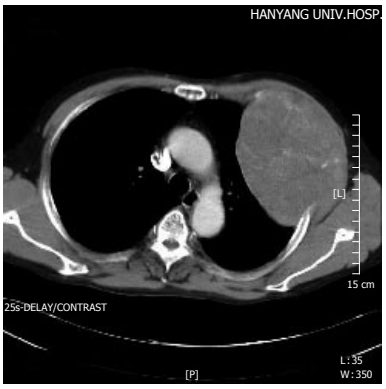

Figure 1 Chest helical CT. A mass on the anterolateral aspect of the left chest wall. The inner part of the mass is heterogeneously attenuated and consistent with mottled calcification. There is no evidence of metastasis in the mediastinum or the lung parenchyma.

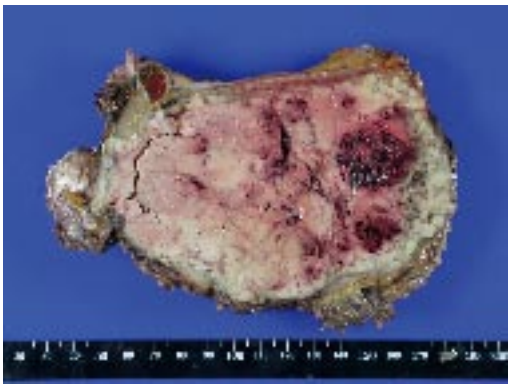

Figure 2 Gross findings of the chest wall tumor. The cut surface shows an encapsulated, yellowish-white solid mass with areas of hemorrhage and necrosis.

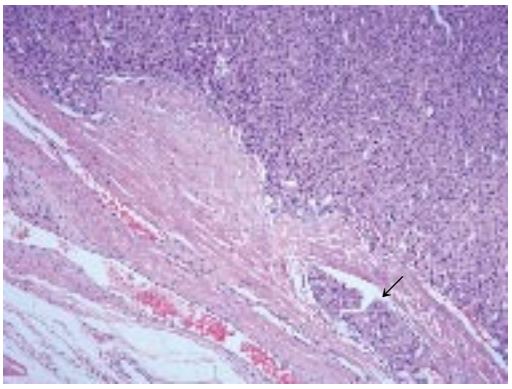

Figure 3 Microscopic findings of the chest wall tumor. The tumor displays typical features of well-differentiated, trabecular liver cell carcinoma with intravascular tumor emboli (arrow) (H&E stain, ×200).

as follows: WBC 10 000/mm^3, hemoglobin 134 g/L, platelets 216 000/mm^3, serum protein 56 g/L, albumin 34 g/L, total cholesterol 214 mg/dL, serum glucose 113 mg/dL, total bilirubin 0.9 mg/dL, AST 38 U/L, ALT 52 U/L, alkaline phosphatase 104 U/L, and prothrombin time 135%. Patient's viral markers were as follows: HBsAg(+), HBcAb(+), HBeAg(-), HBeAb(+) and HBV DNA < 2.5pg. Serum alpha-fetoprotein was 308.3 ng/mL.

The density of the soft tissue protruding inwards towards the lung was noted, with accompanying evidence of rib destruction along the anterolateral aspect of the third left rib on the chest film. A 9.5 cm× 12.7 cm sized heterogeneous tumor with a mottled calcification was noted on the anterolateral aspect of the left chest wall on the CT, but there was no evidence of metastasis within the lung parenchyma or the mediastinum (Figure 1). An additional study of abdominal ultrasonography and whole body bone scan revealed no evidence of an intrahepatic mass, abdominal lymphadenopathy, or bone metastasis. A primary bone tumor on the left upper chest wall was suggested, and radical resection of the tumor around the rib and soft tissue was performed.

The resected tumor was well-encapsulated with dimensions 14 cm× 10 cm× 7.5 cm and weighed 650 mg including the excised rib tissue. The cut surface of the tumor was whitish-brown with accompanying hemorrhage and necrosis, and was solid and hard but tended to crumble easily (Figure 2). Microscopic examination revealed large polygonal cancer cells separated by thin vessels resulting in a pole-like arrangement, and multiple cells undergoing mitosis were seen. Cancer cell emboli were seen inside the vessels surrounding cancer cells, and there was no evidence of normal liver tissue within the microscopic field (Figure 3). Immunohistochemical staining showed positive results for alpha-fetoprotein, hepatocyte specific antigen and cytokeratin, and showed negative results for vimentin and smooth muscle actin (Figure 4).

Abdominal computerized tomography (CT) and abdominal magnetic resonance imaging (MRI) were performed in search of the primary focus of the hepatocellular carcinoma. The surface of the liver was slightly irregular which was suspicious of liver cirrhosis, but there was no evidence of hepatocellular carcinoma, and no lipiodol uptake nor tumor vessels were seen in the hepatic arteriography. Mild lobular necrosis with mild portal inflammation and macronodular cirrhosis on the portal area were noted upon liver biopsy, but there was no evidence of a tumor. Upon esophagogastroduodenoscopy, antral gastritis was present but there were no gastric or esophageal varices, and there was no significant sigmoidoscopic finding. Postoperative alpha-fetoprotein level decreased from 308.3 ng/mL to 42.4 ng/mL. Follow-up abdominal CT was performed three weeks after the first hepatic arteriography, and like before, no lipiodol uptake was seen. There were no operation-related complications, and the patient was discharged to be regularly monitored as an outpatient.

Four months after discharge, the patient visited the emergency room due to back pain radiating to both lower extremities. On physical examination, there was evidence of motor weakness in both lower extremities, and the patient complained of paresthesia and hyperesthesia in the dermatome below the forth thoracic vertebra. Immediate thoracic MRI revealed spinal cord compression due to a metastatic lesion to the third and forth thoracic vertebrae, with spinal deviation to the right of the posterolateral position (Figure 5). Emergent decompressive laminectomy was performed. Further evaluation with a whole body bone scan revealed a newly developed increased uptake at the left side of the third to fifth thoracic vertebrae, while abdominal ultrasonography showed no significant change from the initial diagnosis of metastatic hepatocellular carcinoma in the chest wall. Histological examination of the metastatic vertebral lesions revealed metastatic hepatocellular carcinoma, which was the same finding observed dur-

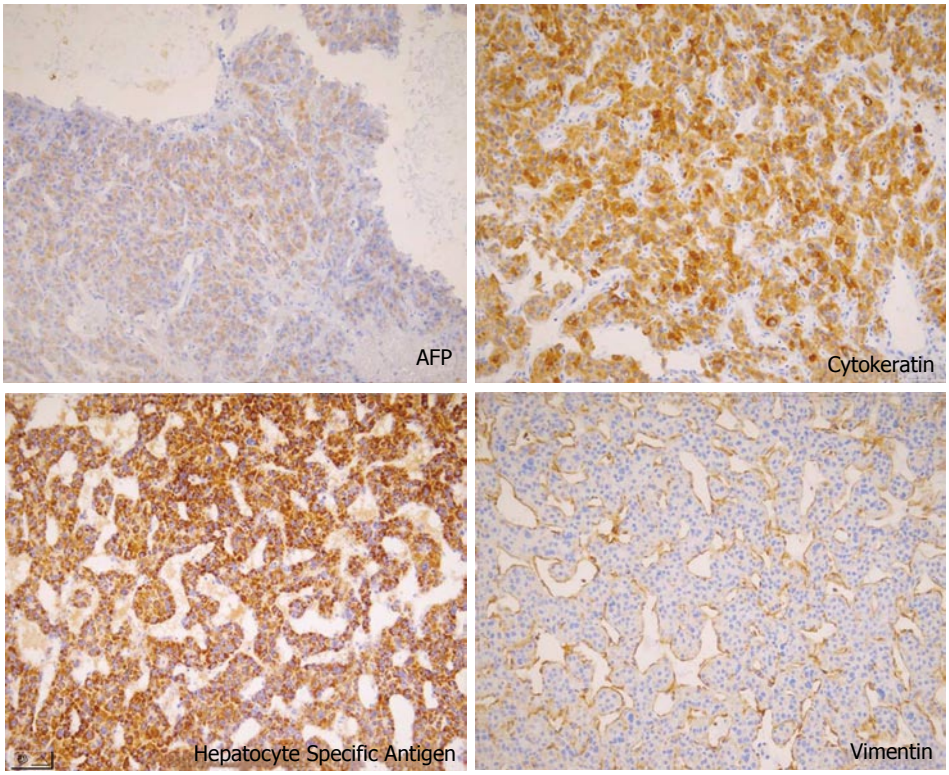

Figure 4 Microscopic findings of the chest wall tumor. The tumor cells are positive for AFP, cytokeratin, and hepatocyte specific antigens. This section shows positive staining for hepatocyte-specific antigens (IHC, ×400).

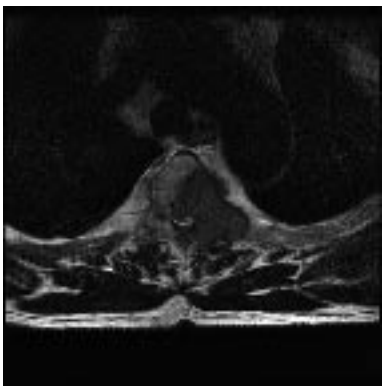

Figure 5 Thoracic spine MRI. The metastatic tumor of the third vertebra compressing the spinal cord (T2 weighted image).

ing microscopic examination of the chest wall mass. Afterwards the patient received radiotherapy and adriamycin-based chemotherapy. One year later, no lesion suggesting primary hepatocellular carcinoma has been found in the follow-up abdominal CT scan.

DISCUSSION

An extrahepatic hepatocellular carcinoma without a primary intrahepatic hepatocellular carcinoma can be explained in two different ways. The first is ectopic liver carcinogenesis and the second is a hepatoid adenocarcinoma.[6-8]

Ectopic liver is a rare developmental abnormality that can originate in the gall bladder, hepatic ligament, omentum, posterior peritoneal cavity, or the thorax.[8-11] Because ectopic liver does not have perfect functional architecture as does a normal liver, it is more likely to develop hepatocellular carcinoma, if carcinogenic factors such as viral hepatitis infection or liver cirrhosis are involved.[6]

Characteristically, the pathologic examination of hepatocellular carcinoma arising from ectopic liver reveals normal liver tissue, including portal triads. It may be connected to the liver by a fibrous stalk, which is composed of the portal vein, hepatic artery, or bile duct. If no evidence of primary cancer of the liver is present after a long term follow-up with various liver imaging studies, a malignancy originating from ectopic liver should be suspected .[7] In Japan, 25 cases of hepatocellular carcinoma of ectopic liver were described. This can be treated with complete resection of the tumor, which yields a good prognosis.

Hepatoid adenocarcinoma is a variant form of adenocarcinoma, characterized by vast hepatic differentiation. It produces alpha-fetoprotein, while having the same function and form as hepatocellular carcinoma, but originates from the endoderm and mucosa of urogenital organs and therefore can manifest itself in the gastrointestinal tract, ovaries, pancreas, lungs, kidneys, uterus or bladder. It contains a characteristic moderately to well-differentiated tubular or papillary shaped adenocarcinoma[8].

The present case differs from the two aforementioned examples because it was discovered as a solitary metastatic hepatocellular carcinoma of the chest wall, with an unknown primary focus. First, it was composed solely of hepatocellular carcinoma, and microscopic examination revealed no evidence of adenocarcinoma nor normal liver tissue including portal triads. This finding was entirely different from that expected in the carcinogenic sequence of ectopic liver or hepatoid adenocarcinoma. Second, cancer cell emboli were found within the vessels near the cancer cells, which is a characteristic microscopic finding of metastatic cancer. Thirdly, there was no evidence of an intrahepatic tumor in the liver imaging studies, and this result was again seen during the follow-up abdominal CT one year later. Fourthly, four months after resection of the chest wall tumor, metastatic hepatocellular carcinoma was found in the thoracic vertebrae. The thoracic vertebrae

are a region of intense bone marrow replication, and are among the most important metastatic sites for hepatocellular carcinoma[12].

In summary, when hepatocellular carcinoma is found outside the liver without a primary focus within the liver, a tissue biopsy must be obtained. Tissue biopsy must confirm the presence of normal liver tissue, including portal triads and cancer emboli within vessels near the tumor, for an accurate differential diagnosis. Chest CT, abdominal ultrasonography, and whole body bone scans must be used in order to confirm the presence of metastasis to other organs. To discern between hepatocellular carcinoma of ectopic liver and solitary metastatic hepatocellular carcinoma without a primary focus in the liver, a follow-up study is essential after the operation in order to find the primary site of origin.

Although the patient had no evidence of an intrahepatic tumor, he was a hepatitis B carrier with liver cirrhosis which suggested a latent primary hepatocellular carcinoma metastasizing to the chest wall that spontaneously regressed. In cases like this, in which extrahepatic hepatocellular carcinoma exists without evidence of tumor within the liver, one has to consider the possibility of a tumor originating from ectopic liver tissue, hepatoid adenocarcinoma, or, although rare, metastatic hepatocellular carcinoma without an intrahepatic mass.

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Meetings

MAJOR MEETINGS COMING UP

Digestive Disease Week
107th Annual of AGA, The American Gastroenterology Association
20–25 May 2006
Loas Angeles Converntion Center, California

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

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Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
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World Congress on Gastrointestinal Cancer
28 June 2006–1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6–8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

7th World Congress of the International Hepato-Pancreato-Biliary Association
3–7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

Society of American Gastrointestinal Endoscopic Surgeons
26–29 April 2006
Dallas – TX
www.sages.org

Digestive Disease Week 2006
20–25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25–26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3–7 June 2006
Seatlle - Washington
www.fascrs.org

EVENTS AND MEETINGS IN 2006

10th World Congress of the International Society for Diseases of the Esophagus
22–25 February 2006
Adelaide
isde@sapmea.asn.au
www.isde.net

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24–25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3–8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26–30 April 2006
Vienna, Austria
Kenes International

VII Brazilian Digestive Disease Week
19–23 November 2006
www.gastro2006.com.br

International Gastrointestinal Fellows Initiative
22–24 February 2006
Banff, Alberta
Canadian Association of Gastroenterology
cagoffice@cag-acg.org
www.cag-acg.org

Canadian Digestive Disease Week
24–27 February 2006
Banff, Alberta
Digestive Disease Week Administration
cagoffice@cag-acg.org
www.cag-acg.org

Prague Hepatology 2006
14–16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1–5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com/

Falk Seminar: XI Gastroenterology Seminar Week
4–8 February 2006
Titisee
Falk Foundation e.V.
symposia@falkfoundation.de

European Multidisciplinary Colorectal Cancer Congress 2006
12–14 February 2006
Berlin
Congresscare
info@congresscare.com
www.colorectal2006.org

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4–5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6–7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

14th United European Gastroenterology Week
21–25 October 2006
Berlin
United European Gastroenterology Federation
www.uegw2006.de

World Congress on Controversies in Obesity, Diabetes and Hypertension
25–28 October 2006
Berlin
comtec international
codhy@codhy.com
www.codhy.com

Asia Pacific Obesity Conclave
1–5 March 2006
New Delhi
info@apoc06.com
www.apoc06.com/

ILTS 12th Annual International Congress
3–6 May 2006
Milan
ILTS
www.ilts.org

XXX Panamerican Congress of Gastroenterology
11–16 November 2006
Cancun
www.panamericano2006.org.mx

Internal Medicine: Gastroenterology
22 July 2006–1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15–18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

Hepatitis 2006
25 February 2006–5 March 2006
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28 June 2006–1 July 2006
Barcelona, Spain
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International Conference on Surgical Infections, ICSI2006
6–8 September 2006
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European Society of Clinical Microbiology and Infectious Diseases
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www.icsi2006.se/9/23312.asp

5th International Congress of The African Middle East Association of Gastroenterology
24–26 February 2006
Sharjah
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7th World Congress of the International Hepato-Pancreato-Biliary Association
3–7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

13th International Symposium on Pancreatic & Biliary Endoscopy
20–23 January 2006
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2006 Gastrointestinal Cancers Symposium
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Society of American Gastrointestinal Endoscopic Surgeons
26–29 April 2006
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Digestive Disease Week 2006
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71st ACG Annual Scientific and Postgraduate Course
20–25 October 2006
Venetian Hotel, Las Vegas, Neveda
The American College of Gastroenterology

AASLD 57th Annual – The Liver Meeting™
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Boston, MA
AASLD

New York Society for Gastrointestinal Endoscopy
13–16 December 2006
New York
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Acknowledgments
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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 Xiaohua Zazhi* 1999; 7: 285-287

Books and other monographs (list all authors)

4 **Sherlock S,** Dooley J. Diseases of the liver and billiary 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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World J Gastroenterol 2006 April 7; 12(13):2147
World Journal of Gastroenterology ISSN 1007-9327

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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_2SO_4	0.5 mol/L H_2SO_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_r18 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 $\cdot L^{-1}$	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	A_{260} nm	*A* should be in italic.
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45	**F*=9.87, $^§F$=25.9, $^\#F$=67.4	^1F=9.87,^2F=25.9, ^3F=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	$10\times10\times10cm^3$	10 cm×10 cm×10 cm	
66	N·km	KN·m	moment
67	$x\pm s$	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 *et al*	
72	EcoRI	*Eco*RI	*Eco* in italic and RI in positive. Restriction endonuclease has its prescript form of writing.
73	Ecoli	*E.coli*	Bacteria and other biologic terms have their specific expression.
74	Hp	*H pylori*	
75	Iga	*Iga*	writing form of genes
76	*igA*	IgA	writing form of proteins
77	-70 kDa	~70 ku	

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10	>	*t*>5	*t* > 5	Space before ">" and after ">" is 0.5 character
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12	⩽	Ages⩽6 mo	Ages ⩽ 6 mo	Space before "⩽" and after "⩽" is 0.5 character
13	()	Bardeen-Cooper-(BCS)theory[4]	Bardeen-Cooper-Schrieffer (BCS) theory[4]	Space before "(" and after ")" is 0.5 character
14	[]	△△Gint = -RTIn [(θwwθmm)/(θwmθmw)]	△△Gint = -RTIn [(θwwθmm)/(θwmθmw)]	Space between "[" and " (" , and between ") " and "] " is 0.5 character
15	,	γ-3,γ-4 andγ-8	γ-3, γ-4 andγ-8	Space between "," and following word is 1 character
16	;	LDT;yellow	LDT; yellow	Space between " ;" and following word is 1 character
17	:	Discovering Dorothea: The life of the Pioneering Fossil-Hunter Dorthea Bate	Discovering Dorothea: The life of the Pioneering Fossil-Hunter Dorthea Bate	Space between ":" and following word is 1 character
18	?	If he had not wavered for two years?We shall never know.	If he had not wavered for two years? We shall never know.	Space between "?" and following word is 1 character
19	…	It is very near to our thoughts, while the ignorance regarding these lands is being very rapidly dispelled…China stands not at the parting of the ways.	It is very near to our thoughts, while the ignorance regarding these lands is being very rapidly dispelled… China stands not at the parting of the ways.	Space between "…" and following word is 1 character
20	' '	Simpler 'prokaryotic'cells	Simpler 'prokaryotic' cells	Space between " ' " and preceding word, and between " ' " and following word is 1 character
21	" "	The PTO calls situation a"workload crisis" in its strategic plan.	The PTO calls situation a "workload crisis" in its strategic plan.	Space between ' " ' and preceding word, and between ' " ' and following word is 1 character
22	.	There are two approaches converging on this issue. One is rooted in social science, the other in evolutionary biology.	There are two approaches converging on this issue. One is rooted in social science, the other in evolutionary biology.	Space between ". " and following word is 1 character
23	℃	3.5℃	3.5 ℃	Space between "℃" and front number is 0.5 character

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EDITORIAL

Role of Toll-like receptors in health and diseases of gastrointestinal tract

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Abstract

The human gastrointestinal (GI) tract is colonized by non-pathogenic commensal microflora and frequently exposed to many pathogenic organisms. For the maintenance of GI homeostasis, the host must discriminate between pathogenic and non-pathogenic organisms and initiate effective and appropriate immune and inflammatory responses. Mammalian toll-like receptors (TLRs) are members of the pattern-recognition receptor (PRR) family that plays a central role in the initiation of innate cellular immune responses and the subsequent adaptive immune responses to microbial pathogens. Recent studies have shown that gastrointestinal epithelial cells express almost all TLR subtypes characterized to date and that the expression and activation of TLRs in the GI tract are tightly and coordinately regulated. This review summarizes the current understanding of the crucial dual roles of TLRs in the development of host innate and adaptive immune responses to GI infections and the maintenance of the immune tolerance to commensal bacteria through down-regulation of surface expression of TLRs in intestinal epithelial cells.

Key words: Toll-like receptor; Gastrointestinal tract; Intestinal disease

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INTRODUCTION

Innate immunity is considered to be important for the elimination of invading microbes from the gastrointestinal tract and for the control of their systemic dissemination. Mammalian toll-like receptors (TLRs) are members of the pattern-recognition receptor (PRR) family and play a central role in the initiation of innate cellular immune responses and the subsequent adaptive immune responses to microbial pathogens[1,2]. The capacity to recognize diverse pathogen-associated molecular patterns (PAMPs) that are unique to microorganisms and therefore absent from host cells makes TLRs well-suited to act as an early warning system against invading pathogens. Activation of the TLR signal transduction pathway leads to the induction of numerous genes that function in host defense, including those for inflammatory cytokines, chemokines, antigen-presenting molecules, and costimulatory molecules[1,2]. Recognition of PAMPs by TLRs differs from the recognition of microorganism-specific antigens by the adaptive immune system, in that PAMPs are typically highly conserved across several species of microorganisms, such as surface lipoproteins common to several bacterial species, or genetic material from an entire family of viruses. The ability of TLRs to recognize a broad spectrum of microbial molecules enables the host to detect the presence of pathogens rapidly, before a more widespread infection occurs.

In this review, we have briefly summarized the recent progress in the understanding of the role of TLRs in the host defense against gastrointestinal pathogens and in the maintenance of immune tolerance to commensal microflora. For more general information on the biological functions of TLRs and the TLR signaling pathway, the readers are referred to a number of excellent review articles in this field[3-7].

TLRs, TLR LIGANDS AND TLR SIGNALING PATHWAYS

To date, 11 related TLR genes have been identified and characterized (*tlr1* to *tlr11*) (Table 1)[3,4,7-9]. Some TLRs, such as TLR3, TLR5 and TLR9, only recognize one type of PAMP, while others, such as TLR2, appear to recognize several different microbial molecules. Among these, TLR4 is the signal-transducing element of the

Table 1 Toll-like receptors and known microbial ligands[4,7,18]

TLR family	Microbial ligands
Lipid ligands	
TLR1	Tri-acyl lipopeptides (bacteria, mycobacteria)
TLR2	Lipoprotein/lipopeptides (a variety of pathogens)
	Peptidoglycan (Gram-positive bacteria)
	Lipoteichoic acid (Gram-positive bacteria)
	Lipoarabinomannan (mycobacteria)
	A phenol-soluble modulin (*Staphylococcus epidermidis*)
	Glycoinositolphospholipids (*Trypanosoma Cruzi*)
	Glycolipids (*Treponema maltophilum*)
	Porins (*Neisseria*)
	Zymosan (fungi)
	Atypical LPS (*Leptospira interrogans* and *Porphyromonas gingivalis*)
	Hemagglutinin (measles)
TLR4	LPS (Gram-negative bacteria)
	Fusion protein (respiratory syncytial virus)
	Envelope proteins (mouse mammary tumor virus)
	HSP60 (*Chlamydia pneumoniae*)
TLR6	Di-acyl lipopeptides (mycoplasma)
Nucleic acid ligand	
TLR3	Double-stranded RNA (virus)
TLR7 or 8	U-rich ssRNA
TLR9	CpG DNA (bacteria)
Protein ligand	
TLR5	Flagellin (bacteria)
Uropathogenic bacteria	
TLR11	Uropathogenic bacteria
Ligand unknown	
TLR10	?

lipopolysaccharide (LPS) receptor complex, and is also involved in the signaling response to other exogenous stimuli [*e.g.*, bacterial HSP60 and fimbriae, *Streptococcus pneumoniae* pneumolysin, lipoteichoic acid (LTA) from gram-positive bacteria, and respiratory syncytial virus coat protein][10,11]. TLR2 binds to bacterial lipoproteins, LTA and peptidoglycan[11-13], although some recent studies have argued that peptidoglycan recognition does not occur through TLR2[14], or that TLR2 alone is not sufficient to detect peptidoglycan[15]. Flagellin, a bacterial protein involved in motility, binds TLR5[16]. CpG, a repetitive sequence of unmethylated nucleic acids found in high quantities in bacterial DNA, is recognized by TLR9[17]. Also, although the specific ligand is not yet known, murine TLR11 is involved in protection from uropathogenic bacterial infection in mice[18]. Certain bacterial virulence factors, such as fimbriae or enterotoxins, have been shown to activate TLR2 and/or TLR4[19-23]. Some viruses are also recognized by TLRs. Double-stranded RNA (dsRNA), which is found in many types of virus, elicits immune responses through TLR3[24] and probably another PRR[25, 26]. Human TLR7 and/or TLR8 are known to bind single-stranded RNA (ssRNA) from viruses, such as human immunodeficiency virus (HIV)-1, influenza and human parechovirus-1[27-29]. TLR specificity is not limited to bacterial or viral PAMPs. TLR2 and/or TLR4 have been implicated in the detection of *Candida albicans* and *Entamoeba histolytica*[30-34]. In addition, some TLRs also bind endogenous molecules, such as HSP60, fibronectin, surfactant protein A, and β-defensin-2[4, 9].

TLRs vary from one another by their ligand specificity, determined by the extracellular portion of the receptor. The cytoplasmic tails of TLRs appear to be associated with the tails of other TLRs in a process known as TLR cooperation[35]. This can occur between receptors of similar or different specificity. For example, TLR2 requires association with TLR6 in order to propagate the correct intracellular signal after binding peptidoglycan or zymosan (a yeast cell-wall particle)[35]. In the cytoplasmic domain of TLRs, the element common to all TLRs is the Toll-interleukin-1-related (TIR) domain. After homo- or heterodimerization of TLRs, the intracellular TIR domains self-associate, and bind TIR domains of intracellular adaptor molecules. All TLRs except TLR3 associate with the TIR-containing myeloid differentiation factor (MyD) 88[36], which upon activation mediates a signaling cascade leading to activation of the NF-κB transcription factor[6]. The end result of TLR signaling is an upregulation of pro-inflammatory cytokines and chemokines, such as TNF-α and IL-8, and the induction of a localized immune response.

TLR4 was the first PRR to be properly identified as having a specific ligand[10], and the mechanism of TLR/LPS interaction is thus the best studied. LPS is transferred to cell-surface CD14 by LPS-binding protein (LBP)[37,38]. CD14 does not signal LPS presence directly to the cell because it lacks a cytoplasmic domain. Instead, the proximity of CD14 to TLR4 allows CD14 to "present" LPS to TLR4[10,39,40], which itself is bound to MD-2 on the cell surface. A physical association on the cell surface between MD-2 and TLR4 is essential for TLR4 function[41], and MD-2 is in fact essential for TLR4 to be trafficked to the cell surface in the first place[42].

TLR ACTIVITY IN THE GASTROINTESTINAL (GI) TRACT

Emerging evidence has shown that TLR expression and activation is specially regulated in the GI tract. This is probably due to the continuous presence of physiological microflora in the gut. It is essential that TLRs do not react to PAMPs expressed by commensal microflora, yet retain the ability to detect and mount effective immune responses against invading pathogens. This is mainly accomplished by the down-regulation of surface expression of TLRs, such as TLR2, TLR4 and MD-2, in the gut epithelium[5,43-47]. Although intestinal epithelial cells (IEC) can and sometimes do express TLR2 and/or TLR4[46,48-50], these TLRs usually relocate to either intracellular compartments such as the Golgi apparatus, or to the basolateral membrane of the cell as a result of the continuous stimulation by varying components of the commensal bacteria[50-53]. Indeed, *in vitro* studies of an IEC line have shown that LPS or peptidoglycan stimulation relocates the constitutive surface expression

of TLR2 and TLR4 into intracellular compartments near the basolateral membrane[51]. Others have shown that both primary and immortalized IEC responded to TLR ligand stimulation, and that prolonged exposure to these ligands reduced surface expression of TLRs without reducing mRNA levels[49]. It is important to note that intracellular TLR4 retains its full signaling capability, and detects both internalized LPS and intracellular bacteria[52,53]. This mechanism allows the host to detect the pathogenic organisms that have penetrated the intestinal epithelium without overreaction to commensal bacteria on the surface of intestinal epithelium.

There have been some debates over the precise cellular localization of TLR5, the receptor for flagellin, in IEC[54-57]. One group has shown that TLR5 was only expressed on the basolateral membrane[55], whereas another group using a different cell line showed both basolateral and apical TLR5 expression following the stimulation with *Escherichia coli* flagellin[54]. Apical TLR5 expression has also been demonstrated *ex vivo* in the murine ileum[54]. In addition, *Salmonella typhimurium* flagellin can translocate across epithelial cells to the basolateral membrane, a process that is essential for *S. typhimurium* flagellin to induce inflammatory responses[55,58,59]. These data strongly suggest the possibility that under normal circumstances TLR5 is only expressed at the basolateral membrane in IEC. The basolateral expression of TLR5 may be important for the maintenance of GI homeostasis since flagellin from commensal bacteria generally does not translocate to the basolateral membrane and thereby does not induce an inflammatory response[58].

The intestinal epithelium also uses specific tissue distribution and compartmentalization of TLR-expressing cells to avoid unnecessary TLR activation and at the same time allow the development of rapid and efficient host defense against invasion by pathogenic organisms. In this regard, intestinal myofibroblasts are capable of upregulating TLR2, TLR3, TLR4, TLR6 and TLR7 expression after LPS or LTA stimulation, thereby allowing a functional TLR response to invasive pathogens in the subepithelial compartment[60]. It has also been shown that crypt epithelial cells express TLR2 and TLR4, whereas mature IEC express TLR3 only[44]. Since crypt epithelial cells do not come into direct contact with commensal bacteria, their expression of TLR2 and TLR4 should not be detrimental to the host. TLR3 expression in the intestinal lumen is also non-detrimental because the TLR3 ligand, viral dsRNA, is not a natural presence in the gut microflora.

Another strategy in the regulation of TLR activities in the GI mucosa is through high expression of TLR-antagonists to suppress the activation of these TLRs still present at the cell surface. For example, TLR9 is constitutively expressed in IEC, but remains completely unresponsive to CpG[61]. In this regard, various proteins, termed TLR-attenuating factors, are known to attenuate TLR signaling, and this was extensively reviewed by Liew *et al*[6]. Some of these TLR-attenuating factors have been shown to be highly expressed in TLR-hyporesponsive IEC, or to be lacking in cases of intestinal inflammation. Toll-interacting protein (TOLLIP) inhibits TLR signaling by interfering with IL-1 receptor-associated kinase (IRAK), an important component of the TLR signaling cascade[62]. TOLLIP was found to be upregulated in TLR-hyporesponsive primary and immortalized IEC after prolonged exposure to TLR ligands[45,49], and TOLLIP mRNA was highly expressed in healthy colonic mucosa[49]. Peroxisome proliferator-activated receptor γ (PPARγ) limits TLR activity by inhibiting NF-κB activation[63,64]. PPARγ was more highly expressed in the colon compared to the small intestine[65], and has been shown to have a crucial role in the induction of tolerance to commensal bacteria[66]. Stimulation of IEC by TLR ligands or by intestinal microflora extracts increased PPARγ expression[67]. Thus, TOLLIP and PPARγ appear to down-regulate TLR activity in direct response to the continual exposure of IEC to commensal bacteria.

It has recently been identified that TIR8/single Ig IL-1-related receptor (SIGIRR) can negatively regulate TLR activity, possibly by interfering with TLR4 and IRAK signaling[68,69]. Studies in TIR8$^{-/-}$ mice showed that these mice developed more severe intestinal inflammation than wild-type control mice after LPS treatment[70], implicating the role of TIR8 in the suppression of the intestinal inflammatory response. In addition, it has been shown in a mouse model of colitis that vasoactive intestinal peptide (VIP) treatment can restore the overexpressed TLR2 and TLR4 to baseline levels[71]. The mechanism of action was unknown, but might involve either VIP-mediated suppression of NF-κB activation (leading to a cessation of further TLR expression) or suppression of cytokines known to contribute to TLR upregulation in IEC[71]. This appears to be a novel mechanism by which a natural intestinal peptide suppresses TLR activity. Finally, macrophages isolated from the intestinal lamina propria of IL-10$^{-/-}$ mice, which develop inflammatory bowel disease (IBD)-like colitis, were shown to express reduced levels of IκBNS, an inhibitor of NFκB activation[72]. IκBNS is responsible for suppression of LPS-induced cytokine production by lamina propria macrophages[72]. The lamina propria macrophages are normally hyporesponsive to TLR stimulation except in cases of intestinal inflammation[73], but these from IL-10$^{-/-}$ mice were responsive.

There are some known cases where commensal bacteria actually enhance anti-inflammatory activity in the intestinal epithelium. One example is the aforementioned upregulation of TOLLIP and PPARγ by commensal bacteria[45,49,66]. Others have shown that non-pathogenic *S pullorum* could block the activation of NF-κB by *S typhimurium*[74]. Furthermore, Backhed *et al*[75] showed that hypo-acylated LPS was less stimulatory towards TLR4 compared to normally acylated LPS, and that it actually inhibited the pro-inflammatory effects of wild-type LPS. Several species of commensal bacteria produce hypo-acylated LPS, which may contribute to the down-regulation of TLR4 activities[75].

TLRs AND INFLAMMATORY BOWEL DISEASE

IBD, comprising Crohn's disease (CD) and ulcerative colitis (UC), is a chronic, relapsing GI disorder of

unknown etiology. The development of IBD is hypothesized to be the result of dysregulated immune responses to one or more intestinal luminal antigens (loss of tolerance) in genetically predisposed individuals. While the pathophysiological features of IBD are uncontrolled, excessive inflammation in the GI mucosa and the upregulation of a host of pro-inflammatory and T cell cytokines[76,77], the root of the problem may lie in the defective immune tolerance to commensal bacteria and other intestinal luminal antigens. Experimental and clinical studies suggest that the over-expression of certain TLRs and down-regulation of TLR antagonists in IEC can be one of the underlying mechanisms leading to an improper reaction to commensal bacteria by the host. In this regard, TLR4 expression was reported to be elevated in colonic tissue of UC and CD patients[47], and TLR4 polymorphisms at Asp299Gly and Thr399Ile have been linked to the development of both CD and UC[78,79]. It was also shown that TLR2 activity was increased in a mouse model of colitis[80]. The presence of high titers of flagellin-specific antibodies in the serum of CD patients raises the possibility that flagellin from commensal bacteria might trigger an improper immune response in the GI mucosa through TLR5[81,82] and that TLR5 may also play an important role in the pathogenesis of IBD. In addition, as discussed above, intestinal myofibroblasts express TLR2 and TLR4 and respond to LPS and LTA stimulation, and have been implicated in the development of CD-associated fibrosis[60]. Moreover, PPARγ was found to be decreased in intestinal epithelial tissue of UC patients[67]. Thus, TLR mutations and dysregulation are likely major contributing factors in the predisposition and perpetuation of IBD.

More recently, it has been shown that TLRs may contribute to the pathogenesis of IBD in conjunction with another family of PRRs termed nucleotide-binding oligomerization domain proteins (Nod). Specific genetic variations in Nod2 have been strongly linked to the development of CD[83,84] and to excessive NF-κB activity[85]. Interestingly, the Nod2 variations may also have a direct effect on TLR-mediated control of intestinal inflammation. In IEC from Nod2-variant patients, TLR2 stimulation led to excessive production of both pro-inflammatory and Th1 cytokines[15,86,87]. These cytokines are heavily involved in the pathogenesis of IBD[77]. It appears that the association between Nod2 and TLRs seen in normal intestinal tissue[88] is important for intestinal homeostasis. Alteration of this association by genetic variation in Nod2 leads to the development of chronic intestinal inflammation. Further exploration into how Nod2 mutations affect TLR function will undoubtedly shed light on novel interactions between Nod1/2 and TLRs in the GI mucosa.

TLRs AND *HELICOBACTER PYLORI* INFECTION

Helicobacter pylori (*H pylori*) is a Gram-negative bacterium that colonizes the gastric mucosa and causes chronic gastritis and gastric ulcers. The bacterium adheres strongly to the surface of gastric epithelial cells (GEC) without actually invading them[89,90]. As is the case with IBD, the host inflammatory response to *H pylori* infection directly contributes to disease pathogenesis[91]. Although the host mounts a strong specific immune response to the pathogen, this response is for the most part ineffective[92]. *H pylori* infection is relatively common worldwide, yet less than one quarter of infected individuals progress to disease[93]. Whether or not an individual proceeds to a disease state might be influenced by any combination of host, bacterial and environmental factors.

Because of the clinical significance of *H pylori* infection, the interaction between TLR and *H pylori* is probably the most extensively studied. Since the first step in *H pylori* infection is the adherence to GEC by the bacterium, it is logical to postulate that TLRs would play a role in *H pylori* detection, as well as the subsequent mounting of the deleterious cellular and inflammatory immune response. Despite extensive studies on this subject, as yet there is no clear consensus as to which TLR(s) is involved in the detection of *H pylori* by GEC. Several groups have shown the apical and basolateral expression of TLR4 in *H pylori*-infected GEC[94,95]. TLR5 and TLR9 were also expressed both apically and basolaterally in the GEC of healthy individuals, but the apical expression of these TLRs was lost in *H pylori*-induced gastritis[95]. GEC expression of TLR2, another important receptor for bacterial PAMPs, has yet to be fully characterized.

Several studies have suggested that TLR4 may play an important role in the recognition of *H pylori* infection by gastric mucosa[94,96] as TLR4 and MD-2 expression, as well as responsiveness to *H pylori* LPS stimulation, in gastric biopsy samples of patients with *H pylori* infection were up-regulated[94]. However, others have reported that the detection of *H pylori* by primary GEC is TLR4-independent[97]. Interestingly, Smith *et al*[98] found that the gastric epithelium recognizes *H pylori* LPS through TLR2 rather than TLR4, suggesting the possible disassociation between the up-regulation of TLR4 and the pro-inflammatory potential of *H pylori* LPS. Similarly, Mandell *et al*[99] showed that whole *H pylori* elicited an immune response through TLR2, not TLR4, in mice. These findings are not entirely surprising since it has been long recognized that *H pylori* LPS does not share all the characteristics of other Gram-negative GI bacteria.

Although *H pylori* flagellin was initially shown to be able to interact with TLR5[100], more recent studies have found that TLR5 was unresponsive to *H pylori* flagellin, suggesting the low immunogenicity of this molecule[101-103]. Anderson-Nissen *et al*[101] have recently mapped low TLR5 responsiveness to a specific area of the amino acid sequence in the *H pylori* flagellin. Introduction of this sequence into *Salmonella* flagellin renders the new construct devoid of all TLR5-activating activity[101]. Thus, it is possible that *H pylori* uses TLR5 evasion to avoid immune detection. The ability of *H pylori* to induce chronic and persistent gastric inflammation suggests that PAMP(s) other than flagellin may be involved in the pathogenesis of the infection. Indeed, Takenaka *et al*[104] have shown that *H pylori* heat shock protein (HSP) 60 is able to activate TLR2 and TLR4 and increase NF-κB activity and IL-8 production in GEC.

Evidently, there is still much to be discovered regarding the interactions of *H pylori* with TLRs in the gastric epithelium. While it is likely that host factors in the

immune response might play a role in disease pathogenesis, there does not appear to be any evidence in the literature demonstrating an association between genetic variation in TLRs and *H pylori* disease progression, as is the case in IBD.

TLRs AND INFECTIONS WITH INTESTINAL BACTERIA

Despite a relatively large amount of information available concerning the roles of TLRs in the GI tract, there is surprisingly little data showing the actual *in vivo* role for TLRs in combating enteric pathogens. The obvious assumption is that invasive pathogens expressing known bioactive PAMPs will trigger a TLR-mediated immune response upon invasion of the IEC barrier. However, *in vivo* models of this scenario are scarce. Of the most common enteric pathogens, the interplay between TLRs and *S typhimurium* has been most extensively studied.

Invasion of IEC by *S typhimurium* leads to bacterial replication in intracellular vacuoles, localized inflammation, and lysis of infected cells. Several TLRs (TLR2, TLR4 and TLR5) appear to play a crucial role in the host defense against *S typhimurium* infection. Allelic variation in chicken TLR4 has been linked to the susceptibility to *S typhimurium*[105]. Studies of systemic *S typhimurium* infection in TLR4-deficient mice have also shown an important role for TLR4 in controlling the infection, TNF-α and chemokine production, and cellular immune responses[106-108]. Moreover, results from several recent studies have implicated TLR4 in the immediate detection of *S typhimurium* and early macrophage responses, and TLR2 as a key player in late responses after cellular invasion and intracellular replication have occurred[109,110].

S typhimurium flagellin induces a strong, TLR5-mediated inflammatory response in IEC[55,59]. Interestingly, this phenomenon does not require cellular invasion; adherence to IEC is sufficient[55,58,111]. The fact that IECs do not express TLR5 on the apical membrane[55,58] implies that *S typhimurium* actually has to translocate flagellin molecules through IEC to the basolateral membrane where TLR5 is expressed[55,58,59]. This process is dependent on the presence of *S typhimurium* pathogenicity island 2 (SPI2)[59,112], and probably also *S typhimurium* guanine nucleotide exchange factor, SopE2[113]. Therefore, it appears that the interplay between TLR5 and *S typhimurium* flagellin is a major determinant in the host response to IEC infection and the clinical outcome of the infection. Indeed, Sebastiani *et al*[114] linked the murine TLR5 gene to an *S typhimurium* susceptibility locus, and showed that susceptible mice expressed decreased levels of TLR5. Also, Zeng *et al*[115] found that *S typhimurium* strains lacking flagellin expression induced minimal inflammatory responses, suggesting that flagellin is the primary cause of inflammation in enteric *S typhimurium* infection.

The important role of TLRs in the immunopathogenesis of *Salmonella* infection is further verified in infection with *S typhi*, the etiological agent of typhoid fever. Unlike *S typhimurium*, *S typhi* infection fails to induce IL-8 production or neutrophil recruitment to the intestinal epithelium that is characteristic of *S typhimurium* infection, thereby allowing the systematical dissemination of the infection. It has been suggested that the ability of the *S typhi* capsular antigen (Vi, a virulence factor not expressed in *S typhimurium*) to inhibit the TLR4 and TLR5 response to the infection may partially contribute to its pathogenesis[116].

The role of TLRs in the pathogenesis of and immunity to other enteric bacterial infections remains largely unexplored. Recognition of LPS by TLR4 is unlikely to be a major contributing factor in diarrheagenic *E coli* infection because lipid A, the structure within LPS which activates TLR4, is highly conserved, and is therefore common to both pathogenic strains and non-pathogenic commensal strains of *E coli*. Although the O antigen of *E coli* LPS is more variant between strains, this antigen does not activate TLR4[75]. In addition, commensal bacteria-derived LPS is known to induce the intracellular relocalization of TLR4 in IEC[51]. It is, therefore, reasonable to assume that IECs do not react to LPS from *E coli* adhered to the outer apical membrane of the cell. However, other *E coli* PAMPs may play a role in the up-regulation of TLR activities in IEC. In this regard, it has been shown that flagellin from several strains of pathogenic *E coli* can induce NF-κB activation and IL-8 production through TLR5[117-119]. In addition, it has recently been shown that aggregative adherence fimbriae (AAF), an EAEC virulence factor, is involved in cell adhesion and contribute to inflammation and IL-8 production in IEC[120], although it is unclear whether this effect is TLR-mediated. Since both *Porphymonas gingivalis* fimbriae and *E coli* P fimbriae, a virulence factor in uropathogenic *E coli*, can activate TLR2 and/or TLR4[20,22,121,122], it is possible that the inflammatory response induced by EAEC AAF is mediated through TLR recognition as well. Furthermore, it has been shown that the *E coli* type II heat-labile (LT-II) enterotoxin, expressed by ETEC, activates TLR2 *via* its B subunit[21].

Campylobacter jejuni infection is one of the most common causes of food-born gastroenteritis. *C jejuni* infection leads to adhesion to IEC, followed by cellular damage due to invasion, toxins and excessive inflammation[123,124]. Infection of IEC by *C jejuni* leads to an enhanced IL-8 production, which is dependent on bacterial adhesion to IEC[125]. However, it is not known whether this inflammatory response is TLR-mediated and, if so, which TLR(s) and ligand(s) are involved. Studies of TLR4 and CD14 polymorphisms commonly associated with susceptibility to other infections showed no link to *C jejuni* infection or disease progression, suggesting that TLR4 does not play a role in the immune response to this pathogen. Moreover, *C jejuni* flagellin failed to stimulate TLR5[101,125], as it possesses the same site-specific mutations as *H pylori* that allow it to avoid TLR5 recognition[101]. One possible candidate for the induction of the inflammatory responses seen in the above study could be *C jejuni* fimbriae, as is the case with the fimbriae of other bacterial species[20,22,121,122]. However, it remains controversial whether *C jejuni* expresses any sort of fimbriae[126,127].

Shigella flexneri , the causative agent of dysentery, is able to survive in a highly acidic environment such as the stomach. As a result, a relatively low dose of *S flexneri* can initiate an intestinal infection[128]. *S flexneri* lipoproteins

can activate TLR2 in non-intestinal epithelial cell lines[129], but TLR2 reactivity to *S flexneri* lipoproteins in IEC remains to be demonstrated. The ability of *S flexneri* to invade IEC plays an important role in the induction of inflammation[130]. Cellular invasion by *S flexneri* induces NF-κB activation and IL-8 production in both IEC and non-intestinal epithelial cells[130-133]. However, this response appears to be independent of TLR and MyD88, and is mediated by Nod1[132]. Some clinical isolates of *S flexneri* have been shown to express a type I fimbriae[134], which could potentially be detected by TLRs similar to fimbriae of other enteric bacteria[20,22,121,122].

TLRs AND INTESTINAL VIRAL INFECTIONS

Viral infection in the GI tract can lead to invasion and destruction of IEC and gastrointestinal inflammation. In most cases, an individual becomes immune to reinfection, suggesting that an effective adaptive immune response occurs in viral gastroenteritis[135]. Although it has been proposed that TLR3, TLR7 and TLR8 are likely to play a major role in sensing the viral infection in the GI tract and initiating an effective mucosal immune response, there is little published evidence to support this notion. The four most common viruses associated with viral gastroenteritis are rotavirus, calicivirus, astrovirus and adenovirus (serotype 40, 41). Of these, only rotavirus infection of IEC has been examined for TLR involvement. It appears that extracellular TLR3 was not involved in the response to rotavirus dsRNA since dendritic cells pretreated with TLR3-blocking antibodies, thereby blocking the surface TLR3, remained responsive to rotavirus dsRNA[136]. Because viruses are intracellular pathogens, the viral genetic material is more likely to be exposed after invasion of the cell. Indeed, intracellular expression of TLR3 has been demonstrated in several cell types[136-138]. However, studies on TLR3-deficient mice showed that responses to infection by reovirus, a dsRNA virus which is known to infect the gastrointestinal epithelium, were TLR3-independent[26]. Therefore, it seems that despite its constitutive expression in IEC[44], TLR3 may not play an important role in the host defense against GI infection by dsRNA viruses.

The role of TLR7 and TLR8 in the GI infection with ssRNA viruses, such as calicivirus, has not been directly investigated, despite the importance of these TLRs in the recognition of ssRNA viruses. It is worth noting that of the four major types of viral gastroenteritis, calicivirus infection tends to occur equally in adults and children, whereas infections with rotavirus, astrovirus and adenovirus are mostly seen in children. Glass *et al*[135] suggested that this could be caused by short-lived immunity to calicivirus or because of antigenic variation, rendering the adaptive immune response less effective in the face of future infection. If the former is the case, it would be interesting to know if the short-lived immune response could be attributed to a unique property of TLR7 and/or TLR8-mediated detection of calicivirus in IEC, compared to detection of the other three dsRNA viruses.

TLRs IN PARASITIC GASTROINTESTINAL INFECTION

Despite the high incidence and economic significance of parasitic GI infections, particularly in the developing countries, there is very limited information in literature on the role of TLRs in the parasitic GI infection, with the exception of *E histolytica* infection. *E histolytica* can be ingested with contaminated food or water, and colonize the colon. The infection can sometimes remain asymptomatic, but can also cause diarrhea, vomiting and ulcers. Studies performed prior to the discovery of TLRs showed that *E histolytica* infection induced neutrophil influx into the site of infection[139,140] in mice and IL-8 production in IEC lines as well as in human IEC xenografted into immunodeficient mice[141,142]. In the IEC cell line, the IL-8 response was contact-independent, and presumably mediated by *E histolytica* soluble factors[142]. It has recently been shown that *E histolytica* lipopeptidophosphoglycan (LPPG) induces TLR2- and TLR4-dependent IL-8 production in human kidney cell lines and monocytes[33,34]. These studies also suggest that LPPG might be a novel PAMP, and the factor responsible for induction of IL-8 and the neutrophil response seen in previous studies of *E histolytica* infection.

CONCLUSION AND PROSPECTIVE

Emerging experimental and clinical evidence have shown that TLR expression and activation are specially regulated in the GI tract, probably due to its unique environment (the presence of commensal microflora and the exposure to invading pathogens). This is mainly accomplished by: (1) the down-regulation of surface expression of TLRs by the gut epithelium; (2) the specific tissue distribution and compartmentalization of TLR-expressing cells in the gut; and (3) the high expression of TLR-antagonists/attenuating factors that suppress the activation of these TLRs still present at the cell surface. These mechanisms render the GI mucosa able to avoid unnecessary TLR activation to commensal microflora yet retain the ability to detect and mount rapid and efficient immunity against the invasion of pathogens.

TLRs are expressed by both epithelial and non-epithelial cells throughout the entire GI tract. The unique patterns of cellular localization and tissue distribution of TLRs in GI tract allow the host to differentiate between commensal non-pathogenic and pathogenic microbes. Recent studies strongly suggest that dysfunction or dysregulation of TLR expression and activation in IEC is one of the underlying mechanisms leading to the development of IBD. Although there is little doubt now that TLRs play important roles in both the predisposition and perturbation of IBD, caution must be exercised in the interpretation of the clinical and experimental data on TLR studies because it remains to be determined whether the TLR dysregulation seen in patients with IBD is the pathological consequence or the underlying cause of the chronic inflammation. In addition, conflicting results have been reported in regard to the TLR4 activity[80], and the

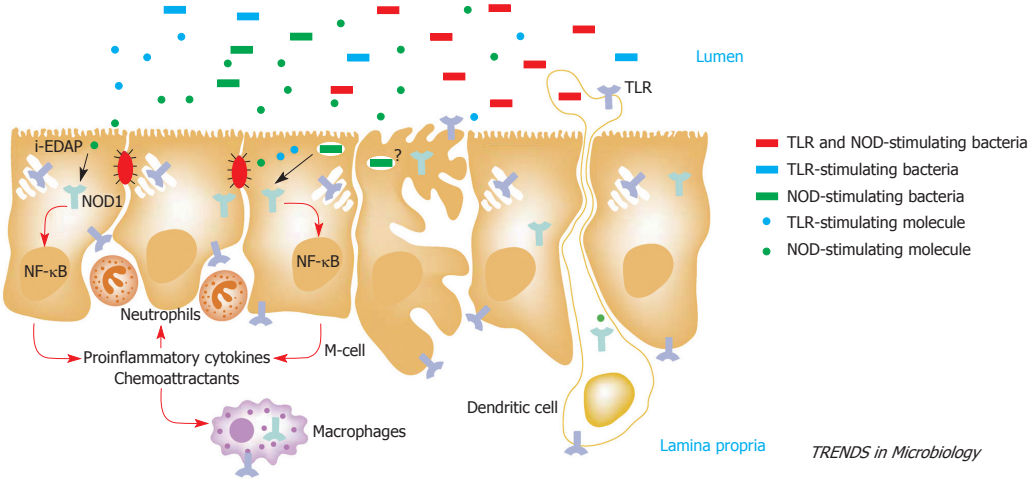

Figure 1 Host sensing of enteropathogenic bacteria. Enteroinvasive bacteria are sensed by specific cells (intestinal epithelial cells, M cells, macrophages and dendritic cells) located in the intestinal mucosa. Resident and invasive bacteria and their molecules released into the intestinal lumen could be recognized by host cells. Sensing of bacteria and their products are mediated by surface Toll-like receptors (TLRs) and cytosolic Nod1 receptors. Intestinal epithelial cells lack functional TLR2 and TLR4 but they might express TLR5 at the basolateral surface. Thus, some entero-invasive flagellate bacteria might stimulate epithelial cells through both TLR5 and Nod1 (depicted in red), whereas other invasive bacteria might activate Nod1 but not TLRs (depicted in green). Flagellate Gram-positive bacteria lacking Nod1-stimulating molecules are expected to trigger TLRs but not Nod1 signaling (depicted in blue). Soluble TLR- and Nod1-stimulating products are found in the intestinal contents but their role in host defense is unknown. Certain TLRs might be also localized to intracellular compartments (e.g., Golgi apparatus for TLR4), but the relevance of intracellular TLR signaling in the intestinal mucosa remains elusive. Reprinted from Chamaillard *et al*. Battling enteroinvasive bacteria: Nod1 comes to the rescue.*Trends Microbiol* 12:529-532[154]. Copyright (2004), with permission from Elsevier.

expression of some TLRs by IEC was found unchanged (TLR9) in patients with IBD[47,61]. This is hardly surprising and probably reflects the complexity of the nature of the disease, the diversified patient populations, and the different research approaches employed.

Despite the demonstrated roles of TLRs in host defense against many microbial infections, there is surprisingly little data on the actual *in vivo* role for TLRs in combating GI pathogens, particularly in viral and parasitic infections. For bacterial pathogens, although the interaction between *H pylori* and GEC has been extensively studied, there is no clear consensus as to which TLR(s) is involved in the recognition of *H pylori* by the host, or the role of TLRs in the pathogenesis of *H pylori*-induced gastritis and gastric ulcer. *S typhimurium* is another well-studied GI pathogen although many studies regarding the interaction between TLR and this pathogen were conducted in animal models where the infection was initiated by systemic injection rather than the natural GI route. In this regard, studies on systemic and respiratory infections have shown that the requirement of different subtypes of TLRs in host defense against microbes appears to be dependent on the type of pathogen, the route of infection, and the initial dose of infection[143-145].

Many virulent strains of pathogens have evolved multiple mechanisms to evade recognition by TLRs. In this regard, a new family of PRRs, the NACHT-LRRs (NLRs), which include both nucleotide-binding oligomerization domains (NODs) and NALPs [NACHT-, LRR- and pyrin domain (PYD)-containing proteins], has been recently identified and implicated in the recognition of bacterial components in the cytosol[146]. It has been suggested that the Nod family of proteins is a major contributor to innate immunity in IEC when TLR activity is attenuated[147-149]. The intracellular location of NODs allows the detection of invasive pathogens in a similar fashion to intracellular or basolateral TLR expression (Figure 1). In addition, Nod1/2 can activate NF-κB through a different signaling pathway from TLRs[150-152], thus rendering them functional even in the presence of TLR-attenuating factors such as TOLLIP and TIR8/SIGGIR that are highly expressed in IEC. Furthermore, Nod1/2 can positively influence TLR activity[15,88,153], and may contribute to the pathogenesis of IBD in conjunction with TLRs. The discovery of the NLR family definitely adds further complexities to the host immune regulation but is also likely to shed new insights into the pathogenesis of GI disorders and provide additional opportunities for the development of novel immunotherapeutic strategies.

TLRs were discovered relatively recently, and their involvement in health and diseases of the GI tract remains a new and exciting field of study. Future work in this field will lead to a better understanding of the unique mechanisms involved in the fine balance between tolerance and immune response. An array of new treatment options for IBD, *H pylori* infection, and other GI disorders could involve tissue-specific suppression of TLR signaling pathways by either chemical means, introduction of natural TLR suppressors and antagonists such as PPARγ, or use of gene therapy to correct TLR gene defects. In this regard, further exploration of the recently characterized negative regulatory mechanisms, that have evolved to attenuate TLR signaling by the host, may be fruitful for the development of new generation of more effective immunotherapeutic agents for the treatment of GI disorders.

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REVIEW

Treatment of nonalcoholic fatty liver disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is the most common cause for elevated liver enzymes in the developed nations. Beyond prevention programs which are of particular interest because of the increasing number of overweight children, treatment should be focussed on the most important risk factors, obesity and insulin resistance. As a consequence of elucidating the pathomechanisms of NAFLD, the number of potential therapeutic options increased. However, many studies investigating the therapeutic effect show shortcomings in at least one of the following points: lack of a serial liver biopsy, short term of treatment and limited number of included patients. The second generation insulin sensitizer pioglitazone and rosiglitazone show the most promising improvements in NAFLD, but weight gain and potential hepatotoxicity calls for attention. In conclusion, a general recommendation for the application of specific drugs cannot be given. Besides controlled clinical trials, weight reduction and physical activity to improve insulin sensitivity in obese patients should be the priority objective.

Key words: Nonalcoholic fatty liver disease; Treatment

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INTRODUCTION

Insulin resistance and obesity represent the most important risk factors for development of NAFLD[1]. Fatty liver has a benign prognosis, whereas up to 20% of patients with NASH develop cirrhosis[2, 3]. Risk factors for development of fibrosis are age, BMI>30, glutamic-oxaloacetic transaminase (AST)/glutamic-pyruvic transaminase (ALT)>1, and diabetes[4]. In addition, the risk for development of hepatocellular carcinoma (HCC) is comparable to that of patients with hepatitis C infection[5]. The pathophysiology of NAFLD is described as a "two hit model". The first hit is supposed to be the increase of free fatty acids in hepatocytes which results in a decrease of β-oxidation. Downregulation of β-oxidation further aggravates accumulation of fatty acids. The second step includes all mechanisms contributing to the development of inflammation and fibrosis[6]. In detail, increase of fatty acids enhances the expression of cytochrome peroxidase 2E1 (CYP2E1). CYP2E1 stimulates generation of oxidative species and thereby enhances lipid peroxidation of the hepatocyte membrane[7, 8]. Endotoxin and TNF-α have been demonstrated to play a harmful role in development of alcoholic steatohepatitis[9]. Injection of lipopolysaccharide (LPS) in leptin-deficient, obese ob/ob mice resulted in a significantly more severe liver injury probably caused by TNF-α compared to lean control animals[10]. Administration of anti-TNF-α-antibody ameliorated liver damage in this model of NAFLD[11]. Both endotoxin and oxidative stress upregulate expression of CD95 ligand and contribute to apoptotic cell death[12]. In fact, increased hepatocyte apoptosis correlating with disease severity was described in patients with NASH[13].

Here we discuss the role of a drug-free management in improvement of insulin resistance and NASH and give a critical summary of recent data on medical treatment. The potential concepts of treatment are summarized in Figure 1.

REDUCTION OF BODY WEIGHT

Although insulin resistance occurs in patients with normal BMI and anthropometric measurements, the majority of these patients are adipose with increased visceral fat. So, in overweight or obese patients, weight loss is usually recommended as the first line management[14]. The American Gastroenterological Association recommends a target of 10% of baseline weight as an initial goal of weight loss if BMI exceeds 25 kg/m^2. Weight loss should proceed at a rate of 1-2 lb/wk. Rapid weight loss due to a very low energy diet (<500 kcal daily) or jejunoileal bypass has been associated with exacerbation of steatohepatitis in obese patients[15-17]. Weight loss should be achieved by restricting calorie intake and physical exercise. Both have been shown to improve insulin resistance[18]. Physical exercise is proven to be beneficial for coronary heart disease and peripheral vascular disease. Rollins demonstrated that moderate to high-intensity exercise (30 min 3-5 times/wk) reduces the

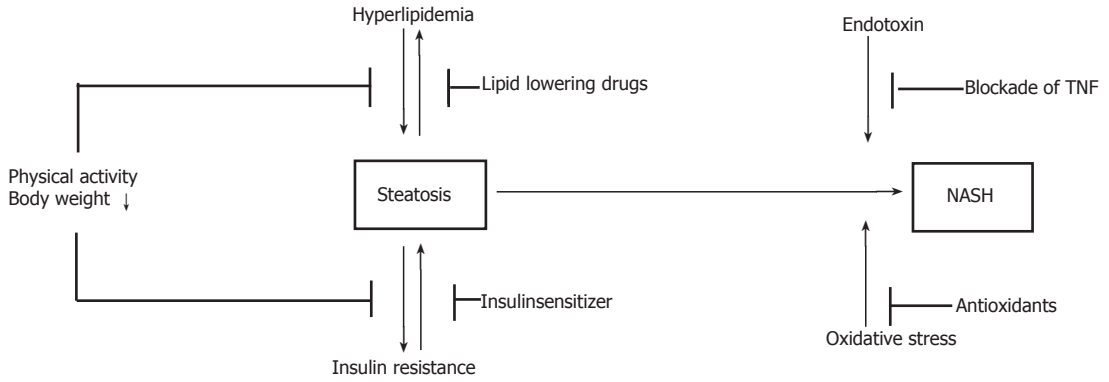

Figure 1 Potential treatment options of NAFLD

risk of complications associated with obesity[19]. However, it is not clear whether patients with NAFLD would benefit from merely increasing physical activity. The goal of weight management is to induce a negative calorie balance. A calorie deficit of 500-1 000 calories/d for those who are overweight or obese appears to be rational, although there are no specific studies on this subject. Dietary recommendation should include reduction of dietary carbohydrates, because lipid profile of overweight patients improves[20, 21]. However, weight loss is rarely achieved or maintained over a long period. Most studies to date have been short term with a small number of patients included. Children with NASH may benefit from weight loss, because serum liver enzymes normalized and sonographic abnormalities disappeared[22]. Another study investigated 48 patients with elevated liver enzymes and clinical, histological, and sonographic characteristics of fatty liver disease[23]. Eighty-one percent of these patients were obese, 73% were glucose-intolerant or diabetic, and 85% had dyslipidemia. Dietary intervention as well as lipid-lowering medication and oral antidiabetics as needed were included into the treatment protocol. These dietary interventions resulted not only in a moderate weight loss, but also in a reduction of serum liver enzymes in 96% of patients. However, it remains unclear, if improved liver enzymes were accompanied by an improvement of liver histology, because serial liver biopsies were not performed. In another study, 15 obese patients followed a restricted diet (25 calories per kilogram ideal body weight) and exercise regimen over a 3 mo period[24]. Liver enzymes decreased in all patients and steatosis determined by biopsy was reduced compared with patients from control group.

A recently published study analyzed the effect of short term weight loss on liver histology[25]. Twenty-three obese patients (BMI>25kg/m^2) with biopsy-proven NASH received standardized nutritional counseling to reduce insulin resistance and body weight. Each subject received individualized nutrition counseling in order to achieve dietary goals. The dietary adjustments included increased intake of fiber and a decreased intake of calories. The daily calory intake consists of 40%-45% carbohydrates with an emphasis on complex carbohydrates with fiber, 35%-40% fat with emphasis on mono- and polyunsaturated fats, and 15%-20% protein. In addition, all participants were encouraged to increase their physical activity to achieve a heart rate of 70% of the calculated target heart rate. Food frequency questionnaires were performed to assess dietary intake and the Paffenberger Activity Questionnaire was used to evaluate the level of physical activity. Sixteen patients successfully completed 12 mo of intense dietary intervention. Mean weight decreased from 98.3 kg to 95.4 kg. There was also a reduction of mean waist circumference, visceral fat, fasting glucose, insulin resistance, triglycerides, serum levels of liver enzymes and histological score, but the differences were not statistically significant. Fifteen patients underwent repeat liver biopsy. Nine of these 15 patients had a histological response, 6 patients had a stable score and none had worsened.

Pharmacological treatment of obesity may be applied to patients with a BMI>27 kg/m^2 and obesitiy-associated comorbidities. Sibutramine is a serotonin reuptake antagonist which should not be used in patients with coronary heart disease and moderate or severe hypertension[26]. The intake of orlistat results in fat malabsorption. Sabuncu and coworkers analyzed the effects of orlistat and sibutramine in obese patients with clinically presumed NASH. Both orlistat and sibutramine improved liver enzymes and decreased the sonographic hallmarks of fatty liver. However, liver biopsies were not performed and the level of alkaline phosphatase increased[27].

Those patients with a BMI>35 kg/m^2 and obesity-associated comorbidities may be considered for more aggressive weight management, including bariatric surgery. Because liver failure occurs after jejuno-ileal bypass, the later has been replaced by the proximal gastric bypass operation. Two studies demonstrated improvement of liver histology after weight reduction and stabilization of weight for long term[28, 29]. However, occasional cases of worsening liver function can also occur during period of rapid weight loss following this procedure. The results of studies investigating the safety of such surgery in patients with severe NASH have to be awaited. Patients considered for this procedure should be monitored carefully and the pros and cons should be discussed with the patient in detail.

ANTIOXIDANTS

Oxidative stress is proposed to act as the "second hit" in

the pathogenesis from steatosis to NASH and fibrosis. Therefore, using antioxidant substances seems to be rational in the treatment of steatohepatitis. Several *in vitro* and animal *in vivo* studies revealed that application of vitamin E decreased levels of profibrogenic TGF beta, improved liver histology and inhibited hepatic stellate cell activation[30-32]. Two open-label pilot trials examined the effect of vitamin E in patients with NAFLD[33, 34]. Eleven pediatric patients with presumed NASH were prescribed 400-1 200 IU of oral vitamin E. Diagnosis was based on the presence of chronically elevated levels of AST and ALT, and fatty liver on ultrasound. Other causes for hepatitis were excluded. Two patients had biopsy-proven NASH. Treatment resulted in normalization of liver function test. However, serial biopsies were not performed, liver remained increased echogenic during treatment and improvement of enzymatic values was not sustained after discontinuation of vitamin E[33]. In another study[34], 10 patients with the clinical diagnosis of NAFLD and 12 patients with biopsy proven NASH were treated with vitamine E (300 mg/d) for 1 year. Treatment resulted in a significant improvement of liver enzymes. In the nine patients with steatohepatitis who had a posttreatment liver biopsy, the degree of steatosis, inflammation, or fibrosis also improved or remained unchanged. The plasma levels of TGF-β decreased significantly with vitamin E. However, these promising results were not confirmed in a subsequent randomized, double-blind, placebo-controlled trial[35]. In this study, vitamin E (1 000 IU/d) in combination with vitamin C (1 000 mg/d) to potentially enhance the regeneration of oxidized vitamin E was given to 23 patients with NASH, while 22 patients were randomized to placebo. The duration of treatment was 6 mo. In addition, a low fat, low calorie diet in combination with increased physical activity was recommended. The degree of adherence to these recommendations remained unclear. The results showed a significant improvement of ALT levels in the placebo group but not in the treatment group. The fibrosis stage of 11 (48%) patients of the vitamin group and 9 (41%) of the placebo group improved by at least one stage. The authors concluded from this within group comparison that vitamin C and vitamin E are effective in improving liver fibrosis, although only two more patients in the vitamin group showed a regression of fibrosis. Adams and Angulo[36] criticized that the effect of placebo treatment was ignored, because no comparison between groups was performed. A between group analysis revealed that 6 mo of therapy with the combination of vitamin E and C is not better than placebo for patients with NASH.

URSODEOXYCHOLIC ACID

This hydrophilic bile acid is approved for the treatment of primary biliary cirrhosis. Ursodeoxycholic acid (UDCA) has been shown to reduce the portion of hydrophobic bile acids which contribute to oxidative stress. This is of particular importance, because fatty hepatocytes reveal an increased sensitivity to hydrophobic bile acids[37]. A pilot study published in 1996 analyzing the effect of UDCA on serum liver enzymes and histology in patients with NAFLD showed promising results[38]. The hepatic steatosis decreased on repeat liver biopsy in 12 of 19 patients and there was also a statistically significant improvement in liver enzymes, but there were no changes in the histological grade of inflammation or fibrosis. In a subsequent controlled trial 166 patients were randomized with liver-biopsy proven NASH to receive 13 and 15 mg/kg of UDCA daily[39]. One hundred and twenty-six patients completed 2 years of therapy and serial liver biopsies were available in 107 patients. Analysis of serum liver chemistry, changes in the degree of steatosis, necro-inflammation or fibrosis revealed no significant difference between the verum and placebo-treated groups. However, the results from this study showed a high rate of spontaneous improvement in hepatic steatosis in the placebo arm probably explaining in part why the data were negative. In addition, the dose of 13 and 15mg/kg pre day was possibly insufficient to improve NAFLD, so effect of higher doses needs to be evaluated in further studies.

INSULINSENSITIZER

The association of insulin resistance and hyperinsulinemia with NAFLD suggests a possibility of therapeutical intervention. The first evidence came from leptin-deficient, obese ob/ob mice. Metformin, a biguanide that reduces hyperinsulinemia and improves hepatic insulin resistance, reduced hepatomegaly and hepatic steatosis in ob/ob mice, whereas caloric restriction did not result in a substantial improvement[40]. The authors postulated that metformin improve hepatic insulin resistance by decreasing hepatic expression of TNF-α, a cytokine that promotes insulin resistance. A pilot study evaluated the effect of metformin in 20 patients with histological proven NASH[41]. When compared with the six patients not complying with treatment, intake of metformin for 4 mo significantly reduced levels of transaminases. They normalized in 50% of treated individuals. Also, insulin sensitivity improved significantly and liver volume decreased by 20%. Metformin was well tolerated and there was no case of lactic acidosis. However, the authors did not provide a serial liver biopsy to evaluate the effect of metformin on liver histology. These promising results were only in part supported by another open label trial [42]. Fifteen patients with biopsy proven NAFLD completed 12 mo of treatment with metformin (20 mg/kg). During the initial 3 mo, liver enzymes improved significantly. There was also an improvement in insulin sensitivity detectable. However, after 3 mo, insulin sensitivity did not further improve and levels of AST and ALT gradually increased back to pretreatment levels. Among the 10 patients with posttreatment biopsy, three showed improvement in steatosis, two showed improvement in inflammation score and one in fibrosis.

Another trial evaluating the effect of metformin was performed by a Turkish group from Ankara[43]. Uygun and coworkers randomized 36 patients with NASH into two groups. The first group was given lipid and calorie-restricted diet alone, while the second group was treated with metformin in addition to the diet for a period of 6 mo. The comparison between both groups showed no

significant differences in inflammatory activity or fibrosis, although more patients in the treatment group showed an improved liver histology. The improvements of liver enzymes, insulin, insulin resistance index and c-peptide levels in the metformin group were significantly greater than those detected in the group with dietary treatment alone. The most recently published controlled trial by Bugianesi and colleagues demonstrated a better effect of metformin on improvement of liver enzymes compared to a prescriptive diet or the administration of vitamin E[44]. Unfortunately, the histological data were limited to support an association between improvement of liver chemistry and histological findings.

Another class of agents presumably improving insulin sensitivity is the thiazolinediones. These compounds are ligands for the peroxisome proliferator-activated receptor gamma (PPAR gamma), which is expressed at high levels in adipocytes. Troglitazone, a first generation thioglitazone, and metformin were shown to inhibit the expression of sterol regulatory element binding protein-1 (SREBP-1), a key regulator of lipogenic enzymes[40, 45]. Troglitazone was investigated in a pilot study including 6 patients with biopsy-proven NASH[46]. Patients received 200 mg of troglitazone twice daily, which was well tolerated. Levels of ALT normalized in 4 of 6 patients on therapy and they persisted in normal range 3 mo after discontinuation of troglitazone. However, the Food and Drug Administration removed troglitazone from the market in March 2000 because of serious hepatotoxicity[47, 48]. The second-generation thioglitazones rosiglitazone and pioglitazone appear to be safer, although their use is currently contraindicated in the presence of active liver disease or of ALT more than 2.5 times normal. An open label trial including 26 biopsy-proven NASH patients analyzed the effect of rosiglitazone, 4 mg twice daily for 48 wk[49]. All patients were overweight, and 23% had a BMI>35 kg/m^2. Twenty-six patients had posttreatment biopsy. The mean necro-inflammatory score significantly improved with treatment and biopsies of 10 patients did not fulfill published criteria for NASH anymore after treatment . Twenty-five patients completed 48 wk of treatment and showed a significant improvement in liver enzymes and insulin resistance. However, 3 patients had to be withdrawn because of adverse events. One of these individuals discontinued because of increased ALT levels. In addition, weight gain occurred in more than two-thirds of participants and liver enzyme levels increased to near pretreatment level 6 mo after discontinuation of study medication. Another pilot study demonstrated similar results in 18 patients with biopsy proven NASH, who were treated with pioglitazone[50]. By 48 wk, levels of ALT normalized in 72% of patients. Hepatic fat content and size decreased which was determined by magnetic resonance imaging. There was also a significant improvement in liver histology regarding features of steatosis, inflammation and fibrosis. Histological improvement occurred in two-thirds of patients. The main side effect in this study was also weight gain and increase in total body adiposity.

In a recently published pilot study by Sanyal and coworkers 20 nondiabetic patients with biopsy-confirmed NASH were randomized to take the combination of pioglitazone (30 mg daily) and vitamin E or vitamin E (400 IU daily) alone for a period of 6 mo[51]. ALT levels normalized in all patients within 6 mo. Compared to baseline, treatment with vitamin E alone resulted in a significant decrease in steatosis, whereas the combination therapy produced a significant improvement in steatosis, cytologic ballooning, Mallory´s hyaline and pericellular fibrosis. Although vitamin E did not have any significant effects on metabolic endpoints, combination therapy improved insulin sensitivity, lowered fasting free fatty acid (FFA) levels and decreased metabolites of FFA oxidation. However, like in the previous trial by Neuschwander-Tetri and coworkers[49] , one of 10 patients receiving pioglitazone plus vitamin E had a significant increase in ALT level and was withdrawn from the study.

LIPID LOWERING DRUGS

Because NAFLD frequently occurs with a disordered lipid homeostasis, lipid-lowering drugs are considered as possible treatment for NAFLD. Hypertriglyceridemia and reduced HDL-cholesterol level are typical dyslipidemias associated with NAFLD. Gemfibrozil reduces very low-density lipoprotein triglyceride production. In a small controlled study of 46 patients with NASH, levels of AST were significantly decreased in 74% of the gemfibrozil group compared with 30% in the control group after 4 wk of treatment[52]. There was no correlation with pretreatment serum triglyceride levels. Posttreatment liver biopsies were not performed and the duration of biochemical response was not evaluated.

In NASH patients with hyperlipidemia statins are another potential treatment option. However, existing data are predominantly uncontrolled with a small number of patients. One study analyzed 28 hyperlipidemic patients with biopsy-proven NASH. Patients were given atorvastatin 20 mg daily for 24 wk. Both significant reduction of LDL-cholesterol and liver enzymes were detectable after treatment[53]. Statin-induced hepatoxicity did not occur and the risk seems to be not increased in patients with presumed NAFLD[54]. However, controlled trials with a bigger number of patients are required to demonstrate the benefit and elucidate potential risks of administrating statins.

BLOCKADE OF TNF-α

Adipose tissue produces several cytokines and biologically active proteins, denoted as adipokines, regulating hepatic and peripheral glucose and lipid metabolism. These adipokines include leptin, resistin, adiponectin and TNF-α. Expression of resistin is not increased in patients with insulin resistance, although resistin inhibits insulin action in animal models[55]. NASH patients show increased serum leptin levels, suggesting the attempt to overcome hepatic leptin resistance to stimulate hepatic lipid turnover[56]. In several studies investigating the pathomechanisms of fatty liver disease increased TNF-α levels have also been demonstrated[57, 58]. TNF-α contributes to insulin resistance and thereby increases hepatic steatosis and plays a

potentially proinflammatory role[59]. This was supported by studies in leptin deficient ob/ob mice. Treatment of anti-TNF-α antibody improved liver histology, reduced hepatic total fatty acid content, and decreased ALT levels[11]. However, studies of ob/ob mice lacking type I and II TNF receptors have suggested that TNF-α is not involved in the liver disease[60]. Further evidence of the involvement of TNF-α came from studies of pentoxifylline which acts as an inhibitor of TNF-α[61, 62]. In these two studies 20 patients and 18 patients, respectively, with biopsy confirmed NASH were enrolled. Pentoxifylline was given for 6 or 12 mo. Both studies demonstrated a significant improvement of AST and ALT levels after application of pentoxifylline in patients with NASH, although histological evidence of its benefit remains unknown.

Adiponectin is exclusively secreted from adipose tissue in inverse proportion to BMI[63, 64]. Although the three dimensional structure of adiponectin closely resembles that of TNF-α[65], these two proteins have completely opposite effects. Adiponectin and TNF-α suppress each other´s production and antagonize their biological effects[66]. Adiponectin acts to reduce body fat[67], improve hepatic and peripheral insulin sensitivity[68] and decrease fatty acid levels[69]. Adiponectin has also antiinflammatory effects which could prevent liver disease. Xu and coworkers replenished recombinant adiponectin in mice fed with a high fat ethanol containing diet and in obese ob/ob mice with NASH. In both mice, administration of adiponectin ameliorated hepatomegaly, steatosis, and elevated ALT levels[70]. Both hepatic TNF-α expression and serum levels of TNF-α significantly decreased, which is further evidence for a harmful role of TNF-α.

This concept was further supported by a study of over 100 patients with NAFLD[71]. Multivariate analysis revealed that decreased serum adiponectin levels and increased TNF-α and soluble TNFR2 levels correlated with the presence of NASH independent of the presence of insulin resistance. NASH patients showed lower adiponectin levels than patients with simple steatosis. Levels of adiponectin correlated with the degree of hepatic necroinflammation. These data provide evidence for the involvement of TNF-α and adiponectin in human NAFLD. Therefore, studies evaluating the effect of adiponectin in treatment of NASH are required.

LIVER TRANSPLANTATION

NASH is considered the most common cause of cryptogenic cirrhosis[72]. Patients with pure steatosis have a benign prognosis, whereas the risk for developing cirrhosis and hepatocellular carcinoma in NASH patients is increased[73, 74]. Complications of cirrhosis or hepatocellular carcinoma may require liver transplantation. In one study by Laurin and colleagues six of eight patients who underwent transplantation for NASH developed recurrent NASH. In three of these six patients, perivenular fibrosis recurred[75]. The patients with recurrences revealed post-transplant hyperlipidemia and increases in body weight. In two subsequent studies the recurrence rate of steatosis was between 60 to 100% of transplant recipients[76, 77]. In one third of these patients progression to steatohepatitis occurred.

CONCLUSIONS

Because of increasing incidence of obesity and insulin resistance NAFLD has become increasingly the focus of basic and clinical research. Whereas fatty liver shows a benign prognosis, patients with NASH should be treated. Progress in understanding the pathomechanisms which contribute to aggravation of fatty liver pathology offers potential treatment options. However, a standard therapy has not been established. The most promising results came from trials with second generation insulin sensitizer in obese patients with insulin resistance. Blockade of TNF-α by adiponectin showed impressive improvement of NASH in an animal model. Clinical trials investigating therapeutic effect of inhibiting TNF-α in NAFLD have to be awaited. So, beyond clinical studies, the first step in treatment should be improvement of insulin sensitivity by weight loss and physical activity.

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GASTRIC CANCER

Mechanisms inactivating the gene for E-cadherin in sporadic gastric carcinomas

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Abstract

AIM: To study the role of *CDH1/E-cadherin* (E-cad) gene alteration profiles including mutation, loss of heterozygosity (LOH), promoter polymorphism and hypermethylation in mechanisms of *CDH1* inactivation in gastric carcinoma (GC).

METHODS: Specimens were collected surgically from 70 patients with GC. Allelotyping PCR and detection of LOH, denaturing high pressure liquid chromatography and DNA sequencing, restriction fragment length polymorphism analysis, methylation specific PCR, and immunohistochemical staining were used.

RESULTS: Promoter polymorphism was not a major mechanism of E-cad inactivation. Only one truncating mutation was found in a diffuse type tumor (3%). Both LOH and promoter hypermethylation were major mechanisms of E-cad inactivation, but interestingly, there was a negative association between the fraction of allelic loss (LOH) in tumors and hypermethylation of *CDH1*. Therefore LOH and hypermethylation were two different tumorigenic pathways involved in GC.

CONCLUSION: Given the findings that somatic mutation was extremely low and the relationship between LOH and hypermethylation was inverse, any two combinations of these three factors cannot fulfill the classical two-hit hypothesis of *CDH1* inactivation. Thus, other mechanisms operating at the transcriptional level or at the post-translational level might be required to induce E-cadherin inactivation.

Key words: Gastric carcinoma; E-cadherin gene alteration profiles; Inactivation of E-cadherin

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INTRODUCTION

CDH1/E-cadherin (E-cad) is a member of the family of transmembrane glycoproteins expressed on epithelial cells and is responsible for calcium-dependent cell-to-cell adhesion[1]. E-cad forms complexes and connects actin filaments with α-, β-, and γ-catenins[2,3], which are essential to neoplastic transformation and metastasis[4, 5]. Loss of cell adhesion may contribute to loss of contact inhibition of growth, which is an early step in the neoplastic process. Furthermore, loss of cadherin activity may result in cancer cell detachment and metastasis[6, 7].

Gastric carcinogenesis is a multi-step process with morphological progression involving multiple genetic and epigenetic events. E-cad gene (*CDH1)* is an important putative tumor suppressor gene. In gastric carcinomas (GCs), the reduction in E-cad expression activation of *E-cad* gene varies from 17% to 92%, and is more frequent in diffuse type than in intestinal type tumors[8-13]. Germline mutation of the *CDH1* gene is found in all familial GCs[14, 15]. Somatic mutations of *CDH1* are found in more than

50% of diffuse type GCs but are not found in intestinal type GCs in Caucasians and Japanese populations[16-19]. The rate of loss of heterozygosity (LOH) ranges from 2.8% to 60% in diffuse and intestinal type tumors[16-20]. In addition to the well-known 'two-hit'inactivation mechanism proposed by Knudson (1971), *CDH1* can be silenced in GC by epigenetic promoter hypermethylation[17, 21]. Besides, Li *et al*[22] reported that the-60C/A polymorphism has a direct effect on the transcriptional regulation of *CDH1*. All above previous studies of the inactivation of this gene in patients with GC have been limited in their analyses. In this study, we investigated a range of alterations in *CDH1* expression profiles, including genetic mutations, LOH, promoter polymorphism, promoter hypermethylation, and immunohistochemical stain of E-cad protein together to determine possible genetic and epigenetic mechanisms of *CDH1* inactivation.

MATERIALS AND METHODS

Patients and samples

Specimens were collected surgically from 70 Taiwanese patients with GC between July 1999 and July 2002 at the Division of General Surgery, Department of Surgery, Tri-Service General Hospital, Taipei, Taiwan. None of the subjects received preoperative anticancer therapy. Clinical information was obtained from medical records. Samples were taken from representative cancerous lesions and the adjacent non-cancerous epithelial parts of the tissues were flash frozen in liquid nitrogen and stored at -80℃. All tumor DNA samples were obtained by micro-dissection from 5-μm thick hematoxylin and eosin stained and paraffin embedded tissue sections[23]. Non-cancerous DNA was extracted from tissues which were flash-frozen in liquid nitrogen and stored at -80℃. All 70 samples were classified according to the Lauren's criteria[23]: 27 were intestinal and 43 were diffuse types. The tumors were staged at the time of surgery using the standard criteria by TNM staging, with the unified international gastric cancer staging classification[24].

Allelotyping PCR and detection of allelic loss or loss of heterozygosity (LOH) of CDH1

DNA samples from tumor and normal mucosal specimens were used for allelotyping PCR with fluorescent primers (markers). Three micro-satellite markers (D16S3043, D16S3050, and D16S3021) at 16q22.1 were used to detect LOH at the CDH1 locus. PCR amplification was carried out as previously described[26]. PCR products were separated electrophoretically on an ABI PRISM 377 DNA sequencer, and fluorescent signals from the differently sized alleles were recorded and analyzed using Genotyper version 2.1 and GeneScan version 3.1 software packages. A given informative marker was considered to display LOH when a threefold or greater difference was seen in the relative allele intensities of the tumor and normal DNA samples.

Denaturing high pressure liquid chromatography (DHPLC) analysis and DNA sequencing for CDH1 mutation analysis

We used DHPLC and direct sequencing to determine inactivating mutations responsible for the loss of *CDH1* expression. The promoter region and 16 exons including the exon-intron boundaries were analyzed using the previously described protocol and primer pairs[26]. The optimal conditions for DHPLC analysis of each amplicon were available as requested. All variants detected by DHPLC were re-amplified and the site of variation was identified by direct DNA sequencing using an ABI PRISM 377 DNA sequencer.

Restriction-fragment length polymorphism (RFLP) analysis to identify nucleotide changes at –160 of the CDH1 promoter

The -160 polymorphic site contained either a C or A residue. The tumor type was determined by *Bst*EII digestion of the PCR products amplified using the primer set 5′-TGATCCCAGGTCTTAGTGAG-3′ (upstream) and 5′-AGTCTGAACTGACTT CCGCA-3′ (downstream). The 318-bp PCR product was cut into two fragments (208 and 110 bp) if it contained the A residue. To ensure that the observed polymorphism was specific and not an experimental artifact, the results were confirmed by direct DNA sequencing.

Methylation-specific PCR (MSP) and bisulfite-modified genomic sequencing to detect promoter hypermethylation of CDH1

Genomic DNA was modified by bisulfite treatment, converting unmethylated cytosines to uracils and leaving methylated cytosines unchanged. MSP was performed on the treated DNA to detect all three CpG islands in the *CDH1* promoter region as previously described[27]. Each unmethylated–methylated primer pair set was engineered to assess the methylation status of 4-6 CpGs with at least one CpG dinucleotide positioned at the 3′ end of each primer to discriminate between methylated and unmethylated alleles following bisulfite modification. Hs578t cells, which contain a heterogeneously methylated CpG island 1 and methylated CpG islands 2 and 3, served as the positive control, and MCF7 cells were used as the negative control.

Immunohistochemical staining and evaluation of E-cad expression

Sections (5 μm thick) were treated with monoclonal anti-E-cad antibody (Cappel, Aurora, OH, USA), then with secondary antibody. The signal was detected using a kit containing avidin–biotin complex and diaminobenzidine (DAB; Vector Laboratories, Burlingame, CA, USA). DAB produced a yellowish brown staining if the sample was positive. If more than 90% of the tumor cells exhibited intense membranous staining similar to that of normal cells, the result was considered positive (++). If the staining intensity was demonstrably reduced relative to that of normal cells and/or the staining pattern was heterogeneous (10%-90% positive), the result was deemed to be weakly positive (+). If IHC expression was completely lost or positive in less than 10% of cells, the result was defined as negative (–).

Statistical analysis

Analyses were performed using S-Plus® 2000 for

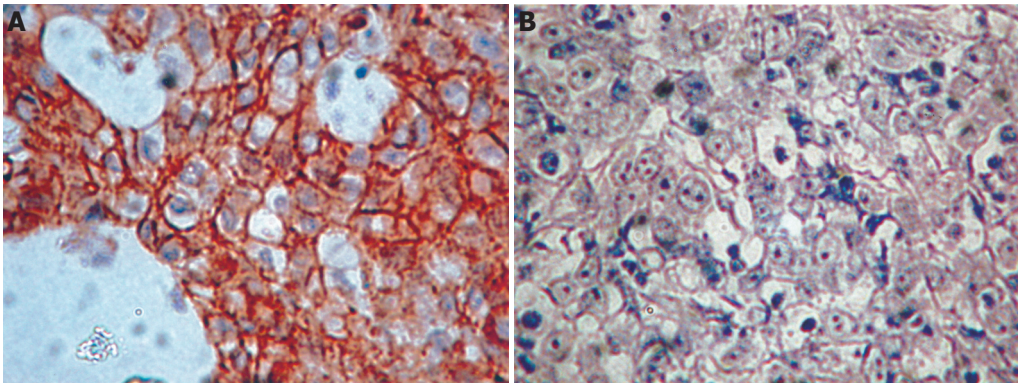

Figure 1 Immunohistochemical staining for positive (**A**) and negative (**B**) E-cad expression in diffuse type tumor.

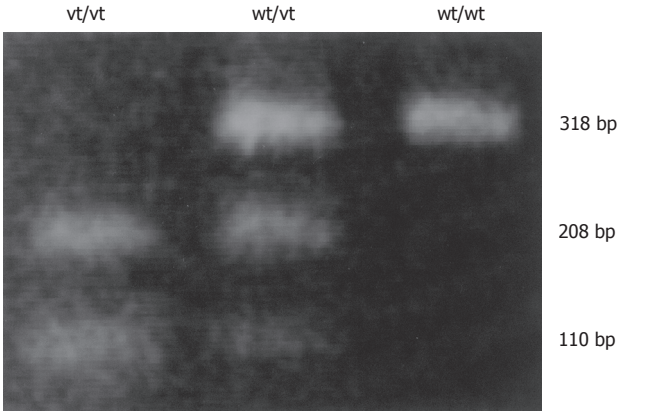

Figure 2 PCR-restriction fragment length polymorphism (RFLP) analysis of genetic polymorphism of the -160 site of the *E-cad* promoter. The C/A polymorphism was differentiated by *BstEII* digestion of PCR products homozygous for the wild-type (high-activity) allele (*wt/wt*, CC gentoype), heterozygous for the variant (low-activity) allele (*wt/vt*, CA genotype), and homozygous for the low-activity allele (*vt/vt*, AA genotype)

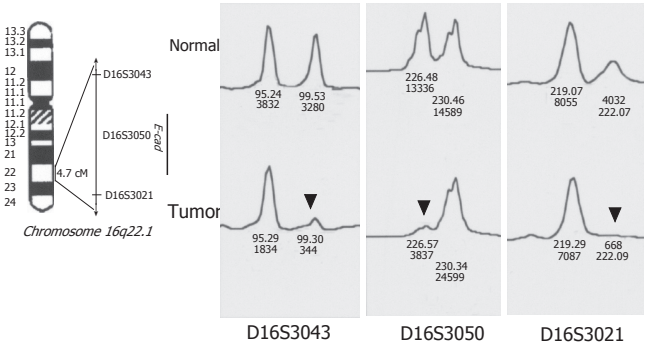

Figure 3 Allelic loss or loss of heterozygosity (LOH) of *CDH1*/*E-cad*. Left panel: *E-cad* detected by allelic loss or loss of heterozygosity (LOH) of the *E-cad* locus, reflected by three microsatellite markers (*D16S3043*, *D16SS3050* and *D16S3021*) at 16q22.1. *Right panel*: LOH in a representative GC. The locius of markers *D16S3043*, *D16S3050*, and *D16S3021* were considered to be informative when they were heterozygous in normal tissue (i.e. two alleles were seen), and showed LOH when a 3-fold or greater difference was seen in the relative allele intensity ratio between the tumor and normal DNA (arrow).

Windows statistical software (CANdiensten, Amsterdam, Netherlands). Significance was assumed at $P<0.05$ for all tests. Categorical variables were tested using Fisher's exact test.

RESULTS

Of the 70 patients, 52 were men and 18 were women. Their median age was 69.7 years (range 32-88 years). According to Lauren's classification, 27 and 43 tumors were intestinal and diffuse histotypes, respectively. Reduced gene expression was more frequent in diffuse type tumors (38/43, 88%) than in intestinal type tumors (13/27, 48%; $P=0.006$). Representative examples of immunohistochemical staining for E-cad expression in diffuse type tumors are shown in Figure 1.

Promoter polymorphism

Three of the 70 patients were omitted from our analysis of the -160C/A polymorphism due to insufficient samples. Among the other 67 patients, 29 were genotype C/C (43%), 24 were genotype A/C (36%) and 14 were genotype A/A (21%) (Figure 2). There was no significant difference in the frequency of the C/A + A/A genotypes between diffuse and intestinal type tumors (27/42, 64% *vs* 11/25, 44%). There was no significant difference in LOH between the C/C and C/A + A/A genotypes (10/25, 40% *vs* 13/33, 39%). There was also no significant difference in hypermethylation between C/C and C/A + A/A genotypes (20/29, 69% *vs* 24/37, 65%). There was no significant difference in the frequency of the C/A + A/A genotypes between tissues with reduced and normal E-cad expression (12/17, 71% *vs* 27/50, 54%).

Loss of heterozygosity

To detect allelic loss at *CDH1*, three micro-satellite markers (D16S3043, D16S3050, D16S3021) at 16q22.1 were used (Figure 3). The allelic status of this gene was reflected well by these three markers, because its locus was very close to the loci of these markers (LOD score > 4 estimated by linkage analysis). We considered the results for all three markers together and found heterozygosity in at least one. Of the 70 samples collected, 10 were omitted from the analysis or homozygous and could not be detected. A high frequency of allelic loss at *CDH1* was detected (23/60, 38%). The frequency of LOH at *CDH1* was similar between diffuse type tumors (15/38, 39%) and intestinal type tumors (8/22, 36%). Reduced E-cad expression was more frequent in LOH-positive tumors (21/23, 91%) than in LOH-negative tumors (24/37, 65%; $P=0.03$).

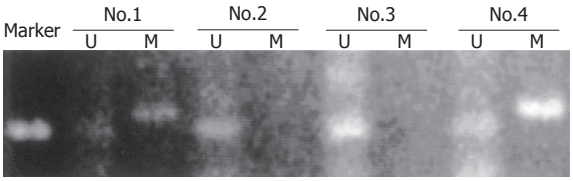

Figure 4 Promoter hypermethylation of the *CDH1/E-cad* detected by methylation-specific PCR (MSP). The presence of a visible PCR product in the lanes marked U indicates the presence of an unmethylated allele, while the presence of the product in the lanes marked M indicates the presence of a methylated allele. The intensity of each methylated band was further semi-quantitated, and as shown in the figure, cases 1 and 4 were defined as "hypermethylation"with "+"and "++", respectively, and cases 2 and 3 were defined as "unmethylation".

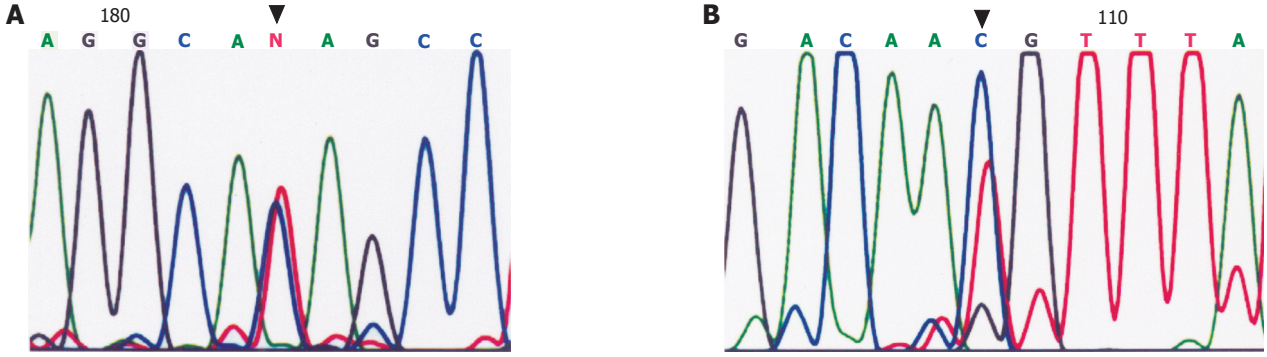

Figure 5 *CDH1*/E-cad mutation and polymorphism detected by direct DNA sequencing. Two tumors subjected to DNA sequencing were found to harbor C-to-T transversion in exon 13 (**A**), resulting in a truncated mutation (Gln to stop condon TAG) and C-to-T transversion in exon 14 (**B**), resulting in no amino acid change, which was considered to be polymorphism.

Promoter hypermethylation

The degree of hypermethylation estimated by MSP was defined as strongly detectable (+++, ++), detectable (+), or not detectable (–)(Figure 4). Three of the 70 samples were omitted from our analysis of hypermethylation due to insufficient samples. The *CDH1* promoter was hypermethylated in 45 of these 67 GCs (67%). Hypermethylation was more frequent in diffuse type tumors (31/41, 76%) than in intestinal type tumors (13/26, 50%; $P=0.03$ by Fisher's exact test). Furthermore, hypermethylation was more frequent in GCs with reduced E-cad expression than in those with normal levels (37/45, 82% *vs* 12/22, 55%; $P=0.02$). The fraction of allelic loss (FAL) of *CDH1*, calculated as the frequency of LOH at *CDH1* locus, was generally inverse to the degree of hypermethylation (Tables 1, 2).

Mutation

In these 70 patients, five diffuse type tumors (Case No. 15, 24, 29, 30, and 39) had a single-nucleotide polymorphism (SNP) at amino acid 692, and four diffuse type tumors (Case No. 35, 40, 59, and 63) had an SNP at position 755 . Case No.15 had a truncated mutation at position 699 (Figure 5). No *CDH1* mutation was found in intestinal type tumors.

DISCUSSION

In this study, 27 and 43 tumors were of the intestinal and diffuse histotypes, respectively. Inactivation of the *CDH1* gene and loss of normal E-cad expression were involved more frequently in diffuse type than in intestinal type tumors (88% *vs* 48%; $P=0.006$). However, the percentage of reduction in E-cad expression of GC varies from 17% to 92% in previous reports[8-13]

Promoter polymorphism

Li *et al*[22] reported that the A allele of the -160C/A promoter polymorphism alters transcriptional binding, resulting in a reduction in transcriptional efficiency of 68% relative to that of the C allele. In our study, there was no significant difference in the frequency of the C/A + A/A genotypes between diffuse and intestinal type tumors. There was no significant difference in LOH and hypermethylation between the C/C and C/A + A/A genotypes. There was also no significant difference in the frequency of the C/A + A/A genotypes between tumors with reduced and normal E-cad expression, suggesting that the A allele does not play a major role in the inactivation of *CDH1* and can not serve as the 'second hit'.

Mutations

Somatic mutations of *CDH1* are found in more than 50% of diffuse type GCs but not in intestinal type GCs in Caucasian and Japanese populations[16-19]. A review by Berx *et al*[28] noted that the predominant defects in diffuse type tumors are splice mutations causing skipping in exon 8 or 9, which account for in-frame deletions, whereas mis-sense and truncating mutations are less frequent in diffuse GCs. Moreover intragenic polymorphisms arise from changes in the third (wobble) position of the respective codons and are more frequent in codons 692 and 751. In the present study, five of the diffuse type tumors had a codon 692 polymorphism and four diffuse type tumors had a codon 755 polymorphism. Only one of 38 diffuse type tumors had a truncated codon 699 mutation. Because consistent findings have been obtained by repeated detection of the same specimens, we considered this finding to be valid. Therefore, this low rate of *CDH1* mutation in the Taiwanese GCs may suggest different tumorigenic mechanisms to inactivate this gene.

Table 1 Fraction of allelic loss (FAL) in tumors with different hypermethylation statuse

Promoter hypermethylation	FAL	
Yes (++,+++)	0.098	
Yes (+)	0.214	
No	0.377	$P=0.03$

FAL was estimated by allelic status at D16S3043, D16SS3050 and D16S3021. FAL (fraction of allelic loss) = number of loci showing LOH / number of informative loci in each tumor. The FALs of tumors with different hypermethylation status were calculated as the mean of FALs of individual tumors with the same hypermethylation status.

Table 2 Association between loss of heterozygosity and promoter hypermethylation of *CDH1*

	Loss of heterozygosity		
	Yes (%)	*n* (%)	Promoter
hypermethylation			
Yes (++,+++)	1 (9.1)	17 (34.7)	
Yes (+)	2 (18.2)	21 (42.9)	
No	8 (72.7)	11 (22.5)	$P=0.001$

LOH status is defined by D16S3043

Loss of heterozygosity

It was reported that the rate of LOH ranges from 2.8% to 60% in diffuse and intestinal type tumors [16-20]. A high frequency (38%) of allelic loss at *CDH1* was identified in our study. The frequency of LOH was similar between the diffuse and intestinal type tumors (39% *vs* 36%). Reduced E-cad expression demonstrated by immunohistochemical analysis was more frequent in LOH-positive tumors than in LOH-negative tumors (91% *vs* 65%; $P=0.03$), suggesting that LOH is a major mechanism for the inactivation of *CDH1*.

Promoter hypermethylation

Tamura *et al*[29] and Graziano *et al*[30] indicated that *CDH1* promoter methylation may play a major role together with mutations or deletions, in causing the inactivation of the *CDH1* gene in GCs, especially in diffuse type tumors. They also reported that *CDH1* promoter hypermethylation is associated with reduced E-cad expression detected immunohistochemically. In the present study, the *CDH1* promoter was hypermethylated in 67% of GCs. Hypermethylation was more frequent in diffuse type tumors than in intestinal type tumors ($P=0.03$). Furthermore, hypermethylation was more frequent in tumors with reduced E-cad expression than in normal E-cad expression (82% *vs* 55%; $P=0.02$), suggesting that *CDH1* promoter hypermethylation is a major mechanism for gene inactivation.

Methylation of the *CDH1* promoter has been documented as the 'second hit' responsible for the development of hereditary diffuse GCs[31] and sporadic diffuse GCs[17] among Caucasians. Because there was only one genetic mutation in diffuse type tumors and no mutation in intestinal type tumors in this series, we examined the hypermethylated status of tumors with or without LOH at the *CDH1* locus. We investigated the relationship between hypermethylation and FAL, which was estimated from the allelic status at D16S3043, D16S3050, and D16S3021. Hypermethylated tumors tended to have significantly lower FAL values (Table 1). This is contrary to the result predicted by the two-hit hypothesis. Further examination using individual markers to redefine the LOH status of tumors yielded similar results (Table 2). Therefore, cancers having lost one *CDH1* allele and those carrying hypermethylated *CDH1* alleles may be involved in two different tumorigenic pathways. Because the somatic mutation rate is extremely low, any two combination of these three factors cannot fulfill the classic 'two-hit' hypothesis. Other molecules involved in the E-cad-mediated cell-cell adhesion complex, such as the intracellular attachment proteins α, β, and γ-catenin, may be subjected to targeted inactivation[32-36]. Receptor tyrosine kinase (RTK), the main positive regulator of progression and tissue expansion, can repress E-cad function by transcriptional repression of *CDH1* via the transcription factor SNAI1[37,38], posttranscriptional repression via direct or indirect phosphorylation of adheren junction components such as β-catenin[39], or RTK-associated endocytosis and degradation of the E-cad protein[40]. This more flexible status achieved either by retaining an intact allele subsequent to LOH or by regulation via epigenetic mechanisms operating at the transcriptional or posttranslational levels, could provide an advantage in counteracting the changing microenvironment during tumor progression. Further investigation is needed at the transcriptional level and the post-translational level into E-cad inactivation of GC.

In conclusion, given the finding that somatic mutation was extremely low and the relationship between LOH and hypermethylation was inverse, any two combinations of these three factors can not fulfill the classical two-hit hypothesis of E-cadherin inactivation. Thus, other mechanisms operating at the transcriptional level or at the post-translational level, might be required to inactivate E-cadherin in GC.

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Helicobacter pylori

Effect of NaCl and *Helicobacter pylori* vacuolating cytotoxin on cytokine expression and viability

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Abstract

AIM: To determine whether *Helicobacter pylori* (*H pylori*) vacuolating cytotoxin (VacA) regulates release of pro-inflammatory cytokines (IL-1β, IL-8, TNF-α, and IL-6) or alters gastric epithelial cell viability and to determine whether NaCl affects these VacA-induced changes.

METHODS: Vacuolating activity was determined by measuring the uptake of neutral red into vacuoles of VacA-treated human gastric epithelial (AGS) cells. AGS cell viability was assessed by direct cell counting. Specific enzyme-linked immunosorbent assays (ELISA) and reverse transcriptase-polymerase chain reaction(RT-PCR) were performed to examine the effects of *H pylori* VacA and NaCl on cell pro-inflammatory cytokine production in AGS cells. Immunohistochemical staining of gastric tissue from Mongolian gerbils was used to confirm VacA-induced pro-inflammatory cytokine production and the effects of NaCl on this VacA-induced response.

RESULTS: Addition of VacA alone reduced AGS cell viability ($P < 0.05$), and this reduction was enhanced by high doses of NaCl ($P < 0.05$). VacA alone induced expression of TNF-α, IL-8 and IL-1β, while NaCl alone induced expression of TNF-α and IL-1β. Changes in mRNA levels in the presence of both VacA and NaCl were more complicated. For the case of TNF-α, expression was dose-dependent on NaCl. IL-6 mRNA was not detected. However, low levels of IL-6 were detected by ELISA. Positive immunohistochemical staining of IL-1, IL-6, and TNF-α was found in gastric tissue of *H pylori*-infected gerbils fed with either a normal diet or a high salt diet. However, the staining of these three cytokines was stronger in *H pylori*-infected animals fed with a 5g/kg NaCl diet.

CONCLUSION: VacA decreases the viability of AGS cells, and this effect can be enhanced by NaCl. NaCl also affects the production of pro-inflammatory cytokines induced by VacA, suggesting that NaCl plays an important role in *H pylori*-induced gastric epithelial cell cytotoxicity.

Key words: *Helicobacter pylori*; Vacuolating cytotoxin; Cytokine; Gerbil; AGS cell

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INTRODUCTION

Gastric cancer is one of the leading causes of cancer-related death[1, 2]. Epidemiological studies in humans and rodents have demonstrated that chronic gastritis caused by *Helicobacter pylori* (*H pylori*) is a strong risk factor for this malignancy[3]. Current models suggest that *H pylori* is not directly carcinogenic but rather acts indirectly on inducing cancer. For example, it may act through inflammatory mediators or by decreasing gastric acid secretion, thus favoring the formation of mutagenic adducts[4]. *H pylori* expresses several major proteins that are critical to the pathogenicity of the bacterium[5]. The vacuolating cytotoxin, VacA, is an *H pylori*-secreted virulence factor that induces the formation of large cytoplasmic vacuoles in epithelial cells, which precedes cultured cell death both *in vitro* and *in vivo*[6-9]. In the past decade, a considerable number of studies have contributed to our understanding of the mechanism of VacA-induced intracellular vacuolation[6].

Salt consumption is considered a risk factor for gastric cancer[10]. *H pylori*-associated mucosal atrophy is partially associated with a high salt diet[11]. Excessive NaCl intake enhances *H pylori* colonization in mice and humans, while chronic salt intake may exacerbate gastritis by increasing *H pylori* colonization[12]. Furthermore, salt intake may interfere with *H pylori* infection and modify the cancer risk[13]. The high prevalence of *H pylori* infection and high salt diets may significantly affect the rate of occurrence of gas-

tric carcinogenesis[14-17].

It has been reported that cytokines play an important role in gastric cancer. Proinflammatory cytokines such as interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) may up regulate cyclooxygenase-2 (COX-2) during gastric carcinogenesis[18]. Genotypes of IL-1β (-511 T/T) and TNF-α (-308 A/A) are associated with the risk of non-cardia gastric cancer. The presence of TNF-α SNPs -308 and -1031 might favor *H pylori* infection and promote an inflammatory response in infected gastric mucosa[19]. IL-1β genotype has been found to increase the risk of distal gastric cancer[20]. IL-1β is one of the potent proinflammatory cytokines elicited by *H pylori* infection[21, 22]. In human gastric cancer cells, IL-1β induces vascular endothelial growth factor (VEGF), a dominant angiogenic factor in gastric cancer[23]. Moreover, levels of IL-1β and IL-8 have been found to be significantly higher in *H pylori*-positive gastric cancers compared with controls, and eradication of *H pylori* can significantly reduce the levels of these cytokines[24]. Genetic polymorphisms identified in *IL-6* can be attributed to ethnicity and appear to be independent of the clinical outcome of *H pylori* infections[25].

The present study was to examine the combined effects of VacA and NaCl on cell viability and pro-inflammatory cytokine expression both in AGS cells and in Mongolian gerbils.

MATERIALS AND METHODS

Preparation of VacA

The toxin-producing strain, *H pylori* ATCC49503, was used as the source of VacA. *H pylori* was grown under microaerophilic conditions on Columbia blood agar plates with vigorous shaking in a controlled microaerophilic atmosphere of 50 mL/L O_2 and 100 mL/L CO_2 at 37℃ for 3-4 d, harvested and re-suspended in brucella broth (Difco) supplemented with 100 mL/L fetal calf serum. *H pylori* cells were grown for 48 h at 37℃ in an Erlenmeyer flask with shaking at 60 r/min. Cells were then pelleted by centrifugation at 500 r/min for 20 min. The culture supernatants were collected and pooled. Solid ammonium sulfate was added to the pooled material at 4℃ to 50% saturation. The precipitate was collected by centrifugation at 12000 r/min for 20 min, dissolved in 60 mmol/L Tris-HCl (pH 7.7) containing 0.1 mol/L NaCl and subjected to liquid chromatography on a Superose 6 HR 10/30 column (1cm × 30 cm) equilibrated with 60 mmol/L Tris-HCl (pH 7.7) containing 0.1 mol/L NaCl. The material was eluted with the same buffer at a flow rate of 0.5 mL/min. The eluted protein was monitored by Western blotting using polyclonal anti-VacA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The fractions containing VacA were collected, pooled, diluted with deionized water to the intended concentration and maintained at -20℃[26]. VacA concentration was determined by Micro-BCA assay (Bio-Rad, Hercules, CA).

Vacuolating activity assay

The human gastric epithelial cell line AGS (ATCC CRL 1739) was maintained in Ham's F-12 medium supplemented with 100 mL/L fetal calf serum and 50 mg/L penicillin-streptomycin (Life Technologies, Inc.) under 50mL/L CO_2 at 37℃. AGS cells were seeded in 96-well culture plates (5×10^3 cells/well in 90 μL) and cultured as monolayers for 20 h in a 50 mL/L CO_2 atmosphere at 37℃. Samples (10 μL) of VacA were added to achieve the indicated final concentrations and cells were incubated for an additional 2-8 h at 37℃[27]. Vacuolating activity was determined by measuring the uptake of neutral red into vacuoles in VacA-treated cells. Cells were incubated for 5 min at room temperature with 50 μL of freshly prepared 0.5 g/L neutral red in PBS containing 3 g/L BSA and washed three times with 0.1 mL of PBS containing 3 g/L BSA. After addition of 0.1 mL 700 mL/L ethanol in water containing 0.4 mL/L HCl, absorbance at 540 nm (A_{540}) was measured. Vacuolating activity was determined by subtracting the A_{540} of cells incubated without VacA from the A_{540} of VacA-treated cells. To evaluate the effect of NaCl on vacuolating activity, AGS cells were incubated with 40 mg/L VacA and 0-10 mmol/L NaCl for the indicated periods.

Assessment of AGS cell viability

AGS cells were seeded to a subconfluent density of 5×10^4 cells/well in 24-well plates and incubated at 37℃ overnight. The supernatant was discarded before co-incubation. AGS cells were grown with NaCl alone or in the presence of VacA and NaCl in F-12 medium supplemented with 100 mL/L FBS. Control cells were inoculated into F-12 and incubated for up to 72 h in triplicate. At the end of each time of incubation, cell viability was determined in a hemacytometer by trypan blue exclusion.

Cytokine and cytokine mRNA measurement

After AGS cells were incubated for 24 h. *H pylori* VacA (40 mg/L) and 10 mmol/L NaCl were added. The cells were incubated for an additional 4 h. The supernatants were then collected and stored at -20℃ until assay. The levels of IL-6, IL-8, IL-1β and TNF-α in the culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (Immunotech, France) according to the manufacturer's instructions. In these assays, the lower limits of detection were 3 ng/L for IL-6, 8 ng/L for IL-8, 1.5 ng/L for IL-1β and 5 ng/L for TNF-α.

Total RNA was extracted from AGS cells (incubated as indicated above, under cytokine measurement) using Isogen (Nippon Gene, Tokyo, Japan). Aliquots (2.5 μg) of total RNA were incubated at 70℃ for 5 min, chilled on ice and reverse transcribed in a final volume of 10 μL of a solution containing 200 MU/L Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Invitrogen Life Technologies), first-strand buffer (250 mmol/L Tris-HCl, pH 8.3 at room temperature, 375 mmol/L KCl, 15 mmol/L $MgCl_2$) containing 0.1 mol/L DTT and 2.5 mmol/L dNTPs plus random primer (6 mer) pd(N)6 (TaKaRa, Japan). Reactions were incubated at 22℃ for 10 min, at 37℃ for 60 min, heated to 80℃ for 5 min, and stored at -20℃ until use.

The resultant cDNA from above (1 μL) was added to a 29-μL reaction containing PCR reaction buffer, 1 μL of 4 nmol of each primer (IL-1 β, IL-6, IL-8, TNF-α),

Table 1 Viability of AGS cells grown in the presence of NaCl or VacA and NaCl (*P* value, *t*-test)

VacA (mg/L)	NaCl (mmol/L)	24 h	48 h	72 h
(-)				
	0	-	-	-
	2.5	0.12	0.07	0.22
	5	0.04	0.09	0.18
	10	0.06	0.11	0.15
50				
	0	0.03	0	0.01
	2.5	0.02	0	0.01
	5	0.01	0.02	0
	10	0	0	0
50				
	0	-	-	-
	2.5	0.45	0.36	0.13
	5	0.27	0.08	0.01
	10	0.12	0.01	0.04

0.1 μL of 5 MU/L of Taq DNA polymerase and water. Oligonucleotide primers were designed based on previous reports[28]. PCR was performed with an automatic thermal cycler using an initial denaturation step at 95 ℃ for 5 min followed by 36 cycles of denaturation at 94 ℃ for 1 min, annealing at 60 ℃ for 1 min, and an extension at 72 ℃ for 1 min. The final cycle included an extension for 7 min at 72 ℃ to ensure full extension of the products. Aliquots (5 μL) of each PCR product were analyzed by electrophoresis on 1.5 g/L agarose S gels (Wako Chemical Co., Ltd., Osaka, Japan) containing ethidium bromide, and the bands were examined under UV light to detect amplified DNAs.

Animal experiment

H pylori-infected and non-infected 7-wk-old male Mongolian gerbils (MGS/Sea; Seac Yoshitomi, Fukuoka, Japan) were housed in steel cages on hardwood chip bedding in an air-conditioned biohazard room with a 12 h light-12 h dark cycle. The animals were classified into four groups according to *H pylori* infection and NaCl diet ($n = 3$). *H pylori*-infected and non-infected gerbils were given autoclaved distilled water *ad libitum* and CE-2 irrigated with 50 g/kg NaCl (Kyudo Co. LTD, Japan) or CE-2 only (normal diet). After 4 mo, the gerbils were sacrificed and stomach tissue was obtained and frozen.

The frozen tissue blocks were sectioned (6 μm thick) as previously described[28] and the sections were fixed with 4 g/L paraformaldehyde and acetone for 10 min each. The sections were treated with 30 mL/L H_2O_2 (Wako, Japan) in methanol for 5 min followed by blocking with 10 ml/L normal rabbit serum (Vector Laboratories, Inc., Burlingame, CA) for 10 min. We used goat polyclonal antibodies as primary antibodies directed against the following mouse proteins: TNF-α, IL-1β, and IL-6 (R & D Systems, Inc.). Tissue sections were incubated for 60 min at room temperature in a moist chamber with the desired primary antibody diluted according to the manufacturer's instructions. Biotinylated rabbit anti-goat immunoglobulin G (Vector Laboratories) was then applied for 30 min at room temperature. After three times of 5-min rinse with PBS, the signals from the antibodies were amplified using an ABC kit (Vector Laboratories) for 30 min and visualized with DAB reagent (Vector Laboratories) for 5 min. The sections were counterstained with Mayer hematoxylin (Muto Pure Chemicals Co., Ltd., Tokyo, Japan), dehydrated and mounted using histological mounting medium (Fisher Scientific). Negative control reactions contained purified goat IgG (Vector Laboratories) instead of specific primary antibodies.

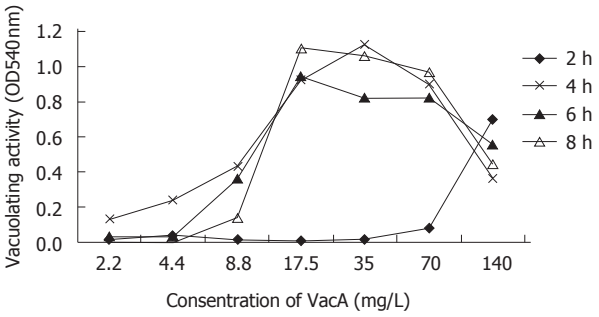

Figure 1 Activity of VacA-induced vacuolization.

Statistical analysis

All data in each experiment were expressed as the mean ± SE. The statistical significance of the response of cytokine production to VacA was evaluated using the Student's *t*-test. Differences between cytokine levels were considered significant at $P < 0.05$.

RESULTS

VacA visualization

VacA, partially-purified from a toxin-producing *H pylori* strain, was visualized on a Western blot with a VacA-specific antibody (Table 1). An antibody-reactive protein at 87 ku, the molecular weight expected for the mature toxin[26] was found.

VacA-induced vacuolation in AGS cells

AGS cells showed dose-dependent vacuolation induced by VacA (Figure 1). Different VacA concentrations were shown for the indicated incubation times. Vacuolization was quantified by measuring the uptake of neutral red into vacuoles. The experiments were carried out in triplicate. When VacA was incubated with AGS cells at 4, 6 and 8 h, its activity was stable in the range of 17-70 mg/L. Vacuolation was not seen in AGS cells in response to VacA after incubation for 2 h, except at the maximum concentration.

VacA-induced vacuolating activity was not influenced by NaCl

The time course for vacuolation within cultured cells in the presence of VacA and NaCl showed the effect of NaCl on the vacuolating activity (Figure 2). The vacuolating activity was shown as a function of incubation time at different NaCl concentrations. Cells were induced with 40 mg/L VacA. The experiments were carried out in triplicate.

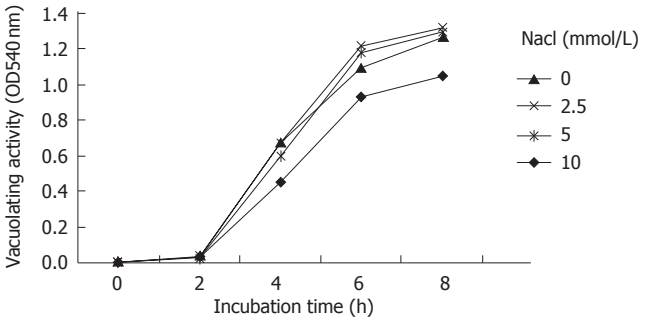

Figure 2 Time course of VacA-induced vacuolization of AGS cells at different NaCl concentrations.

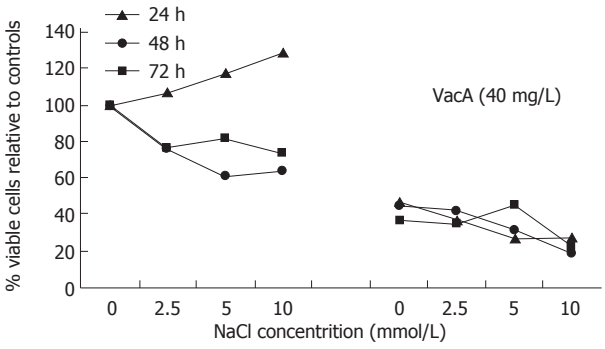

Figure 3 Viability of AGS cells grown in the presence of NaCl alone or VacA and NaCl.

At all NaCl concentrations, the vacuolating activity was minimal after incubation for 2 h. Time-dependent vacuolating activity increased linearly, doubling every two hours between 2 and 6 h of incubation. Because the curves at all NaCl concentrations were similar, NaCl did not appear to significantly affect VacA-induced vacuolation. As a control, the same concentrations of NaCl were added to AGS cells without VacA and no vacuolating activity was observed (data not shown).

Viability of AGS cells was decreased after coculture with VacA and NaCl

The effect of NaCl on AGS viability was assessed with and without VacA at the indicated incubation times. Cell viability was determined by trypan blue exclusion. The experiments were carried out in triplicate. Upon incubation with increasing levels of NaCl for 72 h, there was an apparent slight increase in the viability of AGS cells. However, culture for 24 or 48 h apparently decreased the viability of AGS cells (Figure 3). These results, however, were not statistically significant ($P > 0.05$) compared with control AGS cells at all incubation time (Table 1). Furthermore, the viability of AGS cells cultured with VacA and 10 mmol/L NaCl was significantly reduced ($P < 0.05$) compared with VacA alone after incubation for 48 and 72 h. Although the average viability values after incubation for 24 h were similar to those at 48 and 72 h, the viability at 10 mmol/L and 0 mmol/L NaCl was not significantly different ($P > 0.05$) because large variances were obtained for the 24-h samples. The reduced viability in the presence of VacA was similar at all three incubation times.

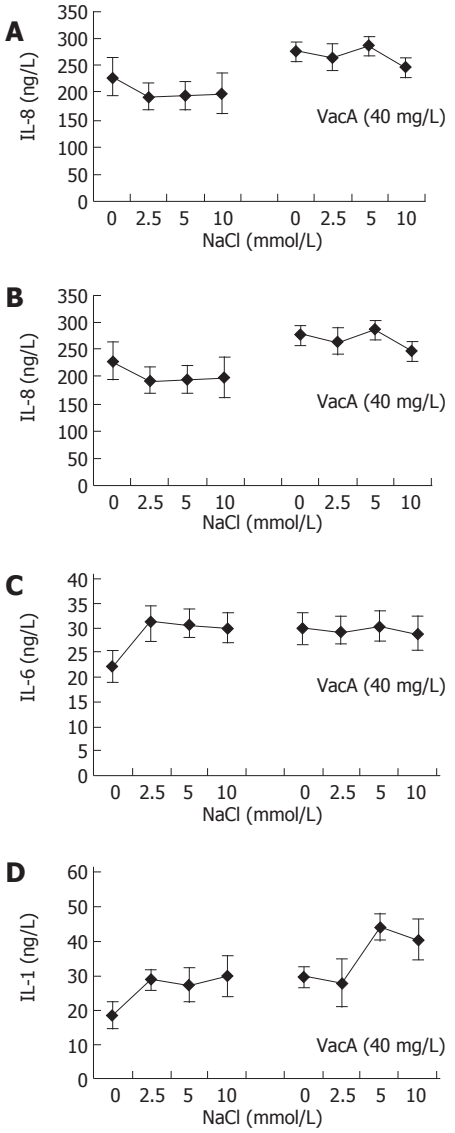

Figure 4 Production of cytokines induced by NaCl alone or VacA and NaCl.

VacA-induced cytokine production in AGS cells

To examine cytokine production after cell culture with VacA combined with NaCl, AGS cells were incubated with VacA or VacA and NaCl. The secretion of TNF-α, IL-8, IL-6 and IL-1β assessed by ELISA, is shown in Figure 4. The experiments were carried out in triplicate. In addition, we assessed the cytokine response of cultured human AGS cells to NaCl alone. The secretion of TNF-α increased in cells exposed to VacA, but this increase was inhibited in the presence of both VacA and NaCl. However, the secretion of TNF-α seemed to increase slightly in culture with NaCl alone ($P > 0.05$, Figure 4A). The secretion of IL-8 increased in cells exposed to VacA compared with cell-only control cultures or those exposed to NaCl alone. The secretion increased when VacA was combined with NaCl. However, NaCl did not appear to increase IL-8 secretion in the presence of VacA. NaCl did not induce IL-8 secretion from AGS cells at any concentration tested (Figure 4B). IL-6 secretion increased in cultures incubated with VacA or NaCl or both VacA and NaCl (Figure 4C). IL-1β secretion increased after culture with VacA or NaCl

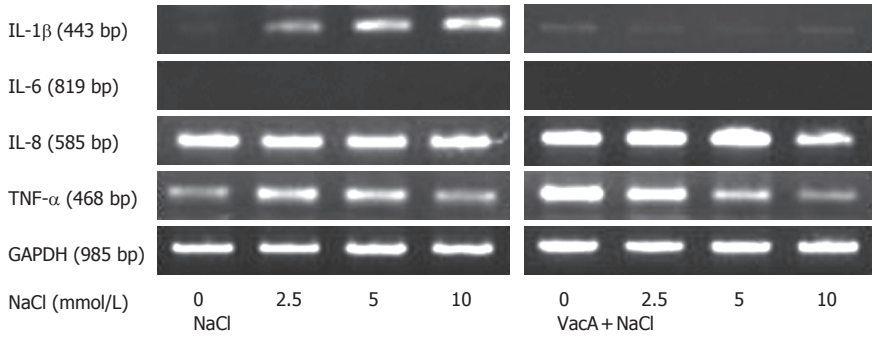

Figure 5 Expression of cytokines induced by NaCl or VacA with NaCl (RT-PCR) in gastric tissue of *H pylori*-infected gerbils (×200) fed with a normal diet (top) and a 50g/kg NaCl diet (bottom).

alone. The addition of 5 or 10 mmol/L NaCl enhanced VacA-induced IL-1β secretion, although it was not dose-dependent (Figure 4D).

Cytokine mRNA expression induced by VacA in AGS cells

To examine the ability of VacA combined with NaCl to induce the production of cytokines, cytokine-specific mRNA expression in AGS cells was analyzed by RT-PCR at 4 h post-induction. The expression of TNF-α, IL-8, IL-6 and IL-1β mRNAs is shown in Figure 5. TNF-α mRNA expression increased slightly after culture with NaCl, and there was a significant increase in TNF-α expression after culture with VacA. TNF-α mRNA expression decreased after culture with VacA combined with NaCl and displayed a significant NaCl dose-dependence. IL-8 mRNA expression was not stimulated by NaCl alone. However, the IL-8 mRNA PCR product increased after culture with VacA especially with VacA combined and NaCl, although a decrease was observed at the highest NaCl concentration. IL-6 mRNA was not detected in the control cells or upon exposure to any of the above conditions. IL-1β mRNA expression was induced in response to NaCl in a dose-dependent manner. Expression was also induced upon exposure to VacA, but this effect was not stimulated by NaCl.

Proinflammatory cytokine production in gastric tissue

Positive staining of IL-1β, IL-6, and TNF-α in gastric tissue was found in all but one of the *H pylori*-infected subjects ($n=6$) regardless of diet. However, we observed more staining of all three cytokines in *H pylori*-infected gerbils on a 50 g/kg NaCl diet than in infected gerbils on a normal diet, suggesting a potential role of NaCl in the up-regulation of VacA-induced pro-inflammatory cytokine production in gastric epithelium. IL-6 displayed less increase in staining than the other two cytokines, consistent with the *in vitro* ELISA results. There was no significant difference between the specific primary antibody staining and the IgG negative control staining in tissues from uninfected animals fed with 50 g/kg NaCl and positive staining was not observed in the untreated gerbils (data not shown).

DISCUSSION

The neutral red uptake assay is often used for quantitatively determining *H pylori* cytotoxicity *in vitro*[9]. Because the shortest time point at which vacuolation was induced in this study was 4 h, 4 h incubation was chosen for further experiments. The appropriate dose of VacA to induce vacuolation was determined to be 17.5 mg/L, and 40 mg/L VacA decreased AGS cell viability to 40% after 24 h. Therefore, 40 mg/L VacA was used in experiments to assess VacA-induced cytokine production.

Significant vacuolating activity is stimulated by acidic ($pH<5.5$) or alkaline ($pH>9.5$) conditions[27]. We evaluated the effect of high Na^+ and Cl^- concentrations on vacuolization induced by VacA. AGS cells exposed *in vitro* to VacA at various concentrations of NaCl had vacuolization rates similar to those exposed to VacA alone, suggesting that VacA may be activated by exposure to acidic or alkaline conditions but not under the osmotic conditions.

H pylori has been shown to either reduce or enhance gastric epithelial cell viability *in vitro*[29]. All strains of *H pylori* decrease cell viability of microvascular endothelial cells after 72 h[30]. Furthermore, co-culture of *H pylori* with AGS cells significantly decreases cell viability[29]. Consistent with these studies, our results demonstrated that VacA alone could reduce AGS cell viability ($P<0.05$), which was enhanced by high doses of NaCl ($P<0.05$).

Increased levels of proinflammatory cytokines induced by VacA in AGS cells and gerbils can be considered a cytotoxic effect. Consistent with our findings, others have noted that the damage caused by *H pylori* is partially attributed to the enhanced secretion of the proinflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α, particularly in response to VacA-expressing *H pylori* strains[31,32]. It has been reported that gastric *H pylori* infections induce mucosal production of various cytokines, including IL-1β, IL-6, IL-8 and TNF-α[33]. Our study further confirmed the cytokine response to the combined effect of NaCl and VacA *in vitro* and *in vivo*. First, we examined the expression profile of proinflammatory cytokines in response to stimulation by NaCl alone. The addition of NaCl alone to AGS cells resulted in a dose-dependent increase in IL-1β mRNA level, whereas expression of the other three cytokine mRNAs did not change significantly both *in vitro* and *in vivo*. It has been reported that normal human dermal fibroblasts ele-vate IL-1β mRNA levels upon exposure to 0.5 mmoL/L NaCl and that normal human epidermal keratinocytes increase levels of IL-6 and IL-8 mRNA in response to NaCl[34]. These reported differences in IL-6 and IL-8 expression in response to NaCl exposure may be attributable to differences in cell types or the higher NaCl concentration used in the other studies, which was fivefold higher than that in our study. We found that NaCl could

not alter VacA-induced IL-8 expression. However, the induction of IL-8 by VacA alone is consistent with previous studies that *H pylori* infection increases mucosal production of IL-8 and interaction between viable *H pylori* and AGS cells increases levels of IL-8 mRNA expression and protein secretion[33, 35].

It has also been reported that polymorphisms within the IL-1β and TNF-α genes are associated with a risk for gastric carcinoma in individuals infected with *H pylori*[36]. Our study demonstrated that VacA-induced expression of IL-1β and TNF-α mRNAs could be up-regulated by NaCl in gerbils, whereas NaCl increased IL-1β expression and decreased VacA-induced TNF-α expression in AGS cells.

IL-6 is a multifunctional cytokine that plays a central role in host defense mechanisms[37-40]. IL-6 production upon *H pylori* infection could be induced in response to urease in human AGS cells[41]. IL-6 can also be induced by VacA in bone marrow-derived mast cells[42]. These findings are consistent with our ELISA results, but we were unable to detect IL-6 mRNA with RT-PCR under any of the conditions we tested.

VacA directly activates AGS cells to produce proinflammatory cytokines, which might be a host early innate immune response, suggesting that VacA plays a role in the pathogenesis of *H pylori*-infected gastritis although the cag pathogenicity island is closely related to induction of proinflammatory cytoikine[43]

High-salt diet (NaCl 75g/kg versus 2.5g/kg) intake enhances *H pylori* colonization in mice and humans, while chronic salt intake might exacerbate gastritis by increasing *H pylori* colonization[12]. Our study indicated that excessive dietary NaCl (50 g/kg) influenced cytokine production in the Mongolian gerbil model of *H pylori* infection. VacA may be regulated by NaCl to influence cytokine production through a variety of mechanisms. One mechanism might be through an effect on VacA channels, which are likely to be an important component in the mode of action of this toxin[36].

In conclusion, the virulent *H pylori* cytotoxin, VacA, decreases the viability of AGS cells, which can be enhanced by NaCl. NaCl also affects the production of proinflammatory cytokines induced by VacA. Our results suggest that NaCl plays an important role in *H pylori*-induced AGS cell cytotoxicity. However, the interactions between *H pylori*, NaCl and gastric cancer are complex, more studies are required to understand the mechanism by which NaCl affects the progression of *H pylori*-related gastric cancer.

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Helicobacter pylori and other *Helicobacter* species DNA in human bile samples from patients with various hepato-biliary diseases

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Abstract

AIM: To investigate the presence of *Helicobacter* species by nested PCR of 16S rRNA genes followed by the presence of *Helicobacter pylori* (*H pylori*) 16S rRNA, *ureA*, *cagA* genes in bile obtained at endoscopic retrograde cholangio-pancreatography (ERCP) from 60 Indian subjects.

METHODS: Sixty bile samples were obtained from patients diagnosed with various hepato-biliary diseases and control subjects at ERCP. PCR analysis was carried out using primers for *Helicobacter* genus 16S rRNA gene and *H pylori* (16S rRNA, *ureA* and *cagA*) genes. Gastric *H pylori* status was also assessed from biopsies obtained at endoscopy from patients with various hepato-biliary diseases and controls. The control group mainly consisted of subjects with gastric disorders. Sequencing analysis was performed to confirm that PCR products with 16S rRNA and *cagA* primers were derived from *H pylori*.

RESULTS No *Helicobacters* were grown in culture from the bile samples. *Helicobacter* DNA was detected in bile of 96.7% and 6.6% of groups I and II respectively. Ten from group I were positive for 16S rRNA and *ureA* and 9 were positive for *cagA* gene. In contrast of the 2 from the control, 1 amplified with 16S rRNA, *ureA* and *cagA* primers used. The sequences of the 16S rRNA genes and *cagA* were 99% similar to *Helicobacter pylori*.

CONCLUSION: *Helicobacters* are associated with the pathogenesis of various hepato-biliary disorders.

Key words: *Helicobacter pylori*; Bile; Hepato-biliary diseases; PCR, Sequence analysis

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INTRODUCTION

The re-discovery of *Helicobacter pylori* (*H pylori*) as a curved bacterium in the stomach by histological examination of gastric biopsies[1, 2] and its subsequent first isolation by Warren & Marshall in 1983 have probably etched new avenues in the management of various gastro-duodenal disorders. Since its discovery, this microaerophilic Gram negative pathogen has been linked to various gastric pathologies including gastric carcinoma and mucosa associated lymphoid tissue (MALT) lymphoma[3, 4]. Numerous other *Helicobacter* species along with *Helicobacter pylori* have subsequently been isolated from sites other than the stomach, including oral cavity, liver and biliary tree of animals and humans[5-7]. Recent studies have implicated the association between *Helicobacter* infections with certain diseases of the liver of some animal species such as *H canis* in dogs[8] and *H hepaticus* & *H bilis* in mice[9-11].

In humans, other *Helicobacter* species including *H pylori* DNA has been detected in the liver of patients suffering from cholestatic diseases[12, 13]. In one study, a high frequency of *H pylori* and *H pullorum* sequences were detected by PCR, in the liver of patients with cirrhosis and superimposed hepatocellular carcinoma[14]. More recently, a study by Pellicano *et al* suggests that presence of *Helicobacter spp* in liver samples could possibly serve as a co-factor in the development of end-stage of liver disease in humans[15]. These concerns have spurred considerable interest in determining the mechanisms by which these extra cellular bacteria and the associated inflammatory response endorse hepatic and biliary disease.

Therefore, we investigated the presence of *Helicobacter*

Table 1 List of primers used for the study

Primer	Sequence (5′→3′)	Product size (bp)
Heli-nest S	5′ATTAGTGGCGCACGGGTGAGTAA 3′	1300
Heli-nest R	5′ TTTAGCATCCCGACTTAAGGC 3′	
Heli-S	5′ GAACCTTACCTAGGCTTGACATTG 3′	480
Heli-R	5′ GGTGAGTACAAGACCCGGGAA 3′	
16S-rRNA.F	5′ TAAGAGATCAGCCTATATGTCC 3′	534
16S-rRNA.R	5′ TCCCACGCTTTAAGCGCAAT 3′	
UreA-S	5′GCCAATGGTAAATTAGTT 3′	411
UreA-R	5′ CTCCTTAATTGTTTTTAC 3′	
CagA-S	5′ CCATGAATTTTTGATCCGTTCGG 3′	349
CagA-R	5′ GATAACAGGCAAGCTTTTGAGAGGGA 3′	

spp genomes in the bile specimens obtained from patients with different hepato-biliary diseases and among control group (without any hepatic and biliary disease but with different gastric disorders) at endoscopic retrograde cholangio-pancreatography (ERCP). We also evaluated the association between the presence of *Helicobacter* species with various hepato-biliary disorders.

MATERIALS AND METHODS

Subjects

The study population consisted of 60 subjects categorized into two groups (thirty in each) *viz*., Group I (those with hepato-biliary disorders) and Group II (those with no pathologically proven liver or biliary disease but with different gastric disorders, served as control). Patients of both sexes and age range: 23-68 years, average age: 48.1 years were included in the study. Subjects from either group underwent ERCP followed by upper gastrointestinal endoscopy at the Department of Gastroenterology, Deccan College of Medical Sciences, Hyderabad. The study protocol was approved by the Institutional Review Board (IRB) and Institutional Ethical Committee (IEC). Approval was obtained from IEC for the initiation of the study. Informed consent was obtained from all the patients before their enrolling in the study according to the Helsinki Declaration. None of the patients included for the study were on antibiotics prior to ERCP or endoscopy.

Patients' details and diagnoses were recorded and 5-10 mL bile samples were collected in glycerol by aspiration during the ERCP procedure and 3 gastric biopsies were collected during endoscopy from the same patient: one in urea solution for rapid urease test (RUT), one in brucella broth supplemented with fetal calf serum (FCS) for culture and 1 in phosphate buffered saline (PBS) for DNA isolation. Aspirated bile samples and the biopsy collected in PBS were stored at -80 °C until DNA was isolated.

Helicobacter culture

The gastric biopsy collected in supplemented brucella broth was immediately transported to the laboratory and streaked on the chocolate brucella agar supplemented with 70 mL/L sheep blood and 6 mg/L-vancomycin, 2 mg/L-amphoteracin-B and polymixin-B 2500/L (Sigma Chemicals, USA) and incubated at 37 °C in microaerobic conditions. A small aliquot (about 0.5 mL) of the collected bile sample was also instantaneously homogenized within half an hour of collection in 0.5 to 1 mL brucella broth (Becton and Dickinson, USA) containing 50 mL/L fetal calf serum (Gibco BRL, Germany) and streaked over the same medium for primary isolation of *H pylori* and incubated as described above. Approximately 50 μL of the sample was plated onto each plate and the remaining sample was used for DNA isolation.

DNA extraction

The genomic DNA from the gastric tissue, isolated culture and bile samples was isolated *as per* the standard protocol previously described[16]. In case of bile sample, briefly 450 μL of the sample was diluted with equal volume of PBS and centrifuged at 15000 g for 20 min. The supernatant was discarded and the pellet was again subsequently mixed with 250 μL of the PBS and DNA isolated by modified cetyl trimethyl ammonium bromide (CTAB) method. The DNA was extracted and preserved at -20°C until amplification was performed. Appropriate care was taken during extraction to remove the PCR inhibiting substances present in the bile[17]. Briefly as *Helicobacter* DNA was isolated from an unusual source, there is possibility of existence of specific inhibitors and competing substrates. For such situations, dilution of inhibited samples provides a rapid and straightforward way of permitting amplification. This dilution exploits the sensitivity of PCR by reducing the concentration of inhibitors relative to target DNA.

PCR amplification

Amplification was performed *as per* standard protocol described previously[13] with minor modifications. All primers were synthesized at Bioserve Biotechnologies Pvt Ltd, Hyderabad, India. Amplification was performed in a PTC 100 thermocycler (M J Research Inc. Water town, USA).

PCR amplification with Helicobacter genus-specific primers

Nested PCR was carried out using two oligonucleotide pairs previously reported by Pellicano *et al*[15] and designated as Heli in Table 1. The primers (Helinest-S & R, Heli-S & R) used in our study were reported to amplify 26 species of *Helicobacter* genus[15]. At each amplification, *H pylori* DNA was used as a positive control, while water instead of DNA served as a negative control.

First amplification

Amplification was carried out in a total volume of 20 μL containing 0.5 μL DNA, PCR buffer (1×), 200 μmol/L dNTPs, 1.5 mmol/L Mg^{2+}, 0.2 μmol/L primers (Heli-nest-S and Heli-nest-R), 1U Taq DNA polymerase (Invitrogen Life Technologies, Germany). Amplification conditions were optimized and are enlisted in Table 2. A sample was scored positive if an amplification product of 1300 bp could be resolved after electrophoresis on 15 g/L agarose gel.

Second amplification

One microliter of amplicon from the first amplification

Table 2 Conditions of polymerase chain reactions used in the study

Target gene	Initial denaturation step	Temperature o denaturation, annealing and	Cycle	Final extension step extension
Helicobacter Spp,16S rRNA	94 ℃ for 5 min	94℃ for 30 s 55℃ for 30 s	35	72℃ for 7 min 72℃ for 1.5 min
Second amplification step	94℃ for 5 min	94℃ for 30 s 60℃ for 30 s 72℃ for 30 s	35	72℃ for 7 min
Helicobacter pylori, 16S rRNA	95℃ f	94℃ for 30 s 56℃ for 30	40	72℃ for 5 min 2℃ for 1 min
ureA	95℃ for 5 min	94℃ for 30 s 52℃ for 30	35	72℃ for 6 min 72℃ for 1 min
cagA	95℃ for 5 min	94℃ for 1 min 52℃ for 1 min 72℃ for 2 min	35	72℃ for 10 min

step was used with primers Heli-S and Heli-R (Table 1) and amplification was repeated with minor alterations (Table 2). The expected product size of the amplicon was 480 bp.

Specificity test of genus specific primers

The specificity of genus specific primers *viz.,* Helinest-S, Helinest-R, Heli-S and Heli-R as well as primers specific to *H pylori* was assessed by using 6 bacterial strains. This included two different *Helicobacter pylori* strains along with 4 other enteric bacteria commonly residing the stomach.

H pylori specific 16S rRNA PCR

The samples positive with *Helicobacter* genus PCR were further analyzed for the presence of *H pylori* DNA in the culture, biopsy and bile samples by targeting the 16S rRNA gene using primers enlisted in Table 1. Amplification was carried out as per the mentioned protocol (Table 2). The amplification product size was 534 bp typical of *H pylori.*

Amplification of ureA gene

Samples positive for *H pylori* were subsequently analyzed with a different set of primers designated *ure*A, in Table 1. The sense and anti-sense primers of this gene were used for PCR as per the mentioned protocol in Table 2. The amplification product size was 411 bp typical of *H pylori.*

Amplification of cagA gene

Samples positive for *H pylori* were subsequently screened for the presence of *cag*A gene using specific primers (Table 1). Amplification was carried out as per the program given in Table 2 with minor alterations increasing the annealing time to 1 min and extension time to 2 min. The expected product size of the primers used was 349 bp.

Sequencing of the 16S rRNA products and cagA amplification product

The *H pylori* positive DNA fragments from the bile samples were sequenced. Comparison of DNA sequences of the genomic 16S rRNA amplicons and *cag*A amplicons with those of *H pylori* was performed using sequence alignment with the BLAST programme. The presence of *H pylori* sequence was thus confirmed in these bile samples.

Statistical analysis

Helicobacter genus positivity and negativity was compared using the Fisher's exact test. $P < 0.05$ was considered as significant.

RESULTS

Culture

Of the 60 gastric biopsies streaked, colonies could be isolated from 54(90%) subjects (26 from group I and 28 from group II). No *Helicobacter pylori* colonies could be grown of the 60 bile samples streaked, even after prolonged incubation for up to 2 wk under microaerophilic conditions.

Specificity test

Helicobacter genus specificity was tested using a panel of *H pylori* strains by PCR with specific primer sets used for this study. All the *H pylori* DNA gave a positive amplification with the expected product size. The non-*Helicobacter* species DNA extracts did not yield any result with the oligonucleotide primers used.

PCR amplification with Helicobacter genus-specific primers

Helicobacter DNA was detected by nested-PCR in 29(96.7%) of the 30 bile samples collected from group I patients and 2(6.6%) of the subjects from group II sub-group respectively. All the 29 samples from group I and 2 from group II amplified at both first and second amplification reactions. The amplification product sizes of both the PCR are shown in Figures 1A and 1B respectively. DNA isolated from 60 biopsy and 54 cultures, gave a positive amplification with the expected product size.

Screening for H pylori DNA in bile

Of the 29 *Helicobacter* genus positive samples from group I subjects, 10(33.3%) bile samples were amplified with the *H pylori* specific 16S rRNA primers, yielding a product size of 534 bp on electrophoresis (Figure 1C). While of the 2 *Helicobacter* genus positive from group II, only 1 was amplified with the *H pylori* 16S rRNA primers used (Table 3).

Confirmation of gastric H pylori colonization

Screening of the 60 biopsy DNA and 54 culture DNA from both the study groups gave positive amplification with the specified primers used, yielding a product size of 534 bp.

Analysis of ureA and cagA gene

Of the 29 subjects analyzed for the presence of *ure*A and *cag*A sequences from group I sub-group, we found that 10 (33.5%) were amplified with *ure*A gene while 9 (30%) amplified with the *cag*A primer. On the other hand, 1 sample which was amplified for *H pylori* 16S rRNA gene in group II, also gave positive amplification for *ure*A and *cag*A respectively (Table 3). The amplified products of these genes are represented in Figure 1D, E.

Table 3 Details of *Helicobacter* genus, *H pylori* 16S rRNA, ureA, cagA positivity in the study subjects

No	Category ($n=60$)	*Helicobacter* genus positivity *n* (%)	*H pylori* 16S rRNA positive *n* (%)	*ureA* positive *n* (%)	*CagA* positive *n* (%)
1	Group I	29(96.7)	10(33.3)	10(33.3)	09(30)
2	Group II	02(6.6)	01(3.3)	01(3.3)	01(3.3)

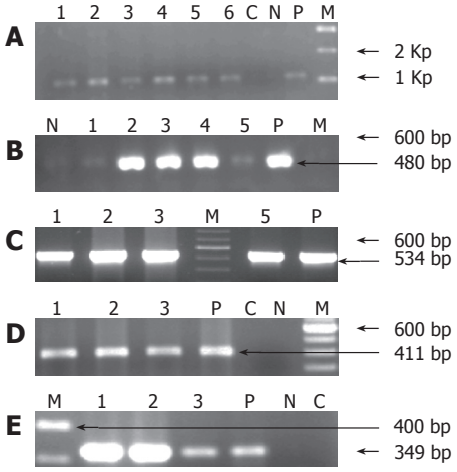

Figure 1 A schematic representation of the PCR products of the *Helicobacter spp* DNA. **A:** Gel image showing first amplification products of 16S rRNA PCR with *Helicobacter* genus specific primers at 1 300 bp. Lanes 1 to 6 represent *Helicobacter* DNA isolated from bile samples, 'P' represents positive control andLanes C, N and M represents negative control, reaction negative control and 1 Kb molecular weight marker. **B:** Second amplification products of 16S rRNA PCR with *Helicobacter* genus specific primers at 480 bp. Lanes 1 to 5 represent bile DNA, 'P' represents positive control. Lanes N and M represent reaction negative control and 100 bp molecular weight marker ladder respectively. **C:** Gel picture showing 16S rRNA amplification products specific to *H pylori* of bile samples at 534 bp. Lanes 1, 2, 3 and 5 represent bile DNA samples while Lanes M and P represent 100 bp ladder and positive control respectively. **D:** *ureA* amplification products at 411 bp. Lanes 1 to 3 represent bile DNA, Lane 'P' represents positive control and Lanes C, N and M represent negative control, reaction negative control and 100bp molecular weight ladder respectively. **E:** Gel picture illustrating the *cagA* amplification products at 349 bp. Lanes 1, 2 and 3 represent bile DNA while Lanes M, P, N and C represent 100bp molecular weight ladder, positive and reaction negative control, negative control respectively.

Sequence analysis of PCR product from bile DNA with 16S rRNA and cagA primers

To confirm that the PCR product obtained with the 16S rRNA and *cag*A primers belonged to *H pylori*, we examined the sequence of the PCR product in 4 bile samples (3 from Group I and 1 from Group II). The nucleotide sequence of the amplified products shared 99% identity with the 16S rRNA and *cag*A gene of *H pylori* respectively.

DISCUSSION

The presence of *Helicobacter* species DNA in the bile samples of patients with different hepato-biliary diseases is interesting since some reports in the past have suggested a positive association of *Helicobacter* and the evolution of liver diseases[9, 15, 18, 19]. Recent studies on *Helicobacter spp* in different diseases of liver and bile ducts have shown that *Helicobacter* can be detected not only in the extremely hostile milieu of the stomach but also in human bile[20, 19]. Although none of the previous studies including the present study have not been able to isolate *H pylori in vitro,* it has been proven that some of *Helicobacter* species live in the gall bladder. In a recent study[15], by PCR and subsequent sequencing of the 16S rDNA and amplification of *cag*A gene, *Helicobacter spp* was detected in 17 of 20 patients operated for hepatocellular carcinoma (HCC). The same study reported the presence of a 290 bp product of 128 KDa CagA protein specific only for type I *H pylori*. Further, it has been shown that several *Helicobacter spp*. secrete a liver specific toxin that causes hepatocyte necrosis in cell culture and might also be involved in damaging liver parenchyma *in vivo*[15]. In contrast, other authors did not detect any *Helicobacter* or *H pylori* DNA in the patients with similar diseases[21, 28]. These observations impelled us to explore a possible association of *Helicobacter* and hepato-biliary disease among Indian patients.

In the present study, which comprised of two groups (I & II), of the 30 subjects from group I with various hepato-biliary ailments, we could detect *Helicobacter* genus specific 16S rRNA sequence in 29 bile samples by nested PCR and only 1 sample did not give any amplification with the specific primers used. By contrast, in Group II subjects, i.e. those with no significant hepato-biliary disease but with various gastric disorders (Control subjects) only 2 subjects gave positive amplification with the *Helicobacter* genus specific primer used yielding the desired fragment. We carried out 16S rRNA amplification specific to *H pylori* on 29 subjects positive from group I and 2 from group II for *Helicobacter* genus, followed by subsequent sequencing of the amplified products to confirm the presence of *H pylori* DNA in the bile samples. We found that 33.3% samples from group I and 3.33% from group II were amplified giving a product of 534 bp (Figure 1C). Further, we also investigated the presence of *ure*A and *cag*A gene in all the bile specimens followed by sequencing of the 16S rRNA and *cag*A amplified products of 4 bile samples (3 from group I and 1 from group II). Sequence comparison of the sequenced samples confirmed the presence of *H pylori* DNA sequence in the bile samples.

The usage of less invasive ERCP procedure to obtain bile is an adequate method for this purpose compared with other invasive approaches currently in practice. This procedure also avoids contamination with *H pylori* colonizing the stomach, as the sampling devices are inserted inside the endoscope and hence never traverse the stomach[28].

This is the first Indian study to simultaneously investigate the presence of *Helicobacter* DNA in bile specimens and gastric tissues and underscore the association of *Helicobacter* in bile obtained from patients with various hepato-biliary disorders. We detected *Helicobacter* DNA by nested PCR using two sets of primer. Further, our study also successfully demonstrated the presence of *ure*A and virulence genes such as *cag*A specific to *H pylori* in the DNA isolated from bile samples. As evident from the results, 33.3% carried *ure*A gene whereas 30% were amplified with the primers used for *cag*A detection (Table 3). Of the 9 positive for *H pylori cag*A

gene, 5 belonged to cholangio-carcinoma, 2 belonged to common bile duct stones and the remaining subjects had pancreatico-biliary malignancies (Data not shown). In addition, we also found that among 10 subjects positive for *H pylori* in group I, *ure*A and *cag*A were simultaneously detected in 9 subjects, 1 subject gave amplification only for *ure*A gene and the only sample which was amplified with the 16S rRNA specific primer of *H pylori* from group II was found to possess both genes respectively (data not shown). The only subject whose bile sample gave positive results with 16S rRNA, *ure*A and *cag*A primers in group II was found to suffer from antral gastritis endoscopically and was co-incidentally positive for *H pylori* by culture and PCR of both the biopsy and culture DNA (data not shown). Every possible precautionary measure was taken to assure that laboratory contamination did not account for the positive amplification results such as diluting the bile with sterile distilled water, centrifuging the samples at 15 000 × *g* for 20 min, and the supernatant being discarded, thus by concentrating the bacterial cells in the pellet, enabling removal of some of the inhibitors predominantly present in the bile. Collection of bile in glycerol and immediate plating nevertheless did not prove to be successful for *in vitro* isolation of *Helicobacters* from any of the bile samples as we could not get any growth even after extended incubations for up to 15 d.

The present study also investigated the gastric *H pylori* status of the patients enrolled in both the sub-groups to see if at all the gastric *H pylori* status had any impact on the etiology of the hepato-biliary diseases. Unfortunately from the results we could neither associate the severity of the hepato-biliary disease with that of the gastric diseases nor could we link the gastric *H pylori* status and the detection of *H pylori* DNA in bile of the hepato-biliary diseases. Even though 29 subjects from group I showed the presence of *Helicobacter* species DNA, only 10 showed the presence of *H pylori* DNA in the bile thus signifying the possibility of the presence of the other bile-resistant *Helicobacters* that normally reside in the liver and biliary tract. Besides the above results obtained in this study, we noticed that patients with various hepato-biliary disorders had much greater probability of positive *Helicobacter* species DNA compared to those with no pathologically proven liver and biliary diseases. Why these intestinal *Helicobacters* have been identified in Chilean patients[26] and *H pylori* has been identified in patients in other geographical regions, as we observed in this study warrant further investigations. In fact, the typical finding of the present study, correlates well with those of Kuroki *et al*[22], who recently demonstrated that the level of epithelium proliferation was higher in *Helicobacter*-positive biliary epithelium than in bacterium-negative epithelium.

The results of our study also suggest that besides *Helicobacter spp* sequences, even sequences pertaining to the virulence genes of *H pylori* are consistently found at a high frequency in bile samples and that they may be a significant cause of biliary diseases. Several hypotheses have been proposed[23-25], by which these *Helicobacters* find their way into the liver and bile duct. But these hypotheses entail strong evidences to underline the precise mechanism by which the *Helicobacter* species anchor the liver and aggravate the clinical outcome.

The findings of this study are in concert to those obtained by Fox *et al*[26] and Linn *et al*[18] who reported *Helicobacter spp* in gall bladder tissue from Chileans with chronic cholecystitis but different from those of Fallone *et al*[28] and Mendez-Sanchez *et al*[21] who were not able to detect *Helicobacter* sequences in the bile samples of North Americans and Mexicans respectively. The raison d'être for these discordant results could be the regional variations in the distribution of bile-resistant *Helicobacter* species[20]. The other alternative reason for the inconsistent results in their studies could be the methodology used, the selection of primers used, as both the studies had used a single primer set for the amplification of the conserved 16S rRNA gene. In contrast, our study used nested PCR for 16S rRNA amplification of *Helicobacter* genus, which according to Stark *et al*[27] is 10^4 times more sensitive than a 1-step PCR. Though we used only one set of oligonucleotide primers each for amplifying *ure*A and *cag*A, the specificity and sensitivity of the selected primers were previously determined during our routine screening. The primers were found to possess the sensitivity and specificity of 90% and 95% for *ure*A and 89% and 85% for *cag*A, particularly in this geographical area.

In conclusion, this study demonstrated that *Helicobacter spp* DNA can be detected in bile by PCR and that gastric presence of *H pylori* in patients with proven hepato-biliary disease had no clinical correlation with the hepatic and biliary disease. However, like other previous studies, we were unable to isolate the bacterium in culture. There are different reasons to justify this finding. Firstly, this could be due to the bacterial conversion from viable helicals to non-viable coccoids in an adverse bile rich environment. It is also possible that the number of bacterium is very few and that they may have been partially inhibited by unfavorable environment that exists in the biliary milieu. In addition, our study also confirmed by DNA sequencing that sequences specific to *H pylori* (16S rRNA, *cag*A) can be found at a high frequency in the bile samples, thus instilling strong evidence that presence of *Helicobacter spp* may in someway aggravate the etio-pathogenesis of hepato-biliary diseases. However, mere detection of *Helicobacter* DNA from patients with different hepatic and biliary disease does not confirm the precise role played by these organisms. Further, future studies unraveling the molecular mechanisms by which these *Helicobacter* members contribute to the clinical outcome of hepato-biliary disorders would be helpful to assess the true impact of enterohepatic *Helicobacters* and its metabolites in the genesis of biliary diseases.

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BASIC RESEARCH

Liver fibrosis and tissue architectural change measurement using fractal-rectified metrics and Hurst's exponent

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Abstract

AIM: To provide the accurate alternative metrical means of monitoring the effects of new antiviral drugs on the reversal of newly formed collagen.

METHODS: Digitized histological biopsy sections taken from 209 patients with chronic C virus hepatitis with different grade of fibrosis or cirrhosis, were measured by means of a new, rapid, user-friendly, fully computer-aided method based on the international system meter rectified using fractal principles.

RESULTS: The following were described: geometric perimeter, area and wrinkledness of fibrosis; the collation of the Knodell, Sheuer, Ishak and METAVIR scores with fractal-rectified metric measurements; the meaning of the physical composition of fibrosis in relation to the magnitude of collagen islets; the intra- and inter-biopsy sample variability of these parameters; the "staging" of biopsy sections indicating the pathway covered by fibrosis formation towards its maximum known value; the quantitative liver tissue architectural changes with the Hurst exponent.

CONCLUSION: Our model provides the first metrical evaluations of the geometric properties of fibrosis and the quantitative architectural changes of the liver tissue. The representativeness of histological sections of the whole liver is also discussed in the light of the results obtained with the Hurst coefficient.

Key words: Liver; Fibrosis; Cirrhosis; Staging; Image analysis; Fractals

Dioguardi N, Grizzi F, Franceschini B, Bossi P, Russo C. Liver fibrosis and tissue architectural change measurement using fractal-rectified metrics and Hurst's exponent. *World J Gastroenterol* 2006; 12(14): 2187-2194

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INTRODUCTION

Background

Experimental medical research based on reductionism retains in principle that the causes governing the processes and controls of the formation, growth and behavioral dynamics of living beings at macro-scalar levels can be derived from the processes and controls that take place at micro-scalar level.

This conception has led the hepatological disciplines to seek exact quantitative descriptions of the contents of liver fibrosis on the basis of the blood molecules related to the presence of the newly formed structure[1-5]. The basic idea is that the laws governing the set of processes and controls acting at the scale of the formation of septal and anular collagen structures can be translated into the set of laws that regulate the dynamics of these molecules at micro-scalar level when they enter the blood stream.

Sophisticated mathematical, physical and environmental studies suggest that these phenomena are governed by different multiple processes and controls (causality) at every larger or smaller scale rather than that of observation. The set of these multiple processes and controls is known as multiple scale causality (MSC)[6]. The macro-scalar growth of liver collagen fibres evolving into the septa and annuli of fibrosis is regulated by the physical events of diffusion, percolation, stratification and contraction of the extra-cellular matrix (ECM), and by the processes of neo-vascularisation. These physical processes are not involved in generation of microfibrils and fibrils, which is limited on ECM deposition. This phenomenon depends on the relationships between the processes of collagen synthesis and lyses, which are different from the biochemical processes that at a lower scale, regulate the quantitative and qualitative behaviors of the molecules formed during fibrogenesis and found in circulating blood. In other words, this means "what happens in the test tubes may be the same, the opposite, or bear no relationship at

all to what happens in the living cells, still less the living organisms".

In brief, the MSC-based conception rejects the idea that the laws regulating phenomena at one scale can in principle be derived from those regulating phenomena at a lower scale. In other words, every scale is characterized by different degrees of entropy[6].

As the characteristics of MSC are not universal, it is almost always impossible to establish a strict connection between the phases leading an observed object from micro- to macro-scalar positions and *vice versa*. Consequently, it is very difficult to identify a one-to-one relationship between the macro- and micro-scalar processes and controls of liver fibrosis production.

These considerations have convinced us to improve a direct, rapid and friendly method of measuring fibrosis using a meter that is suitable for its naturally irregular shape, provided that the biopsy procedures are performed by experts[7-12].

Liver biopsy is the only direct means of discovering the changes in liver tissue induced by chronic B or C virus-dependent inflammation and the fibrosis it causes[12-17]. Although it is the canonical point of reference for establishing the progression of the process, fibrosis has so far only been estimated on the basis of semi-quantitative evaluations[18-21] or morphometric methods[22-26] that must be considered approximate because of their use of the international system (IS) meter, which is unsuitable for measuring irregular shapes[7-11].

Like all natural objects, the collagen islets making up the fibrosis appear to be difficult to measure at all degrees of magnification because of their highly irregular shapes[7-11]. Furthermore, they do not have a single measurement because, at each enlargement (scale), their shape changes as a result of the addition of new details which are imperceptible at smaller scales. Mandelbrot have termed these irregular bodies fractal objects[27].

The studies of Perrin[28] in 1906, Hausdorff[29] in 1919, Richardson[30] in the 1950s, and Mandelbrot *et al*[26] in the 1970s and 1980s suggested a new geometry that has made it possible to measure such objects using a method that can be made simple with aid of computer technology.

The aim of this paper was to present the first results of the quantitative analysis of histological liver bioptical sections made using new metrics suggested by the concepts of fractal theory.

Our contribution to the quantitative evaluation of fibrosis in liver biopsies concerns: (1) the closest to real metric estimate of the surfaces covered by fibrosis and the quantitative classification of collagen islets; (2) the collation of metric and traditional semi-quantitative evaluations of collagen area; (3) considerations concerning the relationships depending on specific estimates defined by real numbers as a means of identifying the stadium of fibrosis; (4) the interior-to-edge metric relationship of the collagen islets used as an index of wrinkledness; (5) the definition of the harmonic state of the architectural structure of liver tissue, and the recognition and classification of its disharmonic states or architectural disorder using Hurst's exponent; (6) the intra-sample variability of fibrosis (area, wrinkledness and Hurst's exponent); and (7) the variability of the latter parameters in eight samples taken from the explanted liver of a transplant patient.

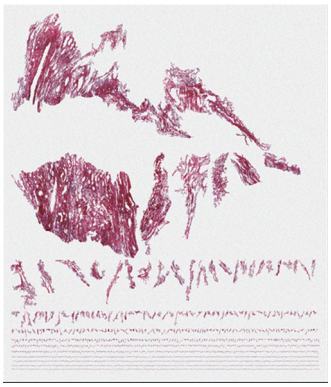

Figure 1 Prototypical catalogues of the Sirius-red stained collagen islets forming liver fibrosis, which consists of a set of irregularly shaped (wrinkled) objects.

Theory and objects

Characteristics of collagen islets The spaces occupied by the collagen islets making up fibrosis (Figure 1), and those occupied by the surviving fragments of parenchymal tissue, are both characterized by irregular shapes that exclude them from the application of Euclidean geometry[7-11]. However, their configurations make them members of the family of asymptotic statistical fractals (also called truncated fractals as they cannot be endlessly fracted)[31].

Dimension The dimension of an object indicates the Euclidean space it occupies. The fractionable "smooth" objects of classic geometry (a point, straight line, plane, and solid figure) occupy spaces whose dimensions are expressed by the whole numbers zo, 1, 2 and 3, whereas the irregular objects that are frangible into irregular pieces (sets of points, broken lines, undulating planes and irregular solids) occupy the intermediate spaces, and therefore have dimensions that cannot be expressed by whole numbers. In 1977, Mandelbrot[27] defined the objects whose dimensions are denoted by non-whole numbers as "fractals". The fractal dimension is indicated by D, and the Euclidean (or topological) dimension by Dγ.

Harmonic state of liver tissue The use of an analytical method based on a new geometry requires a brief formal description of the natural history of chronic virus-related hepatitis starting from the axiom that is defined by the persistence of a discreet necro-inflammatory process maintaining the activity of the ECM→collagen→fibrosis production system. The growth of fibrosis interrupts the continuity of the parenchyma, dissects it into irregular pieces, and compresses and atrophies the pieces enclosed in the fibrotic mass. This has two consequences: 1) it disrupts the natural harmonic state of the metric spaces covered by both natural fibrosis and the parenchyma, and 2) it alters the typical lobular architecture of hepatic tissue.

Using these axioms, the process can be represented by the first-order general equation:

$$z = f(x, y) \qquad 1)$$

in which z denotes the harmony of the natural architecture of liver tissue, x the metric space covered by fibrosis, and y the metric space covered by the parenchyma. The relationship of z to x and y can be indicated by the

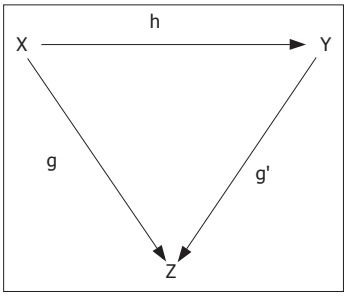

Figure 2 Categorial model of the harmonic state of liver tissue, in which z denotes the harmony of the natural architecture of liver tissue, x the metric space covered by fibrosis, and y the metric space covered by the parenchyma.

categorial representation (Figure 2), which shows the dependence of the state of z on the extension over z of the relationships between the "objects over" indicated by *x* and *y* in $f(x \rightarrow y)$.

As the variations in *x* and *y* are interconnected by the equality $(z\text{-}x)+(z\text{-}y)=1$, we can consider *x* and *y* as complementary spaces, and so the expression $z = f(x \rightarrow y)$ can be simplified to the form $z = f(x)$. The harmony of natural architecture of liver tissue is characterized by 3% fibrosis and 97% lobular parenchyma[32].

The evaluation of the metric space covered by the fibrosis induced by pathogenic agents is therefore sufficient by itself to describe the variations in the harmonic state of liver tissue architecture.

An alteration in the natural quantitative relationship between fibrosis and the parenchyma leads to the loss of the natural lobular architecture of hepatic tissue in a continuous but irregular progression that fractal geometry calls a random walk, or Brownian process[33,34].

MATERIALS AND METHODS

Patients

Two hundred and seven consecutive needle biopsy specimens (≥10 mm long) were taken from 74 female and 135 male patients (mean age: 52 ± 13 years) with chronic C virus-related liver disease. To evaluate the inter-sample variability of geometric characteristics of fibrosis, a total of six biopsy specimens were sampled from a liver explanted from a patient with clinically and histologically proved cirrhosis undergoing orthotopic liver transplantation. Each biopsy specimen was taken from a single segment of the liver.

Histological procedure

The study was performed in accordance with the guidelines of the Ethics Committee of the Istituto Clinico Humanitas, Rozzano and Ospedale Maggiore, Milan, Italy. All the patients were informed of the possible discomforts and risks of bioptic sampling. The liver specimens were fixed in 10% formalin and embedded in paraffin, and 2 μm thick sections were cut and stained with a freshly made 0.1% Sirius red staining solution[35].

Semi-quantitative evaluations

At least three expert pathologists independently staged the biopsies using Knodell's histology activity index (HAI), and the Sheuher, Ishak and METAVIR scoring systems. The number of available portal tracts in the liver fragment was assured on the basis of the indications established for the individual methods[18-21].

Quantitative image analysis

Our specially developed computer program could ensure: computer-driven microscopy focusing, digitation of the whole histological section and its fractal dimension and Hurst's exponent, less-biased measurements of each collagen islet and fibrosis mass raster perimeter length and area magnitude corrected by the fractal dimension, and wrinkledness of the fibrosis[7-11]. The time required for the whole analytical operation using this software was 30 s/mm^2, as determined on a standard reference needle specimen section, and was carried out at a microscopic magnification of ×200 (the measurements were valid only for that scale). The observed objects were measured using the IS meter rectified by their fractal dimension. When present, Glisson's capsule was included in the physiological amount of ECM, which was estimated as being 3% of the true area of the section[32].

Fractal dimension estimates

The fractal dimensions were automatically estimated using the box-counting method and the formula:

$$D_B = \lim_{\varepsilon \to 0} \frac{\mathrm{Log}N(\varepsilon)}{\mathrm{Log}(1/\varepsilon)} \qquad 2)$$

in which, D_B is the box-counting fractal dimension of the object, ε the side length of the box, and $N(\varepsilon)$ the smallest number of boxes of side ε required to cover the surface or the outline of the object completely[7-11,33,34]. As the zero limit could not be applied to biological objects, the dimensions were calculated as D = d, where d is the slope of the graph of log [N(ε)] against log 1/ε. The log-log graphs were plotted, and the linear segments were identified using least squares regression. Their gradients were calculated using an iterative resistant line method.

Computation of Hurst's exponent

Hurst's exponent, which gives a measure of the heterogeneous distribution of a set of irregularly-shaped objects[36-38], was obtained using the general relationship:

$$H = E + 1 - D \qquad 3)$$

where E indicates the Euclidean topological dimension, and D the fractal dimension of the surface covered by the extensions of all the collagen islets making-up the fibrosis. This exponent is capable of evaluating the loss of natural order that occurs in the histological picture as a result of the disruption of the natural quantitative relationship between the metric spaces covered by fibrosis and the parenchyma[11]. This could give the alterations the significance of a physical variable, make Hurst's exponent the descriptor of the configurational disorder of the hepatic tissue, and leave the fractal dimensions of the collagen islets and fibrosis the significance of an evaluation of how "densely" they occupy the metric space in which they lie and their spatial position.

In brief, Hurst's exponent values near to 1 quantitatively indicate a large loss of architectural harmony in the system (*i.e.* disordered states), whereas values near to zero indicate its preservation (*i.e.* states of structural order).

The reduction in Hurst's exponent could also be

interpreted as an index of the limited stability of a fractal object progressing towards more advanced states of stability.

Unit of measure

The construction of a meter with the most favorable spatial dimension for measuring the perimeter and area of the collagen islets was based on the assumptions that broken or mixed (in any case irregular) lines could be described as a "dilatative" (expanded) state of a straight line, and that their irregularity could be measured using the fractal dimension with similar criteria to those with which the coefficient of dilatation (λ) could be used to describe the length of a linear object before (s) and after (S) its exposure to the effect of heat expressed in the following formula:

$$S = s\,(1+\lambda t) \qquad 4)$$

where t is the duration of heat exposure[10].

According to this principle (which has been discussed elsewhere), the meter suitable for measuring a fractal object (m_F) could be obtained using the formula:

$$m_F = m\,[1 + \beta\,(D_P - D\gamma)] \qquad 5)$$

in which, m_F indicates the rectified meter, m the IS meter, β the dilatation constant of the unit of measure, D_P the fractal dimension of the perimeter, and $D\gamma$ the topological or Euclidean dimension[10]. The metric perimeter and area measurements were detected using the true liver biopsy area (indicated by the symbol A_B), by which we meant the surface area excluding any vascular, bile channel, sinusoidal or other empty spaces created by tissue loss during specimen manipulations.

Statistical analysis

All the data were expressed as mean±SD. The linear regression analysis was made using Spearman's correlation and Student's t test with Statistica software (StatSoft Inc., Tulsa, OK, USA). $P<0.05$ was considered statistically significant. The degree of intra- and inter-sample variability was quantitatively evaluated using the coefficient of variation (CV) given by the formula CV = SD/mean. Quantitative measures of fibrosis surfaces were compared (collated) with those of the score categories of the four currently used semi-quantitative methods.

RESULTS

Measurement of fractal dimension

By evaluating this parameter in our case-list of patients with chronic C virus-related hepatitis, we obtained dimensional values (D) ranging from 1.22 to 1.79. The value of this parameter increased depending on the extension of the fibrosis, with a regression curve ratio of $r=0.81$.

Perimeter measurements

The fibrosis perimeter (P_C) defined the sum of the perimeters all of the Sirius Red-stained areas of collagen in each biopsy section measured using the IS meter. The fractal fibrosis perimeter (P_{CF}) defined the same sum of perimeters measured using the fractal-rectified meter. The mean P_C was 860.64±588 μm, and the mean P_{CF} was 2966.1±2304.4 μm ($P<0.0005$). The marked difference

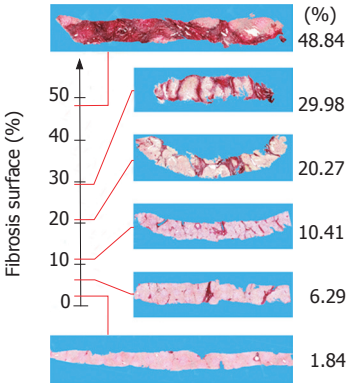

Figure 3 Trajectory of the dynamics of the fibrotic process considered as a continuous straight line going from *0%* to *48.84%* (the highest empirically observed percentage), without any jumps or abrupt swervings. The interaction of every experimental scalar expressing the state of the propositus fibrosis and the points of the geometric model gives the staging of fibrogenesis.

Δ was 225.21±38.69% due to the rectification, which included most of the fine, irregular particulars escaping the IS meter, thus making the result very close to reality. In this study, we only used the measurements corrected using the fractal dimension. The scalars indicating the length of the perimeters of fibrosis in each of the sections of the 209 bioptic samples fell within a range that was only closed at the zero point because the maximum value was not defined and could theoretically be greater.

Area measurements

The area of fibrosis (A_C) defined the sum of the areas of the Sirius Red-stained collagen islets measured using the IS meter. The fractal area of fibrosis (A_{CF}) was the sum of the areas of Sirius Red-stained collagen islets measured using the fractal-rectified meter. Both were expressed as a percentage of the total true area. The minimum and maximum values of A_C were 1.01% and 48.47% of the specimen area, the corresponding A_{CF} values were 1.34% and 48.84%. In this case, although statistically significant ($P<0.005$), the Δ(6.61%±4.79%) was small because histological sections were planar and the fractal measure of area depended only on its irregular contour. The scalars indicating the extent of the area of fibrosis in each of the sections of the 209 bioptic samples also fell within a range that was only closed at the zero point because the maximum value was not yet defined and could theoretically be greater.

Fibrosis staging of histological section

We constructed a geometrical model of the set of all states of ECM deposition on the line of real numbers as their state space using the scalars obtained from the fibrosis measured in our biopsies. The α (start) of the fibrotic process was fixed at 0% and the ω (end) at 48.84% of the area of the section (Figure 3). This model could represent a conventional interpretation of the ideal path that could be covered by potential fibrosis deposition in liver tissue. The staging observed in the histological section under examination was given by the relationship between the scalar expressing the actual fibrotic state of the section and the point indicated by this geometric model.

Classification of collagen islets

During the course of chronic hepatitis, fibrosis appeared under the control of stellate cells in the form of thin

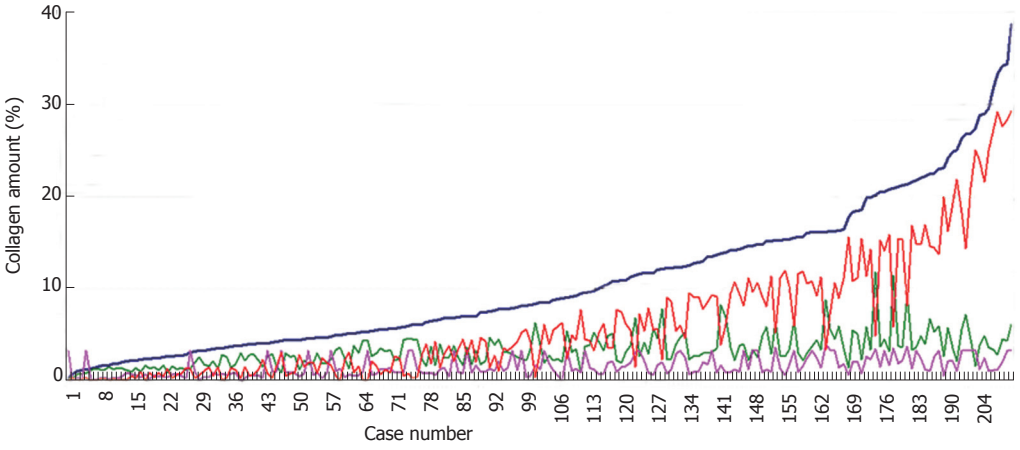

Figure 4 Comparative analysis of Markovian sequence of the amount of fibrosis, and the three sequences expressing the classes of magnitude of the collagen islets identified in 209 needle biopsy specimens. (1). Markovian sequence of the total amount of fibrosis in a histological biopsy section (blue line); (2). Percentage of collagen islets with a magnitude of 10^4-10^6 μm^2 (red line); (3). Percentage of collagen islets with a magnitude of 10^3-10^4 μm^2 (fuchsia line); (4). Percentage of collagen islets with a magnitude of 10^1-10^3 μm^2 (green line).

fibers that tended to grow in width and length within the portal space, around these structures and the central veins, and along the capillarised sinusoidal network. The three-dimensional interconnections of these collagen structures led to the formation of a fibrous network in which irregular meshes appeared as a set of collagen islets on different magnitudes on planar histological sections.

The logarithmic distribution of this set of 2.8 million collagen islets from 209 cases showed that it could be divided into three classes of elements of different magnitude: the collagen islets of 10-10^3 μm^2, those of between 10^3 and 10^4 μm^2, and those between 10^4 and 10^6 μm^2. All the histological sections contained islets with these classes of metrical values. In order to clarify the meaning of their presence, we first ordered the results of the fibrosis surface areas and constructed them in function of their magnitude as a Markovian curve (Figure 4).

The values of the islets belonging to the three subclasses in each bioptic section were plotted on the basis of the order of a Markovian curve, which generated the three irregular curves formed by the sets of islets belonging to each subclass. The fractal dimension of these curves showed that the Markovian reference curve had a fractal dimension of 1.1, whereas the curves of the three subclasses of the largest, medium-sized and smallest islets were respectively 1.9, 1.99 and 1.95. Fractal geometry suggested that the high fractal dimensions of the collagen islet subclasses could describe the instability of the collagen structure as it passed towards more stable fibrotic states[33].

Collagen area metric and semi-quantitative evaluations

The relationships between our measurements of fibrosis, the semi-quantitative Knodell histology activity index, and Sheuer, Ishak and METAVIR scores were investigated by comparing the portraits of each category of elements obtained by labeling the different semi-quantitative values on a geometric model using the line of real numbers as state space (Figure 5). The overlapping metric measures showed that samples with very similar metric surface areas fell into different categories while specimens with different metric surface areas fell into similar categories. Incidentally, our results reinforced the criticism by Rousselet *et al*[39] who have recently criticized the METAVIR system for evaluating fibrosis.

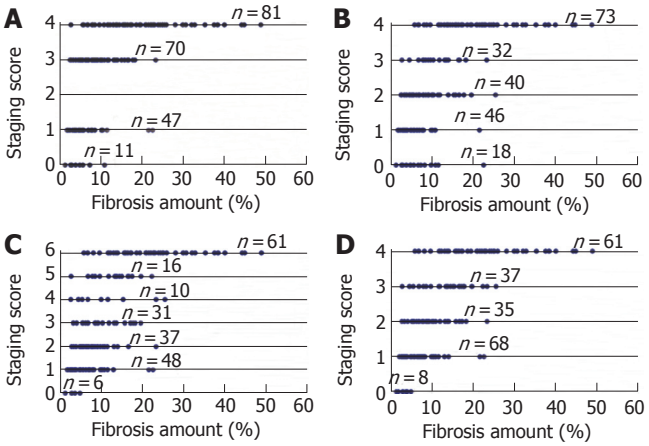

Figure 5 Comparison of the phase portraits obtained using the scalar values of the rectified areas of fibrosis projected onto the state spaces of each Knodell HAI (**A**), Sheuer (**B**), Ishak (**C**), and METAVIR category (**D**). All the graphs highlight the considerable overlapping of the data referring to different categories.

Wrinkledness

Wrinkledness (W), a typical characteristic of all natural fractal objects[11], could be obtained using the formula:

$$W = \frac{P_{CF} - R}{2\sqrt{\pi A_{CF}}} \qquad 6)$$

where R is the roundness coefficient of each islet.

The mean W value obtained by measuring the 209 biopsy sections was 731.3±298.72. As expected, this variable did not depend on the area of collagen (r=0.14), but was highly dependent on the length of its perimeter (r=0.80). Preliminary data showed that W was independent of the degree of necro-inflammatory activity (data not shown).

Measurement of Hurst's index

The same 209 patients provided us with H values ranging from 0.21 and 0.71. As said in the premises, we considered this index a quantitative variable of the alteration in hepatic tissue architecture. The ratio between H and the extension of the area of fibrosis indicated that the increased fibrotic area could lead to an extended loss of the natural harmony of this architecture (r=-0.81).

Table 1 Intra-sample variability in the area and wrinkledness of fibrosis

Patient number	*n*	A_{CF}(%)	CV	W	CV	H	CV
1	30	5.97±4.95	0.83	788.63±590.48	0.75	0.51±0.14	0.27
2	30	9.77±2.64	0.27	830.52±124.93	0.15	0.475±0.04	0.08
3	30	20.15±5.51	0.27	69±140.88	0.18	0.38±0.03	0.09

Note: Patient No.1 = Without pathology; Patient No.2 = Chronic hepatitis with intial fibrosis; Patient No.3 = Cirrhosis; *n* = number of sequential sections; A_{CF} = Fractal fibrosis area; CV = Coefficient of variability (standard deviation/mean); H = Hurst' exponent.

Intra-sample variability in area, wrinkledness and Hurst's exponent of fibrosis

In order to define the usefulness of generating numbers (or other kinds of invariants) to label the investigated structures in such a way that those with the same label could be considered alike and those with different labels could be considered different, the irregular tectonics of fibrosis was investigated in 30 sections taken at equal intervals from two patients with different degrees of disease severity and one was considered normal. The data (Table 1) showed the highly multifarious areas and wrinkledness of the collagen islets in the different sections. However, the Hurst's exponent was found less variable.

Inter-sample variability in area and wrinkledness of fibrosis

There was a high degree of inter-sample variability in the area (CV = 41%), outline perimeter (CV = 61%), and wrinkledness of fibrosis (CV = 38%). The inter-sample variability in Hurst's exponent was less (CV = 13%).

DISCUSSION

We have described a purely quantitative method of assessing liver fibrosis based on the theory of measure, which is the point of departure of all our concrete knowledge of the physical world, and the main reference for the construction of predictive and monitoring models[40].

During this study, our first difficulty was to measure the collagen islets making up liver fibrosis using standard metrics, because their irregularity excluded them from Euclidean geometry, which was created to describe smooth objects. Collagen islets can therefore only be measured using the principles of fractal mathematics and geometry[7-11].

Unlike the regular objects of Euclidean geometry, fractal objects do not have a single measure because it changes at every scale of observation as the result of the appearance of details that are imperceptible at lower magnifications, thus measurements have to be made at a well-defined scale[41].

The second difficulty was to identify the epistemological difference between the meanings of quantitative metric measures and the definition of a diagnosis of fibrosis in chronic inflammation or its progression to cirrhosis, which could be considered a radically different clinical condition. After making numerous experiments with our computer program, to analyze entire Sirius red-stained biopsy sections we established that the most favorable magnification was 200×, thus this magnification was used to digitize the microscope images.

After this methodological study, which considered the process of fibrogenesis as nothing rather than a physical phenomenon, we asked ourselves three basic questions, namely what have we learned about the meaning of liver tissue fibrosis? what new knowledge have we acquired after having measured the new canons of liver tissue architecture and classified their changes with scalars? and how much can these new notions be used in clinical practice to discriminate the progression or regression of fibrosis?

The first new notion was to extend the concept of the fractal dimension to metric measurements that might closely approximate the real magnitude of the irregular objects under observation.

Sensu stricto, the results of the intra-sample behavior and inter-sample variability study showed the non-representativeness of the perimeter and areas of fibrosis: the former due to its dependence on the extension of the bioptic specimen, and the latter for the high variability of the distribution of the inflammation process in the organ.

We added the staging of the evolution of ECM deposition towards stages of non-tolerability using the scalars obtained from the two-dimensional measurements of the area of the fibrotic mass. Although hardly representative and no more than a resonance of the mesomorphic process taking place in the environment, this information has some clinical interest if only as a very generic quantitative portrait of the general condition of the liver.

The quantitative relationships between the edge-interior of the fibrosis mass generated the new concept of its wrinkledness.

The collagen islets were categorized into three classes on the basis of the magnitude of their areas, which provided clues to their state of instability that indicated the activity of the fibrogenetic process. High presence of islets belonging to these subclasses according to the fractal geometry, is a signal of collagen structure instability in transition towards more stable states.

The distribution of the quantitative values of the areas covered by fibrosis in each category of the four semi-quantitative methods of evaluation clearly and simply confirmed the unreliability of the semi-quantitative systems themselves. This inadequacy could also be seen even when the histological preparations were analyzed by expert hepato-pathologists whose experience encompasses a large number of observed cases.

Interest in the collagen/parenchyma ratio led us to recognize the canons (mathematical laws) governing the natural "harmonic state of liver tissue", by which the order that characterizes its organized fibrous and parenchymal components (considered prime bodies) in such a way can provide a definition of the natural histological architecture of liver tissue. This can be defined in two dimensions on the basis of the extension of the metric spaces covered by fibrous tissue (no more than 3%) and the parenchyma (97%). It can be intuitively understood

that altered proportions between these spaces disrupt the natural harmonic state by creating disorder in the natural lobular structure of the liver tissue. In this sense, the words "measure" and "architectural alteration" in Ishak' s classical semi-quantitative definition (1995) …"[Staging] … is a measure of fibrosis, and architectural alteration, i.e. structural progression of the disease…" really indicate a rigorous metric value, and the term architectural alteration of this definition acquires the consistency of a measurable, scientific variable of state.

In order to quantify the architectural alteration, we used Hurst's exponent, and analysis of its variability gave the following promising results: the least variability in thirty sequential sections (intra-sample variability), the greatest representativeness in six samples of hepatic tissue obtained from different parts of the liver (it was not possible to speak of lobes because of the advanced structural alterations induced by cirrhosis). This last result is currently further investigated in our laboratories.

In conclusion, although a number of serum fibrosis markers have been discordantly proposed[1-5,42-46], the results of this study strongly underline the primary role of liver biopsy in the qualitative diagnosis of chronic liver disease and its usefulness in evaluating fibrosis in a metrically rigorous manner. The use of quantitative descriptors of irregularly-shaped fibrosis should shed new insights into the dynamics of viral and non-viral liver diseases. We are concentrating on the clinical use of Hurst's exponent after antiviral drug treatment as it can quantitatively demonstrate a drug's effect on fibrosis with a very low error.

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BASIC RESEARCH

Comparison of protocatechuic aldehyde in *Radix Salvia miltiorrhiza* and corresponding pharmacological sera from normal and fibrotic rats by high performance liquid chromatography

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Abstract

AIM: To observe the effect of protocatechuic aldehyde on the proliferation of hepatic stellate cells (HSCs).

METHODS: Liver fibrosis was induced in rats by carbon tetrachloride (CCl_4). Then normal and fibrotic drug sera were extracted from rats. The effects of protocatechuic aldehyde, raw *Radix Salvia miltiorrhiza* and drug sera of *Salvia miltiorrhiza* on HSC growth were determined by CCK-8. The protocatechuic aldehyde was separated by high performance liquid chromatography (HPLC) in a Alltima C18 column (250 mm × 4.6 mm, 5 μm) with a mobile phase of acetonitrile-4% glacial acetic acid solution (gradient elution) at the wavelength of 281 nm.

RESULTS: Protocatechuic aldehyde, raw *Radix Salvia miltiorrhiza* and drug sera of *Salvia miltiorrhiza* were found to have inhibitory effects on proliferation of rat HSCs. Raw Radix Salvia miltiorrhiza had a stronger inhibitory effect than the drug sera. The fibrotic drug sera showed a higher suppressive effect than the normal drug sera ($P < 0.05$). Protocatechuic aldehyde was found in crude materials of both *Radix Salvia miltiorrhiza* and its corresponding drug sera. The average recovery ($n = 6$) was 110.5% for raw *Salvia miltiorrhiza Bge,* 102% for normal drug sera and 105.2% for fibrotic drug sera. The relative standard deviation (RSD) was 0.37%, 1.96% and 1.51%, respectively ($n = 6$). The contents of protocatechuic aldehyde were 0.22%, 0.15% and 0.19%, respectively ($n = 6$) ($P < 0.05$). The RSD was 0.33%, 0.75% and 1.24% ($n = 6$) for raw material of *Radix Salvia miltiorrhiza,* normal drug sera and fibrotic drug sera, respectively. The samples were stable for 6 d.

CONCLUSION: Protocatechuic aldehyde can inhibit the growth of HSCs. HPLC is suitable for the determination of virtual bioactive components of Chinese herbal medicines *in vitro.*

Key words: *Radix Salvia miltiorrhiza;* Protocatechuic aldchyde; Seropharmacological method; High performance liquid chromatography

Lv T, Yao XX. Comparison of protocatechuic aldchyde in *Radix Salvia miltiorrhiza* and corresponding pharmacological sera from normal and fibrotic rats by high performance liquid chromatography. *World J Gastroenterol* 2006; 12(14): 2195-2200

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INTRODUCTION

Radix Salvia miltiorrhiza is an important traditional Chinese medicine (TCM) for activating blood and eliminating stasis[1]. Its effects on chronic hepatic diseases, such as liver fibrosis and cirrhosis, have been proved in experiments *in vitro* (cell culture) and *in vivo* (animal experiment)[2-4]. Liver fibrosis is a necessary stage during the development of liver cirrhosis. The activation and proliferation of hepatic stellate cells (HSCs) are the critical steps in hepatic fibrogenesis[5,6]. *Radix Salvia miltiorrhiza* could markedly inhibit the activation and proliferation of HSCs. Protocatechuic aldchyde is one of the bioactive components of *Radix Salvia miltiorrhiza*, which could inhibit the proliferation of HSCs and might be one of the efficient components of *Radix Salvia miltiorrhiza* acting on HSCs.

"Traditional seropharmacological method" usually utilizes healthy animals (such as rats) as medicine takers. The effect *in vitro* can be studied by isolating the drug sera (normal drug sera) from healthy animals to clarify the functions of the components in drug sera [7]. But different functional status of liver, which is the biggest organ for biological metabolism, could lead to some differences in the conversion of the same drug. Therefore, we used rats with liver fibrosis as the drug receivers and drug serum resources in our study. The corresponding

drug sera (pathological drug sera) were drawn after oral administration of the herbs. We termed it "modified seropharmacological method".

We compared the contents of protocatechuic aldchyde in raw *Radix Salvia miltiorrhiza* in normal and fibrosis rats before and after administration. Whether this method could predict the curative effects of *Radix Salvia Miltiorrhiza* was evaluated.

MATERIALS AND METHODS

Materials

Twenty male Sprague-Dawley (SD) rats weighing 200-250 g were provided by the Laboratory Animal Center of Hebei Medical University. All rats were randomized into group A (normal rats) and group B (liver fibrotic rats), 10 in each group. The rats in group A were treated with saline, while the rats in group B were treated with 40% carbon tetrachloride (CCl_4). Hepatic fibrosis was induced in rats of group B by 40% CCl_4 4 mL/kg body weight for the first injection, then 2 mL/kg body weight, twice a week, for 9 wk.

HSC cell line was presented by Professor Greenwell, Marion Bessin Liver Research Center, Albert Einstein College of Medicine. The phenotype of *CFSC* was obtained from CCl4-cirrhotic liver of rats after spontaneous immortalization in culture.

RPMI-1640 was from GIBICO. CCl_4 was of analytical grade. *Radix Salvia miltiorrhiza* was provided by Hebei Lerentang Chinese Medicine DrugStore Co. Drug sera were drawn from rats.

Methods

Preparation of samples for HSC cultivation

Radix Salvia miltiorrhiza was ground into powder and filtered with a sieve plate. The powder was dissolved into pure RPMI-1640 media to make the concentration 15 times that of the decoction for adults (within the range of safe concentration for HSCs). Then the dissolution was filtered to remove the bacteria for HSC cultivation.

SD rats weighing 200-250 g were randomized into group A and group B, 10 in each group. Liver fibrosis was induced in rats of group B by 40% CCl_4, 4 mL/kg body weight first, then by 2 mL/kg body weight, twice a week, for 9 wk. The rats in group A were treated with saline. The decoction of *Radix Salvia miltiorrhiza* was administrated to all rats for 5 d. The quantity of raw *Radix Salvia miltiorrhiza* in solution was 1.33 g/mL. Blood was drawn from inferior vena cava 2 h after the drugs were given on the fifth day. Then drug sera were obtained by centrifugation at 3000 r/min for 20 min at 4 ℃. The sera were deactivated at 56 ℃ for 30 min. Then the drug sera were dissolved in RPMI-1640 to obtain 10% drug sera-1640 medium for analysis.

Detection of inhibitory rate of protocatechuic aldchyde, raw Radix Salvia miltiorrhiza and drug sera of Salvia miltiorrhiza on HSC growth by CCK-8

Cell suspension (100 μl) was incubated on a 96-well plate. When HSCs grew to 90% confluence they were incubated in pure RPMI-1640 overnight to synchronize HSCs into the G_0 period. The solutions of protocatechuic aldchyde, raw *Radix Salvia miltiorrhiza* and the corresponding 10% normal and fibrotic drug sera-1640 media in the wells were changed. The results were obtained. A group of controls was set at the same time. One hundred microliters of CCK-8 solution were added to each well of the plate after the drug sera were allowed to work for 24 h. Then the plate was incubated for 4 h. The absorbance (A) at 450 nm was measured with a microplate reader. The inhibitory rates (IR) of all solutions on the growth of HSCs were calculated (IR = experiment group-control group /control group×100%).

Systems for HPLC

Waters 810 controller, Waters 486 tunable absorbance detector, Waters 510 HPLC pump, Millipore Waters U6K, Waters system interface module, Waters baseline 810 chromatography workstation (America) were used for HPLC. Sep-Park C18 purification column, ultrafree-MC filters (10000 NMWL filter unit) and Autoscience ultrasonic producer were all from Millipore Corporation, America. Standard of protocatechuic aldchyde (NO. 110810-200205), glacial acetic acid and methanol were all special for HPLC. All reagents used in HPLC were filtered and deprived of vapor by ultrasound.

Preparation of samples for HPLC

Radix Salvia miltiorrhiza (100 g) was ground into powder and filtered with a sieve plate. The powder of *Radix Salvia miltiorrhiza* was put into a volumetric flask containing methanol. Then ultrasound was used to facilitate the dissolution of the powder for 5 min and methanol was added to obtain 100 mL solution. This solution was then filtered through filters with 0.45 μm Millipore, purified by Sep-Park C18 column, and repeatedly filtered through Ultrafree-MC filters. Finally, the filtered solution was used as samples of raw *Radix Salvia miltiorrhiza* and kept in darkness at 4 ℃.

Samples of Radix Salvia miltirrhiza drug sera (normal and fibrotic drug sera)

The preparation of drug sera was the same as that of the sera for HSC cultivation. After the drug sera were obtained by centrifugation at 3000 r/min for 20 min at 4 ℃, acetonitrile was mixed with the sera to get rid of protein by centrifugation. Then the supernatant was filtered through filters with 0.45 μm Millipore, purified by Sep-Park C18 column, and filtered through filters. Finally, the filtered solutions were kept in darkness at 4 ℃.

Parameters of HPLC

The parameters for HPLC included: HPLC column: Alltima C18 column (250 mm × 4.6 mm, 5 μm); velocity of flow: 0.5 mL/min; detection wavelength: 281 nm; temperature for HPLC column: 30 ℃; mobile phase: A: acetonitrile; B: 4% glacial acetic acid; gradient elution: A-B (2:98): 0-10min; A-B (10:90): 10-26 min.

Linear relation

Protocatechuic aldchyde (4.125 mg) was added into a volumetric flask. Then methanol was used to dissolve the powder to a final volume of 100mL (final concentration was

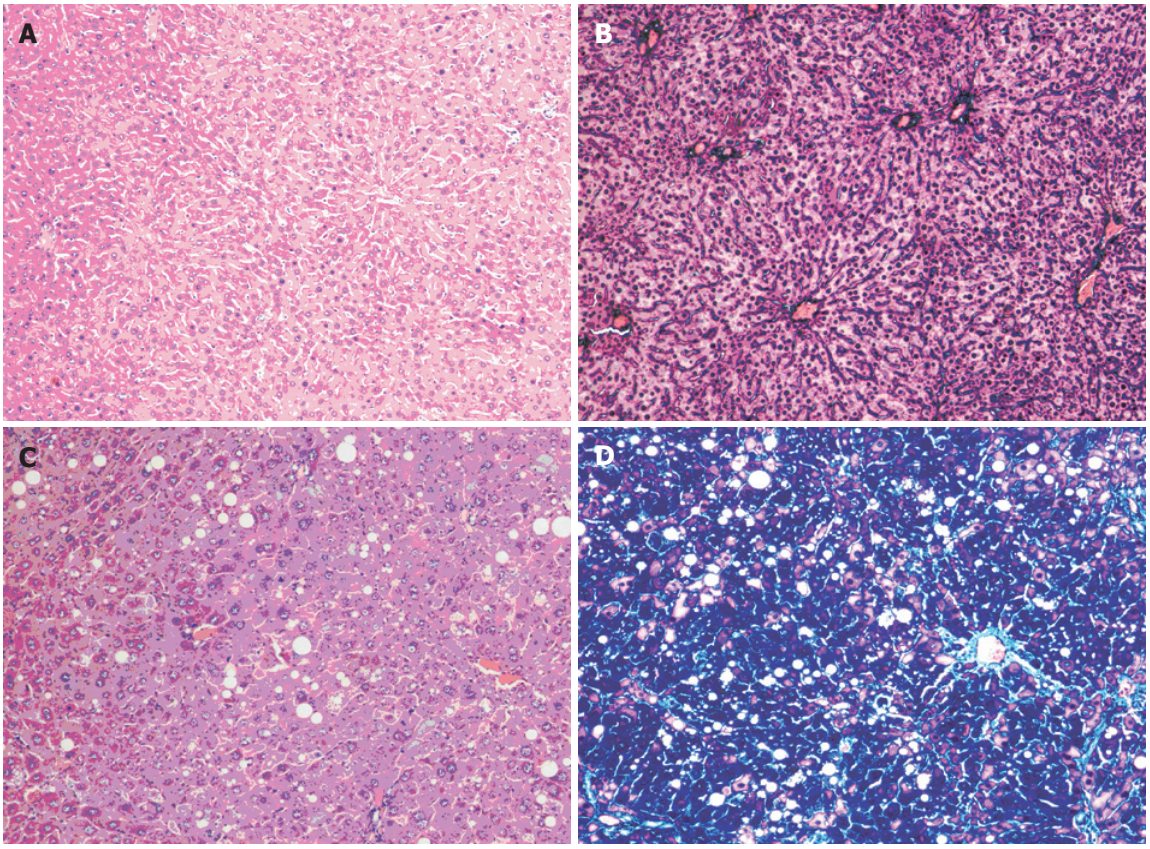

Figure 1 Healthy liver tissue of rats of group A (**A**, **B**) and fibrotic liver tissue of rats of group B (**C**, **D**). **A** and **C** (HE, 100×); **B** and **D** (Masson, 100×).

0.04125 mg/mL). Afterwards, 2, 5, 8, 10, 12, 15, 18 and 20 μL of protocatechuic aldchyde standard solution were injected into U6K for detection. Each volume was detected 3 times by HPLC under the conditions as described above. The standard curve was plotted with X axis (μg) to Y axis (mv.min).

Experiment of exactitude and evaluation of stability of samples

For the experiment of exactitude, 10 μL of protocatechuic aldchyde standard solution (41.25 μg/ml) was injected into U6K for detection. The detection was repeated 6 times, 10 μL for each time. Then the relative standard deviation (RSD) of the peak areas was calculated to evaluate the exactitude of the experiment.

For the experiment of stability, the same volume of standard solution was injected into U6K for detection, 6 times each day for 9 d. The RSD of the peak areas each day was calculated to evaluate the stability of the samples during the 9 d.

Experiment of recovery rate

In brief, 0.05 mg of protocatechuic aldchyde was put into 25 mg powder of raw *Salvia miltiorrhiza*. Then the samples for research were injected into U6K for detection. The average recovery rate ($n = 6$) was calculated.

Six samples of drug sera (400 μL for each sample) were taken out and 10 μL of protocatechuic aldchyde standard solution (0.04125 mg/mL) was dispensed into each sample of drug sera. Then the products were detected by HPLC ($n = 6$) to calculate the average recovery.

Experiment of repeatability

Radix Salvia miltiorrhiza (100 mg) was disposed as in preparation of samples of raw *Radix Salvia miltiorrhiza*. Then the samples were detected by HPLC for the content of protocatechuic aldchyde ($n = 6$). The detection was repeated 6 times independently. RSD of the peak areas was calculated to evaluate the repeatability of the experiment.

Four hundred μL of a certain drug serum was disposed as in preparation of samples of drug sera. The samples were detected for the content of protocatechuic aldchyde by HPLC ($n = 6$). RSD of the peak areas was calculated to evaluate the repeatability of the experiment.

The content of protocatechuic aldchyde in samples was detected by HPLC.

Statistical analysis

SPSS 10.0 was used to perform χ^2 test and $P < 0.05$ was considered statistically significant.

RESULTS

Obvious proliferation of fibers appeared in the livers when the rats in group B (Figures 1C and 1D) were treated with 40% CCl_4 for 9 wk. There were obvious signs of vacuole degeneration, lobular inflammation and necrosis in the tissues of fibrotic liver. No evident changes appeared in the rat liver of group A (Figures 1A and 1B).

It showed that protocatechuic aldchyde, raw *Radix Salvia miltiorrhiza*, the corresponding normal and fibrotic drug sera could all inhibit the proliferation of HSCs compared with blank control group ($P < 0.05$). The raw *Radix Salvia*

Table 1 Inhibitory rates of protocatechuic aldchyde, raw Radix Salvia miltiorrhiza and corresponding normal and fibrotic drug sera on proliferation of HSCs

Group	IR (%)	A
A (Protocatechuic aldchyde)	24.36	1.565±0.08[a]
B (raw Radix Salvia miltiorrhiza)	36.10	1.322±0.10[a]
C (Normal drug sera of Salvia miltiorrhiza)	16.14	1.735±0.12[ab]
D (Fibrotic drug sera of Salvia miltiorrhiza)	21.41	1.626±0.53[ab]
E (Control)	–	2.069±0.37

[a]$P < 0.05$ *vs* group E; [b]$P < 0.05$ *vs* group D

IR = (experiment group–control group) ÷ control group × 100%.

miltiorrhiza had a stronger inhibitory effect than the corresponding drug sera. The fibrotic drug sera of *Salvia miltiorrhiza* from fibrotic rats showed a higher suppressive effect on HSC growth than the normal drug sera from healthy rats ($P < 0.05$) (Table 1). The contents of protocatechuic aldchyde showed a nice linearity within the range of 0.1-0.8 μg ($Y=2.14\times10^4X+2.77$, $r=0.9999$).

Protocatechuic aldchyde standard solution was detected 6 times. The RSD was 0.33% for the peak areas of protocatechuic aldchyde. Within the 9 d of observation, the peak areas of protocatechuic aldchyde in standard solutions did not change significantly in the first 6 d. The RSD was 0.45% ($n=6$) for the peak areas of protocatechuic aldchyde in the first 6 d. It indicated that the solutions of standards could be kept in darkness at 4 ℃ for 6 d.

The average recovery of raw *Radix Salvia miltiorrhiza* was 110.5%. RSD was 0.37% ($n=6$). The average recovery of drug sera of *Salvia miltiorrhiza* was 102%, and the RSD was 1.96% ($n=6$). The average recovery of fibrotic drug sera was 105.2%, and the RSD was 1.51% ($n=6$).

Protocatechuic aldchyde in raw *Radix Salvia miltiorrhiza* was 0.22%, and the RSD was 0.33% ($n=6$). The ratio of protocatechuic aldchyde in normal drug sera was 0.15%, and the RSD was 0.75% ($n=6$). The ratio of protocatechuic aldchyde in fibrotic drug sera was 0.19% and the RSD was 1.24% (Table 2, Figures 2A-2C).

Table 2 Results of sample determination (mean±SD, %)

Group	Content of protocatechuic aldehyde
Salvia miltiorrhiza Bge	0.22±0.07
Normal drug sera of Salvia miltiorrhiza Bge	0.15±0.01[a]
Fibrotic drug sera of Salvia miltiorrhiza Bge	0.19±0.01[a c]

[a]$P < 0.05$ *vs* Salvia miltiorrhiza Bge; [c]$P < 0.05$ *vs* Normal drug sera of Salia miltiorrhiza Bge.

DISCUSSION

Radix Salvia miltiorrhiza is the dried root of labiate-*Salvia miltiorrhiza Bge,* an important drug in traditional Chinese medicine for activating blood flow and eliminating stasis. *Radix Salvia miltiorrhiza* has certain curative effect on coronary heart diseases and chronic liver diseases with few adverse effects. No ideal anti-fibrosis drugs in Western medicine have been developed so far. A series of blood-activating and stasis-eliminating Chinese medicines such as *Radix Salvia miltiorrhiza*, have a positive effect on prevention and reversion of fibrogenesis. However, it is difficult to clarify the working mechanism and real active components of Chinese anti-fibrosis herbs. *Radix Salvia miltiorrhiza* can improve liver function and ameliorate hepatic pathological changes in rats and humans. In our study, when the aqueous extract of *Radix Salvia miltiorrhiza* was given to rats before the injection of CCl_4, liver fibrosis was produced. On the other hand, in cell culture *in vitro*, *Radix Salvia miltiorrhiza* showed its inhibitory effect on lipid peroxidation and some key signal transduction circuits in HSCs, delaying the activation and proliferation of HSCs. A kind of extract from *Radix Salvia miltiorrhiza*-monomer IH764-3 could activate caspase-3 and induce apoptosis of HSCs.

Many bioactive components in *Radix Salvia miltiorrhiza*, including soluble and insoluble parts have various pharmacological effects such as blood-activating and stasis-eliminating and facilitate the circulation of blood and oxygen. The insoluble components mainly include compounds of phenanthrenequinone (PAQ), such as tanshinones I and II cryptotanshinone and isotanshinone. The soluble components are mainly composed of phenolic acids. Salvianolic acid B[8] is one of the bioactive parts of *Radix Salvia miltiorrhiza* to inhibit the activation and proliferation of HSCs by blocking the intracellular signal transduction of transforming growth factor-β1 (TGF-β1).

Protocatechuic aldchyde is one of the water-soluble components of *Radix Salvia miltiorrhiza* and can be used to treat dysmenorrhea in gynaecology. Protocatechuic aldchyde can inhibit the proliferation of HSCs (CCK-8) *in vitro* and might be used to treat liver fibrosis. In this study, the peak areas maintained stable for 6 d. Since the stability of protocatechuic aldchyde is much better than other components (such as salvianolic acid B) in *Radix Salvia miltiorrhiza*, protocatechuic aldchyde performs the pharmacological effects for a longer time *in vivo* and *in vivo*. The content of protocatechuic aldchyde in raw *Radix Salvia miltiorrhiza* is higher than that in the corresponding drug sera, which could explain the phenomenon that the inhibitory effect of raw *Radix Salvia miltiorrhiza* was stronger than that of drug sera. It indicates that only part of metabolites of protocatechuic aldchyde in raw *Radix Salvia miltiorrhiza* could enter blood to exert pharmacological effects after metabolism *in vivo*. If crude medicines are used to work on HSCs directly *in vitro*, the pharmacological effect of the medicine would be exaggerated. On the other hand, some other herbs could perform their pharmacological effect only after decocted *in vitro* and metabolized *in vivo*, because the process of decoction and metabolism would promote the activation of the bioactive components in this kind of crude medicines. Under this condition, the herbs seem useless if they are directly used on cells *in vitro*, suggesting that the effect of raw *Radix Salvia miltiorrhiza* is not equal to

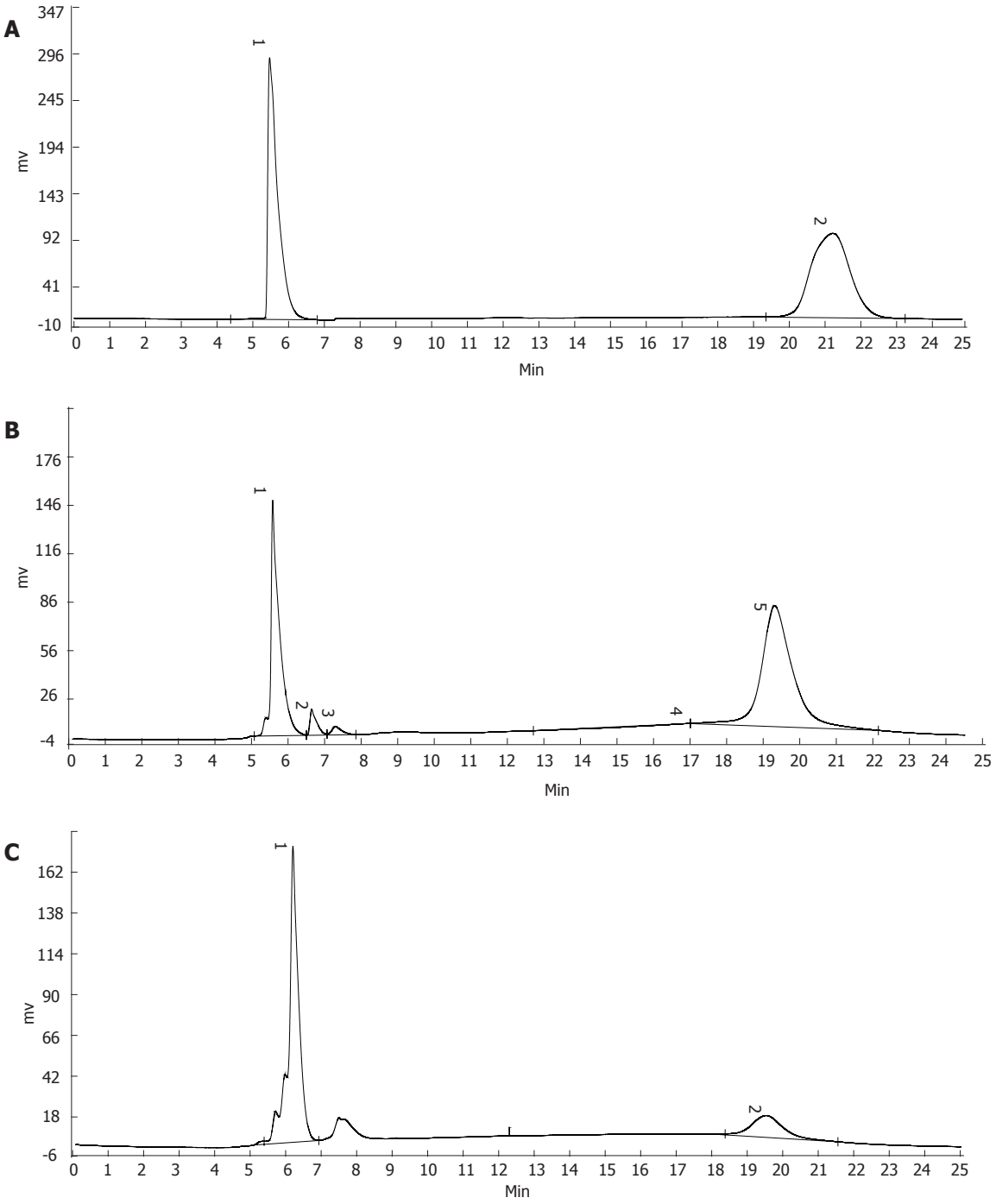

Figure 2 Chromatography of raw *salvia miltiorrhiza Bge* (**A**), pathological drug sera of *Salvia miltiorrhiza Bge* (**B**) and normal drug sera of *Salvia miltiorrhiza Bge* (**C**).

the effect of *Salvia miltiorrhiza in vivo*. The differences are related to the characteristics of herbs. Active constituents of TCM are influenced by soils, climates, growth stages, and factors *in vitro* and *in vivo*. So the "Pharmacological method" is more suitable for researching herbs.

Protocatechuic aldchyde in pathological drug sera is more than that in normal drug sera, which is in accordance with the different effects of normal and fibrotic drug sera [3]. Because of this difference between the two kinds of drug sera and the phenomenon that most of the medicine takers are patients with hepatic diseases, it is better to use "modified seropharmacological method" and " pathological drug sera" to carry out research on herbs *in vitro* (*vs* " traditional seropharmacological method" and "normal drug sera"). In this way, the impact of different liver functional status on herbs' metabolic process could be included as a parameter in experiment. The phenomenon that the content of protocatechuic aldchyde is higher in fibrotic drug sera than in normal sera, coincides with the working characteristics of herbs, which might be caused by different "first pass effect" of the liver under different liver conditions and metabolism of drugs. From the traditional Chinese medicinal theory of "diagnosis and treatment on the basis of an overall analysis of the illness and the patient's different symptoms and pathogenesis" and "bidirectional regulation", TCM works best only when

the symptoms and etiology are just their corresponding indications. *Radix Salvia miltiorrhiza* is one of the blood-activating and stasis-eliminating drugs. Liver fibrosis is considered as "blood obstruction"[9] in traditional Chinese medicine. Undoubtedly it is the indication for *Radix Salvia miltiorrhiza*. So the blood-activating and stasis-eliminating herbs such as *Radix Salvia miltiorrhiza* have better pharmacological effects on fibrotic rats. The active components in *Radix Salvia miltiorrhiza* might be activated and released more efficiently to perform stronger pharmacological effects. The ability of fibrotic liver to transform and inactivate drugs might decrease because the metabolic functions of fibrotic liver decrease, thus making the concentration of the effective components higher, prolonging the effective working period.

The value of this research lies in that we could get the real active components of *Radix Salvia miltiorrhiza* by "modified seropharmacological method" after they are metabolized *in vivo*. HPLC is a quick and sensitive method the determination of effective components in drugs *in vitro* and *in vivo*.

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CLINICAL RESEARCH

Treatment of active steroid-refractory inflammatory bowel diseases with granulocytapheresis: Our experience with a prospective study

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Abstract

AIM: To report our experience with the use of granulocytapheresis (GCAP) in 14 patients with active steroid-refractory inflammatory bowel disease (IBD) in order to evaluate its efficacy in achieving remission and maintaining a long lasting symptom-free period.

METHODS: The activity of the disease was evaluated by clinical activity index (CAI) and endoscopic index (EI) in ulcerative colitis (UC), while by Crohn's disease activity index (CDAI) in Crohn's disease (CD). The patients were treated using the Adacolumn™ system, an adsorption column which selectively binds to granulocytes and monocytes. One session/week of GCAP was performed for 5 wk. Steroids were stopped during apheresis.

RESULTS: All the patients completed the five-week course showing no complications. At the end of the last session, 93% of patients showed a clinical remission of the disease that persisted for 6 mo. Nine months after the end of the treatment, 60% of the cases maintained remission, while 23% of the patients were still in clinical remission after 12 mo.

CONCLUSION: Even if the number of our patients with steroid-refractory IBDs was not big, we can assert that GCAP is well tolerated and effective, especially in the first six months after the treatment, in a significant percentage of cases. The rate of sustained response drops slightly after 6 mo and significantly after 12 mo, however the absence of severe side effects can be a stimulus for further evaluating new schedules of treatment.

Key words: Granulocytapheresis; Ulcerative colitis; Crohn's disease; Steroid-refractory

INTRODUCTION

The term "inflammatory bowel disease (IBD)" usually means two similar but distinct chronic diseases of the gut: ulcerative colitis (UC) and Crohn's disease (CD) characterised by episodes of remission and exacerbation with systemic complications[1-4].

During the last 20 years the treatment of IBD has been greatly improved although it is still empirical due to its unknown aetiology[5-8]. It seems that both ulcerative colitis and Crohn's disease are multifactorial in origin. However, regardless of the cause, the final pathway of tissue damage in IBD is mediated by the cellular immune response through white blood cells in the intestinal mucosa. Corticosteroids are a mainstay of acute therapy for moderate to severe ulcerative colitis or Crohn's disease. However, up to 40% of patients do not respond to the high-dose steroid therapy. So, new therapeutic approaches are needed to improve the clinical outcome of active steroid-refractory IBD.

Classic immunosuppressant drugs like azathioprine or mercaptopurine need some weeks to exert their full activity, so they are useless during the acute phases of the disease. Moreover, newer immunosuppressants like cyclosporine A have shown only a temporary benefit and often serious side effects.

In recent years some trials have suggested that leukocytapheresis can be a useful and safe way to induce clinical remission in patients with IBD[9-11, 15]. Subsequent trials have proved that granulocytapheresis (GCAP), a technique that sequestrates much selectively granulocyte and monocyte subpopulations, is equally effective in patients with active IBD[16, 17].

Even if with a limited casuistry, we aimed at bulking the published data by referring our experience with GCAP. We analyzed the clinical, endoscopic and laboratory parameters of patients with active, steroid-refractory IBDs,

Table 1 Baseline characteristics of UC patients ($n = 8$) (mean ± SD)

Female/Male	3/5
Age (yr)	35.2±7.4
Colonic involvement	All PanUC
Disease duration (yr)	7.8±3.0
Basal CAI	10.0±3.4
Basal EI	10.5±2.5

CAI:clinical activing index;
EI: endoscopic index.

Table 2 Baseline characteristics of CD patients ($n = 6$) (mean ± SD)

Female/Male	3/3
Age (yr)	37.8±2.5
Disease location	All ileal CD
Disease duration (yr)	7.1±3.2
Basal CDAI	213.3±32.0

before and after the completion of GCAP during a follow-up period of 12 mo. This work was to get more information about the real effectiveness of GCAP in achieving remission and possibly in maintaining it.

MATERIALS AND METHODS

All the patients with UC or CD treated with GCAP in our department because of steroid-resistant IBD were followed up by monitoring clinical, laboratory and endoscopic parameters for UC.

Before admitted for GCAP, all patients signed an informed consent and underwent routine laboratory tests and basal ECG. Moreover, they were visited by a cardiologist in order to exclude serious cardiovascular diseases.

All patients received a 5-session (1 session/wk) treatment with GCAP. This was an extracorporeal procedure in which 1.8 L of blood was filtered through an Adacolumn™ (JIMRO, Takasaki, Japan). Adacolumn™ is a 335 mL capacity column filled with 35 000 cellulose diacetate beads (2 mm in diameter) that bind to granulocytes and monocytes via the CR3 receptors present on these cells. Each apheresis procedure required the addition of 1500 UI of sodium heparin as an anticoagulant. Blood was obtained by antecubital vein puncture. Methylprednisolone daily dose was progressively reduced until discontinuation in a 6-wk period (one week after the last apheresis procedure). Concomitant treatment with aminosalicylates was maintained during the treatment and follow-up at the same dosage.

In ulcerative colitis patients, the activity of the disease was evaluated by clinical activity index (CAI): Clinical remission was defined if less than 6 and endoscopic index (EI): endoscopic remission was defined if less than 4. In the subjects with Crohn's disease, the activity was measured by Crohn's disease activity index (CDAI): clinical remission was defined if less than 150.

After the end of the five-week course of granulocytapheresis and every 3 mo, each patient was visited for a clinical and endoscopic assessment, paying particular attention to the activity indices. The subjects who achieved clinical remission were followed up for 12 mo by the last session of GCAP.

Relapse was defined as an increase of clinical and endoscopic scores (CAI, EI, CDAI) more than 6, 4 or 150 respectively. Patients who were on 5-aminosalicylic acid continued this medication but no additional treatment.

Hemograms, biochemistry and coagulation were recorded during the apheresis treatment.

Statistical analysis

Data were expressed as mean ± SD if required. Student's t test for paired variables was performed. Statisticcal analyses were analysed with SPSS 11.5 (®SPSS inc. 2002).

RESULTS

We treated and followed up 14 patients (8 with ulcerative colitis and 6 with ileal Crohn's disease).

Baseline characteristics

Baseline characteristics of the 8 UC patients were mean age: 35.2±7.4 years; female/male ratio: 3:5; mean time of disease history: 7.8±3 years. All patients had an active pancolitis with a mean CAI of 10.0±3.4 at baseline and a mean endoscopic index (EI) score of 10.5±2.5 (Table 1). All the patients showed an active disease and were treated with 0.8-1 mg/kg pre d of i.v. or i.m. methylprednisolone and 2.4 g/d of oral mesalazine during the 8 wk period prior to GCAP initiation, without achieving response. None of the patients was under inmuno-supressor therapy at baseline but all of them were on mesalazine at 2.4 g/d.

Baseline characteristics of the 6 CD patients were mean age: 37.8±2.5 years; female/male ratio: 3:3; mean time of disease history: 7.1±3.2 years. All patients had an active ileal CD with a mean basal CDAI of 213.3±32.0 (Table 2).

Induction of remission in UC patients

One week after GCAP treatment, all UC patients achieved remission (Figures 1A and 1B) and stopped methylprednisolone treatment. The mean CAI decreased from 10.0 ± 3.4 to 3.5 ± 1.2 ($P < 0.001$). Also EI decreased from 10.5±2.2 to 2.6 ± 0.5 ($P < 0.001$). The mean values of CRP and ESR decreased from 32.5 ± 11.2 to 4.5 ± 4.7 mg/L ($P < 0.001$) and 48.5 ± 8.6 to 15.0 ± 2.3 mm/h respectively ($P < 0.001$) (Figures 3A and 2B).

Induction of remission in CD patients

One week after GCAP treatment, 5 out of 6 patients achieved remission (Figure 3A) and stopped methylprednisolone treatment. The mean CDAI decreased from 213.3 ± 32.0 to 135 ± 50 ($P < 0.015$). The mean CRP values decreased from 39.0 ± 12.6 to 9.5 ± 9.1 mg/L ($P < 0.01$) and ESR values from 52.3 ± 9.8 to 21.7 ± 18.0 mm/h ($P < 0.05$) (Figures 3B and 3C).

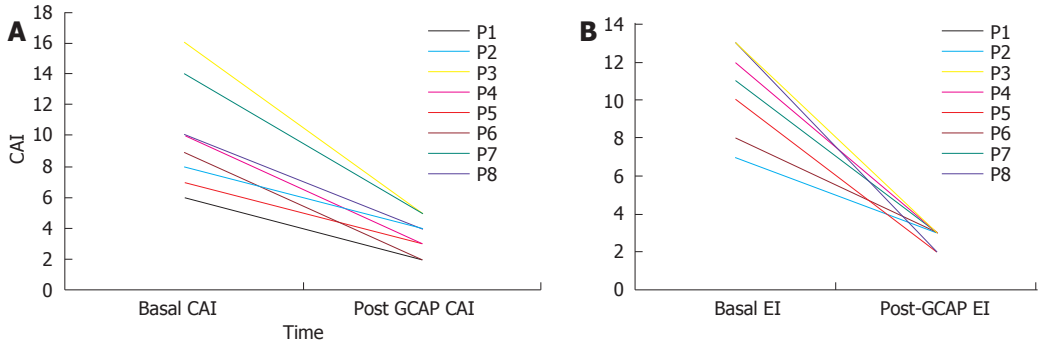

Figure 1 Evolution of CAI (**A**) and EI (**B**) in UC patients.

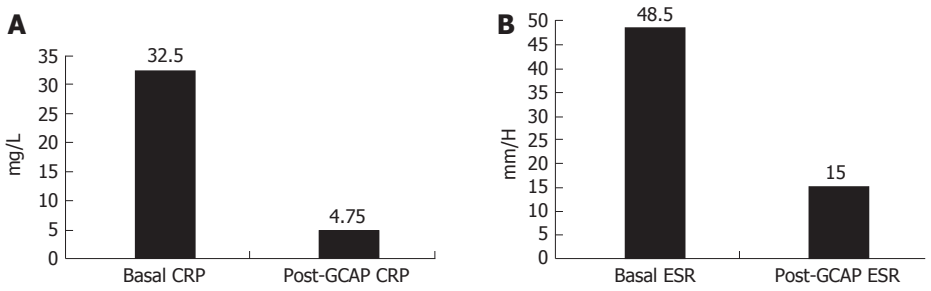

Figure 2 Mean evolution of CRP (**A**) and ESR (**B**) in UC patients.

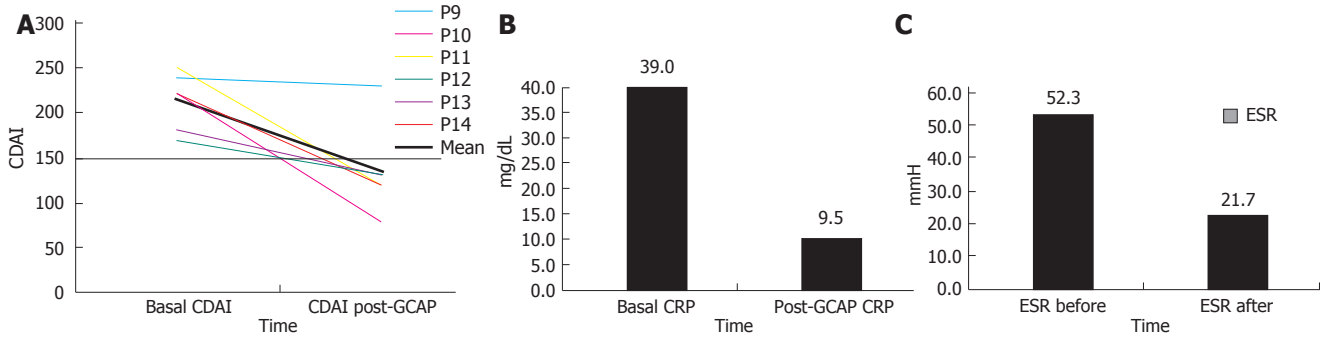

Figure 3 Mean evolution of CDAI (**A**), CRP (**B**) and ESR (**C**) in CD patients.

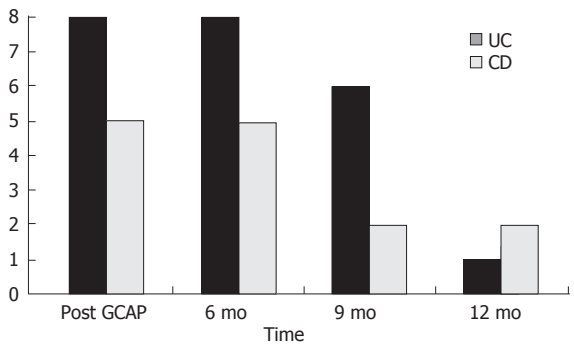

Figure 4 Remission during follow-up.

Adverse events

All the patients completed GCAP without severe side effects. Only a transient mild headache was recorded in two patients during the procedure.

Follow-up

All the patients were followed up for 12 mo after GCAP treatment and no additional treatment was added. Clinical remission persisted for 6 mo in all the patients and was achieved early after apheresis (93%). At the 9th mo of follow-up, 6 of 8 UC patients and 2 of 5 CD patients were still in clinical remission. At the 12th mo of follow-up, only 1 of 8 UC patients and 2 of 5 CD patients were in remission (Figure 4).

DISCUSSION

Currently, the use of steroid drugs is a common strategy for the treatment of acute inflammatory bowel disease. However, large doses of steroids are often necessary to control active diseases and some patients do not respond to this conventional treatment. Colectomy rate varies from 10% to 40% in these patients[12]. The treatment with cyclosporin in these patients can avoid acute colectomy in 57% of cases, but after a 6-mo follow-up period 73% of patients undergo surgery[13]. Azathioprine or 6-mercaptopurine has been reported to be beneficial for steroid resistant cases, but their use requires several weeks to get full effects[14].

Recent data from literature have pointed out that

granulocytapheresis, which acts on specific subpopulations of leukocytes involved in the inflammatory process, can represent a useful and probably much safe treatment for patients with ulcerative colitis or Crohn's disease[16, 17].

In fact it is well known that inflammatory bowel disease is associated with elevated circulating and tissue levels of leukocytes. Granulocytes are the first cells mobilizing to inflammation sites and interact with lymphocytes to orchestrate the inflammatory response. For these reasons the removal of granulocytes may be a logical therapeutic manoeuvre. Moreover, the good results obtained so far by granulocytapheresis have been attributed not only to the removal of granulocytes and monocytes, but also to its immunomodulatory effect[10, 11, 15, 16].

The goal of this study was to assess the safety and efficacy of GCAP in patients with active inflammatory bowel disease that was refractory to conventional drug therapy. In our small casuistry, the procedure was well tolerated and 93% of cases showed remission at the end of a five-week course of GCAP. No serious side effects were recorded and clinical/endoscopic remission lasted for six months in all the patients, achieved at the end of the therapeutic course ("responders"). Nine months after GCAP treatment, about 60% of responders were still in remission, without any significant difference between UC and CD patients. The remission rate dropped dramatically at the 12th mo, with slightly better outcome in CD patients.

In conclusion, this new approach to active steroid-refractory IBD seems an important innovation and a useful therapy after the failure of conventional treatments. GCAP is able to achieve clinical remission in a large proportion of "difficult" IBD patients. This new technique is virtually free of severe side effects and could be repeated more times if necessary.

We hope that our data could represent a stimulus for further trials aimed to evaluate the real usefulness of apheresis in IBD, to clarify the optimal length of treatment and to know which patients could get most benefit from it.

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CLINICAL RESEARCH

Echo-enhanced ultrasound with pulse inversion imaging: A new imaging modality for the differentiation of cystic pancreatic tumours

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Abstract

AIM: To describe and discuss echo-enhanced sonography in the differential diagnosis of cystic pancreatic lesions.

METHODS: The pulse inversion technique (with intravenous injection of 2.4 mL SonoVue®) or the power-Doppler mode under the conditions of the 2nd harmonic imaging (with intravenous injection of 4 g Levovist®) was used for echo-enhanced sonography.

RESULTS: Cystadenomas frequently showed many vessels along fibrotic strands. On the other hand, cystadenocarcinomas were poorly and chaotically vascularized. "Young pseudocysts" were frequently found to have a highly vascularised wall. However, the wall of the "old pseudocysts" was poorly vascularized. Data from prospective studies demonstrated that based on these imaging criteria the sensitivities and specificities of echo-enhanced sonography in the differentiation of cystic pancreatic masses were > 90%.

CONCLUSION: Cystic pancreatic masses have a different vascularization pattern at echo-enhanced sonography. These characteristics are useful for their differential diagnosis, but histology is still the gold standard.

Key words: Cystic pancreatic lesions; Differential diagnosis; Echo-enhanced sonography

Rickes S, Mönkemüller K, Malfertheiner P. Echo-enhanced ultrasound with pulse inversion imaging: A new imaging modality for the differentiation of cystic pancreatic tumours. *World J Gastroenterol* 2006; 12(14): 2205-2208

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INTRODUCTION

Cystic tumours of the pancreas are rare, accounting for about 1% of all pancreatic neoplasms[1]. Cystadenomas and cystadenocarcinomas are the most frequently encountered cystic lesions[1-3]. There are problems in the differentiation of cystic pancreatic masses. It is obvious that the discrimination between these lesions is critical for prognosis and therapy[4].

Conventional transabdominal ultrasound displays no characteristic signs for the differentiation of cystic pancreatic tumours and the diagnostic accuracy is low[5-7]. The vascularization pattern is helpful for the tumour differentiation and can be investigated by fundamental power and colour Doppler sonography. However, there is a low sensitivity of these procedures for detecting low blood flow velocity or small vessels. The sensitivity can be increased by echo-enhancers, such as SonoVue®. Therefore, echo-enhanced sonography is an increasingly used procedure for the differentiation of pancreatic tumours[8, 9].

In this review, echo-enhanced sonography in the differential diagnosis of cystic pancreatic tumours is depicted and discussed.

MATERIALS AND METHODS

All patients were investigated first by conventional sonography using a dynamic sector scanner. A special preparation of the patients was not necessary. The pulse inversion technique or the power-Doppler mode under the conditions of the 2nd harmonic imaging was used for echo-enhanced sonography. The pulse inversion mode was used more frequently than the 2nd harmonic imaging.

For echo-enhanced sonography, the pulse inversion 2.4 mL SonoVue® (sulfur hexafluoride gas-based contrast agent, Bracco International B.V., Amsterdam, Netherlands) was injected intravenously, and the mechanical index varied between 0.1 and 0.2 (low MI procedure). The investigation could be done in real time, and lasted for approximately 2 min.

Echo-enhanced power Doppler sonography was started immediately after intravenous injection of 4 g

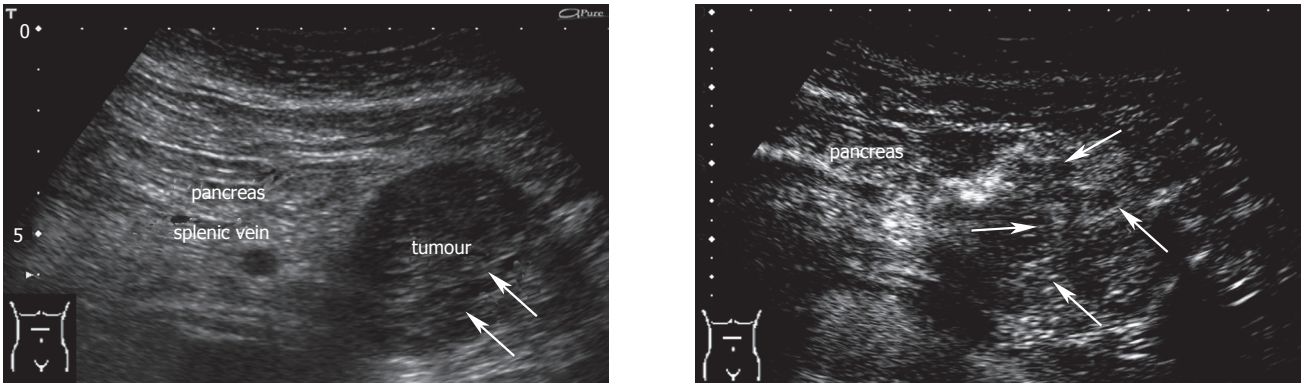

Figure 1 Cystadenoma at conventional and echo-enhanced ultrasound. **A**: A tumour at the pancreatic tail (5 cm in diameter) with small cystic areas (small arrows) and thin fibrotic strands; B: Highly vascularized tumour arteries (large arrows) along the fibrotic strands (maximum of contrastation 15 s after injection of the echo-enhancer).

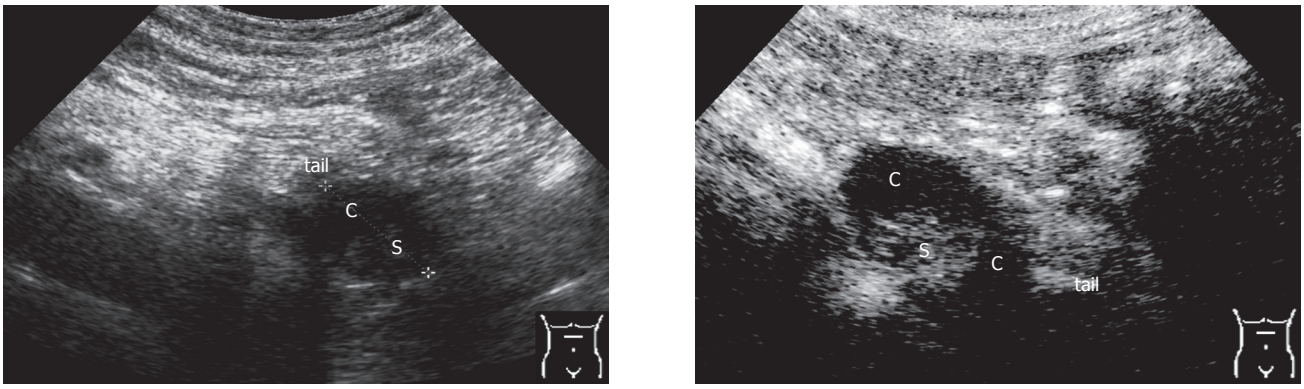

Figure 2 Cystadenoma at conventional and echo-enhanced ultrasound A: A tumour (7 cm in diameter) at the pancreatic tail with large cystic (c) and solid areas (s); **B**: A poorly vascularized solid (s) tumour (maximum of contrastation 15 s after injection of the echo-enhancer).

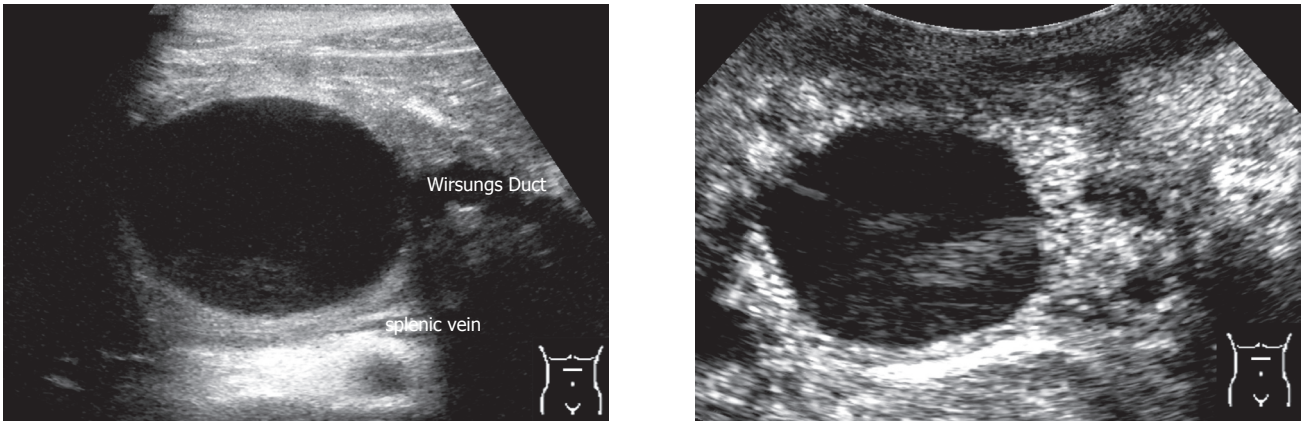

Figure 3 Pseudocust at conventional and echo-enhanced ultrasound A: A lesion with an echo-free pattern and a sharply delineated wall. The Wirsungs Duct is dilated; **B**: A highly vascularized wall (maximum of contrastation 20 s after injection of the echo-enhancer).

Levovist® (galactose-based contrast agent, concentration 300 mg/mL, Schering AG, Berlin, Germany). Intermittent sweeps were done, and the investigation lasted for approximately 2 min. One focus zone with depth adapted to the area of interest and a mechanical index of 0.8-1.3 (high MI procedure) should be used.

RESULTS

Criteria for the differentiation of pancreatic masses by conventional and echo-enhanced sonography[10, 11] are shown in Table 1. Cystadenomas consisting of small cystic areas (< 3 cm) and thin fibrotic strands are shown in Figure 1A. At echo-enhanced sonography highly vascularized solid tumour parts and arteries along the fibrotic strands are shown in Figure 1B. On the other hand, cystadenocarcinomas were found to have large cystic areas (about 5 cm) and solid tumour parts in the conventional ultrasound examination (Figure 2A). After injection of an echo-enhancer, poorly vascularized solid

Table 1 Criteria for differentiation of cystic pancreatic tumours with conventional ultrasound, fundamental power Doppler sonography, and echo-enhanced ultrasound[10, 11]

	Conventional ultrasound	Fundamental power Doppler sonography	Echo-enhanced sonography
Cystadenoma	• small cystic areas (often < 3 cm) • spoke-like pattern of fibrotic strands with small calcifications • no dilated Wirsung's duct	• no tumour vessels detectable	• highly vascularised tumour arteries along the fibrotic strands
Cystadeno-carcinoma	• large cystic areas (often > 5 cm) • solid areas • no dilated Wirsung's duct	• rarely tumour vessels with chaotic pattern detectable	• poorly and chaotic vascularised solid areas
Pseudocyst	• often echo-free pattern • sharply delineated wall • features of acute and/or chronic pancreatitis • signs of bleeding and/or calcifications • bowel infiltration is possible	• rarely tumour vessels detectable in "young cysts"	• "young cysts" (a few weeks of age) show often a highly vascular-ised wall • "old cysts" (a few months of age) show often a poorly vascularised wall

areas could be detectable (Figure 2B). Pseudocysts were found to be characterised by an echo-free pattern and a sharply delineated wall (Figure 3A). In the remaining pancreatic parenchyma features of chronic inflammation such as calcifications and a dilated Wirsung's duct could also be found. After injection of an echo-enhancer the wall of the pseudocysts was highly ("young cyst", Figure 3B) or poorly vascularized ("old cysts").

A recently published study with 31 patients showed that echo-enhanced sonography could differentiate cystic neoplasms from pseudocysts[12]. The sensitivity of echo-enhanced sonography with respect to diagnosing cyst adenomas was 95% and its specificity was 92%. The corresponding values for pseudocysts were both 100%. However, one cystadenoma was misdiagnosed as a cystadenocarcinoma, and vice-versa. The morphological variability of these cystic lesions at conventional ultrasound and the difficulties in the evaluation of the vascularization of cystic masses might be responsible for the false results. On the other hand, only 27% of cystadenomas and 67% of pseudocysts could be correctly classified by conventional ultrasound[12].

DISCUSSION

So far not a single ideal diagnostic procedure is available for the differentiation of cystic pancreatic tumours. Histology is the gold standard but nevertheless can produce false negative results. The correct differential diagnosis of cystic neoplasms of the pancreas has proven to be difficult and imaging techniques have low correct diagnosis percentages[5, 13-15], which can be attributed to the particular anatomicropathological features of these tumors and the difficulty to discriminate them from pseudocysts.

Conventional ultrasound cannot provide the reliable characteristics of different cystic pancreatic lesions. Thus, it is difficult to distinguish these tumours. Cystadenomas often consist of small cystic areas and a spoke-like pattern of fibrotic strands. In contrast, cystadenocarcinomas often show large cysts (frequently larger than 5 cm in size). Pseudocysts are frequently echo-free and have a sharply delineated wall.

The angiographic vascularization pattern contributes to the differentiation of pancreatic tumours[16-18]. Since cyst-adenomas are characterised by their hypervascularization, they are often found to be hypovascularized with a chaotic pattern. However, the diagnostic accuracy of angiography is low because it is not possible to investigate the macroscopic tumour features[17].

The vascularization of tumours may also be studied by fundamental power and colour Doppler sonography. However, the sensitivity of these procedures is low in detecting low blood flow velocity or small vessels. This sensitivity can be improved by echo-enhancers, such as SonoVue® and Levovist®. Levovist® consists of air-filled microbubbles which enhance the Doppler signal at 20-30 dB[19-21]. However, the signal intensity of echo-enhanced sonography from flowing blood is lower than that of tissue movements. To overcome these difficulties the technique of the 2nd harmonic imaging has been developed based on the property of microbubbles to resonate and emit harmonic waves in an ultrasound field with a frequency of 1-5 MHz. If the harmonic frequency is to be detected at twice the transmitted frequency, the procedure is called the 2nd harmonic imaging. Since tissue particles have fewer of the 2nd harmonic waves than microbubbles, the signals of echo-enhancers are better distinguishable[19].

The new contrast agent Sonovue® is used more frequently for echo-enhanced sonography. Furthermore, the 2nd harmonic imaging can be replaced partially by the pulse inversion imaging technique. There are observations that with this new procedure more favourable results can be achieved than with the 2nd harmonic imaging. The 2nd harmonic imaging cannot separate the transmitted and

received harmonic signals completely because of limited bandwidth. However, pulse inversion imaging avoids these bandwidth limitations using characteristics specific to microbubble vibrations to subtract rather than filter out the fundamental signals. Because this imaging technique transmits two reciprocal pulses, leading to a subtraction of the two fundamental signals, it allows the use of broader bandwidths for the transmission and reception yielding an improved resolution and can provide an increased sensitivity to contrast[22]. However, comparative results of large prospective studies are missing.

We want to point out that according to our experience, conventional ultrasound, power and colour Doppler sonography, and echo-enhanced sonography should not be used as single imaging techniques, exclusively. Conventional ultrasound is the basic sonographic method, and tumour differentiation is hardly possible based on an echo-enhanced sonographic examination alone. Echo-enhanced sonography offers more diagnostic criteria than conventional ultrasound alone, but similar to the angiography it is impossible to investigate macroscopic tumour features with this procedure alone. Therefore, all sonographic procedures should be performed in combination.

The characteristic signs of pancreatic tumours at echo-enhanced sonography have been published [10, 11]. Solid areas of cystadenocarcinomas and the wall of the " old pseudocysts" are found to be hypovascularized. In contrast, solid parts of cystadenomas and the wall of the "young pseudocysts" are mostly hypervascularized. A recently published study showed that echo-enhanced sonography can differentiate cystic neoplasms better than conventional ultrasound alone[12].

The successful treatment of cystic pancreatic tumours requires a highly sensitive and specific diagnostic procedure. Echo-enhanced sonography can fulfil this requirement. However, histology is still the gold standard for the differentiation of cystic pancreatic lesions.

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CLINICAL RESEARCH

Surgical anatomy of the innervation of pylorus in human and *Suncus murinus*, in relation to surgical technique for pylorus-preserving pancreaticoduodenectomy

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Abstract

AIM: To clarify the innervation of the antro-pyloric region in humans from a clinico-anatomical perspective.

METHODS: The stomach, duodenum and surrounding structures were dissected in 10 cadavers, and immersed in a 10mg/L solution of alizarin red S in ethanol to stain the peripheral nerves. The distribution details were studied to confirm innervations in the above areas using a binocular microscope. Similarly, innervations in 10 Suncus murinus were examined using the method of whole-mount immunohistochemistry.

RESULTS: The innervation of the pyloric region in humans involved three routes: One arose from the anterior hepatic plexus via the route of the suprapyloric/supraduodenal branch of the right gastric artery; the second arose from the anterior and posterior gastric divisions, and the third originated from the posterior-lower region of the pyloric region, which passed via the infrapyloric artery or retroduodenal branches and was related to the gastroduodenal artery and right gastroepiploic artery. For Suncus murinus, results similar to those in humans were observed.

CONCLUSION: There are three routes of innervation of the pyloric region in humans, wherein the route of the right gastric artery is most important for preserving pyloric region innervation. Function will be preserved by more than 80% by preserving the artery in pylorus-preserving pancreaticoduodenectomy (PPPD). However, the route of the infrapyloric artery should not be disregarded. This route is related to several arteries (the right gastroepiploic and gastroduodenal arteries), and the preserving of these arteries is advantageous for preserving pyloric innervation in PPPD. Concurrently, the nerves of Latarjat also play an important role in maintaining innervation of the antro-pyloric region in PPPD. This is why pyloric function is not damaged in some patients when the right gastric artery is dissected or damaged in PPPD.

Key words: Innervation; Quality of life; Pylorus-preserving pancreaticoduodenectomy; *Suncus murinus*; Whole mount immunohistochemistry

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INTRODUCTION

With the concept of less invasive surgery, pylorus-preserving pancreaticoduodenectomy (PPPD) has taken the place of conventional Whipple pancreaticoduodenectomy as a standard operative procedure for the treatment of various benign and malignant diseases in the periampullary region, even pancreatic head carcinoma, since 1978[1-5]. Early delayed gastric emptying has been described as a common and frustrating complication after this procedure, which occurs in 20% to 46% of patients[6-16]. Although delayed gastric emptying is self-limiting, not life-threatening, and can be treated conservatively[7,9,17], it results in discomfort and a significant prolonging of

the hospital stay, and so contributes to increased hospital costs[7,8,18,19]. However, the pathogenesis of delayed gastric emptying after PPPD remains controversial.

The procedure may impair gastric peristalsis. The duodenal pacemaker, which is located 0.5 to 1 cm distally from the pylorus, should therefore be preserved to avoid disturbances in normal gastric peristalsis[20]. Gastric arrhythmias may be another cause of delayed gastric emptying in the early postoperative period, probably exacerbated by intra-abdominal complications[21]. Problems caused by the surgical procedure itself include injury to the nerves of Latarjat or the placement of suture material through the pyloric muscle. In addition, ischemia of the duodenal stump and antropyloric muscle mechanisms could influence gastric emptying, although mucosal edema at the site of anastomosis and peri-anastomotic fluid collection seem to be more common problems[22]. In regard to maintaining the pylorus as a functional unit, it is of paramount importance to maintain normal innervation of the antro-pylorus, an adequate blood supply and a pyloric muscle unimpeded by sutures[1,23].

Moreover, there has been no detailed description concerning clinico-anatomical and morphologic studies of the innervation of the antro-pyloric region in other literature, although there are some records of human pyloric innervation in literatures[24-27]. Therefore, we attempted to clarify the innervation of the antro-pyloric region in humans from a clinico-anatomical point of view, and to evaluate the innervation-preserving procedure of PPPD and its modified procedures, by using the peculiar method of labeling and dissecting the autonomic nerves of the viscera, not the current dissection of gross anatomy as in our previous studies[28-30]. Furthermore, the experimental animal, *Suncus murinus*, has been used for a comparative study to demonstrate general morphologic characteristics more similar to humans than other current laboratory animals, e.g., mouse, rat and rabbit[28-31].

MATERIALS AND METHODS

Cadavers

The study was performed on 10 cadavers (5 men and 5 women) with a mean age of 79.8 (range, 50 to 94) years. All cadavers were selected from bodies used for research and practice of anatomy at Kanazawa University School of Medicine during the years 1999-2000, and were free from diseases of the liver, stomach, duodenum, pancreas, and their surrounding areas (Table 1).

Animals and tissue preparation

Adult laboratory house musk shrews, *S. murinus*, were obtained and maintained from a closed breeding colony bred in our laboratories in the Department of Anatomy and Neuroembryology, Kanazawa University, Japan. The animals were housed and handled in accordance with the Guide for the Care and Use of Laboratory Animals and the Guide for the Care and Use of Experimental Animals of the Canadian Council on Animal Care. Briefly, all shrews were kept individually after weaning (20 d after birth) in plastic cages equipped with a wooden nestbox containing paper strips, and were kept in a conventionally conditioned animal room: 23 ℃ to 27 ℃, no humidity control, and 14 L: 10 D. Commercial trout pellets containing 45.0% protein, 3.5% fat, 3.0% fiber, 13.0% ash and 26.2% complex carbohydrate (Nippon Haigou Shiryou, Tokyo, Japan) and water were supplied *ad libitum*. The mother colony, JIc: CR, is maintained in the Central Institute for Experimental Animals, Kawasaki, Japan[28]. Adult animals (6 females and 4 males, weighing 45-80 g) were first anesthetized with ether and received an intraperitoneal injection of a solution of urethrane (sodium ethyl carbamate, 900 mg/kg). After each *S. murinus* was completely narcotized, the abdominal cavity was opened, and a catheter was inserted retrogradely into the abdominal aorta at the level immediately above the bifurcation of this artery into the common iliac arteries. Perfusion was commenced with normal saline containing heparin (10 KU/L), and thereafter with phosphate-buffered saline (PBS) containing 40 g/L paraformaldehyde. After perfusion, the animals were injected with neoprene latex to label the blood vessels in the pylorus region. Thereafter, the abdominal organs including the stomach, duodenum, common bile duct, and pancreas were extracted en bloc with the related nerves and vessels, postfixed with 40 g/L paraformaldehyde in PBS (pH 7.4) at 4 ℃ overnight to prepare for whole mount immunostaining.

Anatomical procedures of under-stereoscopic microscopy

The anatomical procedures of the cadavers were performed, according to our previous description[29,30], as follows. From the adult autopsy, the viscera of the upper abdomen (including the liver, pancreas, lower esophagus, stomach, and duodenum) were resected en masse with the abdominal aorta (region including the celiac artery and superior mesenteric artery), portal system, and nerves (including the vagus nerve, celiac ganglion, and plexus). The resected specimens were immersed in a 10 mg/L solution of alizarin red S (Wako, Osaka, Japan) in ethanol to melt the fat tissue and stain the peripheral nerves. The solution was changed 3 times every 2 to 3 d, in principle, but this process may be prolonged if necessary depending on the degree of progression of elimination of fat and staining. The area of each sample surrounded by the horizontal plane that passed through the portal region and the lower margin of the horizontal part of the duodenum and the sagittal plane that passed through the descending part of the duodenum and hilum of the spleen was dissected to the depth of the celiac plexus with the aid of a stereoscopic microscope (magnification, ×40), keeping the sample immersed completely in 100% ethanol. In dissection, the lymphatic vessels and lymph nodes were removed, and particular attention was paid to preserve not only the nerves but also the arteries and veins around the stomach including the hepatogastro mesenteriolum, hepatoduodenal ligament and lower esophagus. Figures 1 and 2 show two such dissections.

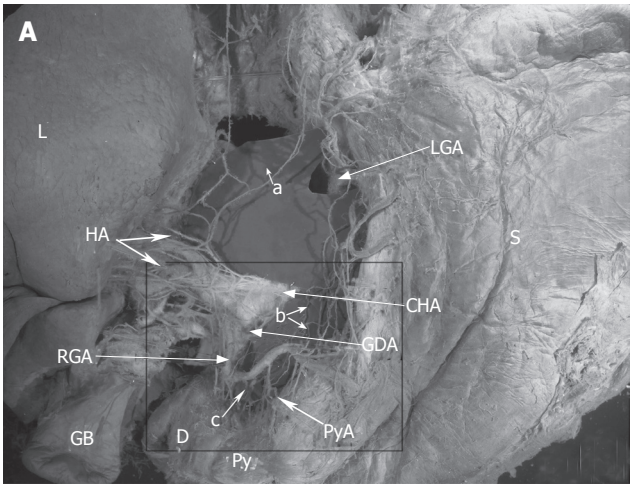
Whole mount immunohistochemistry

The whole mount immunostaining procedures for the *S. murinus* were performed as previously described[28,30]. Briefly, after rinsing in PBS, the fixed specimens were treated with 10 g/L periodic acid for 20 min to prevent

Table 1 Cadavers used in this study and pyloric innervation in the superior part

Case	Sex	Age	Death	Number[1]	Route I	Route II	Route III
A	F	94	Pneumonia	1	O		O
B	F	81	Cerebral hemorrhage	2	O	O	O
C	F	83	Myocardial infarction	1	O		O
D	M	87	Myocardial infarction	4	O	O	O
E	M	86	Pneumonia	4	O		O
F	M	73	Cerebral hemorrhage	3	O	O	O
G	M	87	Cerebral hemorrhage	4	O		O
H	M	75	Myocardial infarction	4	O		O
I	F	82	Subarachnoid hemorrhage	5	O	O	O
J	F	50	Pneumonia	3	O	O	O

[1]The number of hepatic divisions, a total of 46 hepatic divisions (average of 4.6). Route I, from the anterior hepatic plexus via the right gastric artery to the pyloric region; Route II, descending division from the hepatic division to the right gastric artery directly; Route III, from the nerves of Latarjat.



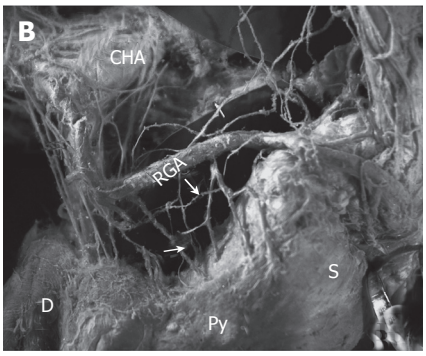


Figure 1 The innervation of the pyloric region from the view of the superior part of the pylorus, its schematic shows in Figure 3A. Hepatic divisions (a) join in the anterior hepatic plexus in the proper hepatic artery or the left/right hepatic artery (HA). The nerves ran along the right gastric artery (RGA) and its branches (PyA), and reached the pyloric region (Py) (c). The nerves (b) of Latarjet ran along the lesser curvature or the branches of the left gastric artery (LGA), intended for the antro-pyloric region. **B** is an enlargement of the box in **A**., the arrows show the nerves innervating the pyloric region from the right gastric artery. CHA, common hepatic artery; D, duodenum; E, esophagus; GB, gallbladder; GDA, gastroduodenal artery; L, liver; S, stomach.

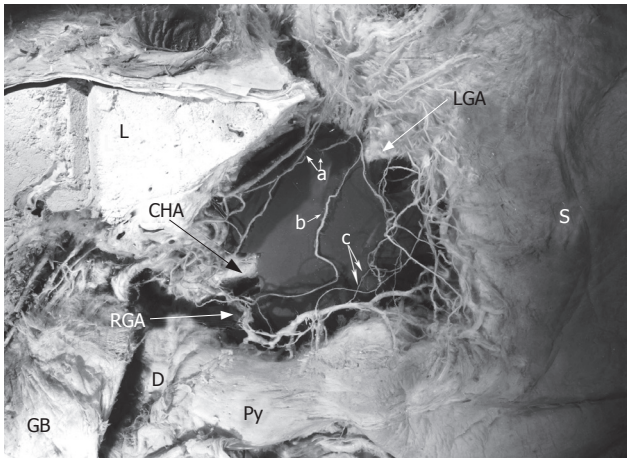


Figure 2 Another specimen, its schematic shows in Figure 3B, showing a descending branch (b) originating from the hepatic division of the anterior vagal trunk passing through the hepatogastric ligament, obliquely downward, reaching the right gastric artery (RGA), innervating the pyloric region (Py). a is similar to a in Figure 1A, indicating the hepatic divisions. c, indicating the branches for the pyloric region from the nerves of Latarjet. CHA, common hepatic artery; D, duodenum; GB, gallbladder; L, liver; LGA, left gastric artery; S, stomach.

any intrinsic peroxidase reaction. They were then incubated in freshly prepared 5 g/L papain (Sigma) in 0.025 mol/L Tris-HCl buffer (pH7.6) for 1 h and 25 g/L, 50 g/L, and 100 g/L sucrose in PBS for 30 min, respectively, followed by freezing and thawing thrice. The specimens were incubated with primary antibody (NFP-Ab) in PBS containing 2 g/L bovine serum albumin (BSA), 3 g/L Triton X-100, and 1 g/L sodium azide for 3 d at 4 ℃. After a thorough wash in PBS, the specimens were then incubated with secondary antibody labeled with peroxidase-conjugated affinity-purified sheep anti-mouse IgG (HRP) in PBS containing 2 g/L BSA and 3 g/L Triton X-100 for 3 d at 4 ℃. After a thorough wash in PBS, coloration was performed in 0.05 mol/L Tris-HCl buffer containing 20 mg/L 3,3'-diaminobenzidine (DAB) and 0.1 ml/L H_2O_2 for 1 to 3 d at 4 ℃. The stained preparations were then stored in glycerin to obtain transparency. The primary antibody was anti-neurofilament protein (NFP) antibody, a monoclonal mouse anti-all neurofilament consisting of three subunit proteins: NF-H (200 ku), NF-M (160 ku), and NF-L (70 ku) (M0762, lot 089, clone: 2F11, Dako).

RESULTS

In human

The innervation of the pyloric region in human involved three routes: via the right gastric artery, the nerves of Latarjet, and the infrapyloric artery.

Via the suprapyloric/supraduodenal branch of the right gastric artery

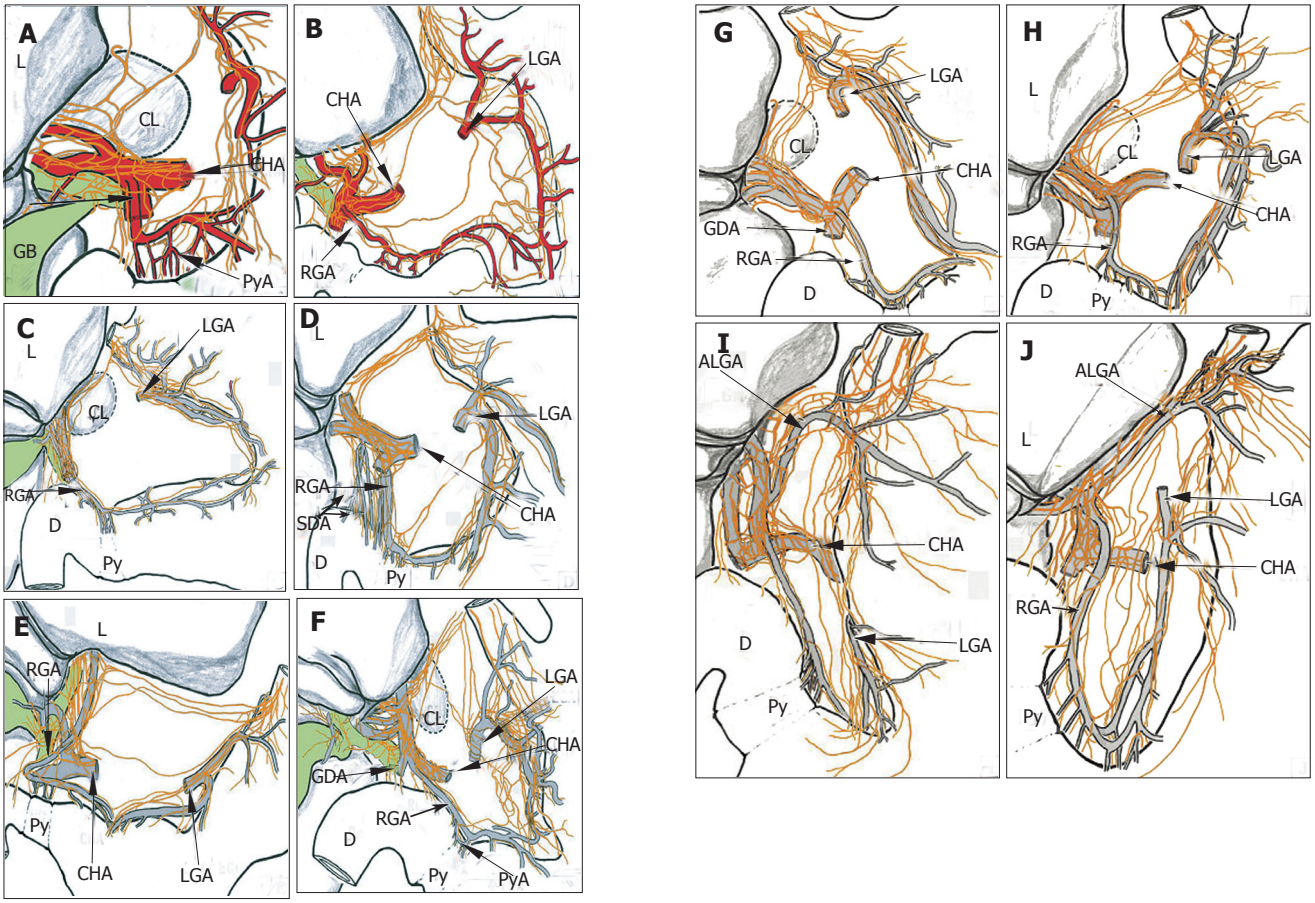


Figure 3 Diagram indicating the distribution in and around the cardia, the lesser curvature, the porta hepatica and the antro-pyloric region in 10 cadavers. Among them, **A** and **B** are diagrams of Figures 1A and 2, respectively. Five specimens, **B, D, F, I** and **J**, showed hepatic divisions joining directly to the right gastric artery, while, for the other specimens, after joining to the proper hepatic or hepatic artery, the nerves sent off some offshoots to the right gastric artery, and innervated the pyloric region. ALGA, accessory left gastric artery; CHA, common hepatic artery; CL, caudal liver; GB, gallbladder; L, liver; LGA, left gastric artery; Py, pylorus; PyA, pyloric artery; RGA, right gastric artery; SDA, supra-duodenal artery.

The hepatic division of the vagus, arising from the anterior vagal trunk, ran through the hepatogastric ligament near the edge of the liver (caudal liver), and joined the anterior hepatic plexus in the hepatoduodenal ligament. The plexus, containing the parasympathetic and sympathetic fibers (the latter originate from the celiac plexus), wound around the proper hepatic artery, then the suprapyloric or supraduodenal branch which comes from the right gastric artery, sent some branches to the pyloric region in the superior part of the pyloric regions (Figures 1, 2). This route was displayed in all ten specimens.

There was variation in the number of hepatic divisions. The total number of hepatic divisions was 46 (average, 4.6) in the 10 specimens in this study. The position where the hepatic division joined the anterior hepatic plexus occurred at different levels between the hepatic hilum and the root of the right gastric artery(Table 1, Figure 3). There were 2 cases of accessory left gastric artery in the 10 specimens. The hepatic division ran along the arteries to the hepatic hilum in these 2 cases(Figures 3I and 3J).

The branches, arising from the origin of the hepatic division, sent single or double descending branches of the hepatic division, ran in the lesser omentum, did not reach the proper hepatic artery or join the anterior hepatic plexus, joined directly to the right gastric artery, and then entered the pyloric region. Five cases of this route were observed in the 10 cadavers (Table 1, Figures 3B, 3D, 3F, 3I, and 3J).

Via the anterior and posterior gastric divisions

The anterior and posterior gastric divisions, namely the nerves of Latarjet, extended to and ran along the gastric lesser, while giving off some offshoots to the lesser curvature, sending some peripheral nerve fibers to the antro-pyloric region. The nerves of Latarjet passed through the lesser omentum, which lies 0.5 to 1.0 cm from the lesser curvature, or extended and anastomosed with the offshoots along the right gastric artery, or lay beneath the serosa of the gastric wall, entered the antro-pyloric region. The route was observed in all cases (Table 1, Figures 1, 2, and 3).

Via the infrapyloric artery or retroduodenal branches

There is always one or several infrapyloric arteries or/and retroduodenal branches, intended for the posterior-lower region of the pylorus. All of these arteries arise either from the gastroduodenal artery, or from one or the other of these two terminal branches, the right gastroepiploic

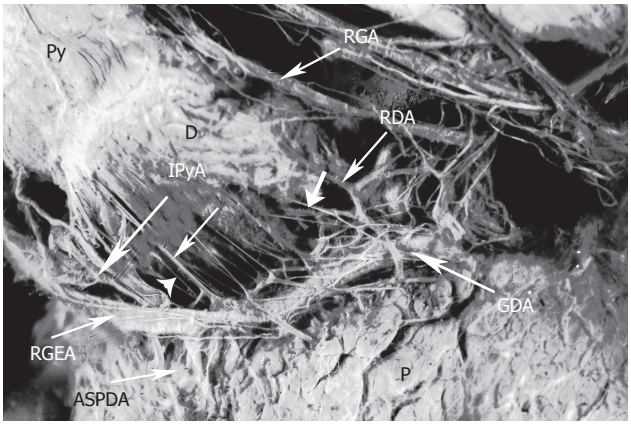


Figure 4 An example of innervation in the posterior part of the pylorus. The stomach was raised. The nerves originating from the right gastroepiploic artery (RGEA) or the gastroduodenal artery (GDA) running along the retroduodenal artery (RDA) (white arrow) or the infrapyloric artery (IPyA) (white arrowhead) reached the first duodenum posterior part and the pylorus of the posterior part.

artery, or the cranial and ventral pancreaticoduodenal artery.

This route with the arteries supplying the posterior-lower region of the pylorus, passed the nerve branches to the pyloric region. The nerve branches, arising from the anterior hepatic plexus, ran along the gastroduodenal artery, or the right gastroepiploic artery, to the infrapyloric artery or/and retroduodenal branches, reached the posterior-lower region of the pylorus. The route was observed in all cases (Figure 4).

In suncus murinus

Firstly, it is necessary to describe the morphological characters of the pyloric region in *S. murinus*. The pylorus closed the duodenal papilla, supplied by the branches of the gastroduodenal artery. The latter sent the branches to the duodenal papilla and the pyloric region. The arterial branch that supplied the pyloric region corresponded to the right gastric artery in humans. However, there existed no ramus anastomoticus between this artery and the left gastric arteries in *S. murinus* (Figure 5). There was the lesser omentum in this animal, and the right gastric artery sent anterior and posterior branches to the anterior and posterior of the pyloric region. Furthermore, there was no infrapyloric artery arising from the gastroduodenal artery in contrast to humans. Even though there are these differences, the innervation of the pyloric region in *S. murinus* was very similar to that in humans.

Via the branches of the right gastric artery

In *S murinus*, the nerves originating from hepatic plexus, ran along the gastroduodenal artery, the right gastric artery, and reached the pyloric region. This route was simple, and did not form the ramus anastomoticus with the anterior or posterior gastric branches, which originate from the vagal trunk or the left gastric artery. The innervation was observed in all cases (Figure 5).

Via the lesser omentum

In *S murinus*, the anterior and posterior gastric branches, corresponding to the Latarjet nerves in humans, while giving off some divisions to the lesser curvature and the gastric wall, sent some branches to the pyloric regions in all cases (Figure 5). The posterior-lower region of the pylorus in *S murinus* was innervated by nerve branches originating from the right gastric artery, in constrast to humans. The anterior and posterior gastric branches ran along the lesser omentum and close to the lesser curvature, or lay beneath the serosa of the gastric wall. Similarly, the route passing through the lesser omentum was also observed in some specimens, and these nerves originated from the anterior or posterior vagal trunk directly. The innervation was observed in all cases (Figure 5).

DISCUSSION

The present paper is concerned with a detailed observation of the innervation of the antro-pyloric region, with the objective of providing an anatomical basis for surgical operations in this region, especially concerning 'organ and function-preserving surgical procedures' like PPPD.

There are three routes of pyloric innervation in humans. One is the superior region of the pylorus, which is related to the hepatoduodenal ligament, whereby the nerve branches arose from the anterior hepatic plexus containing the branches coming from the hepatic division of vagus. The nerves ran along the right gastric artery, via the suprapyloric or supraduodenal branch, intended for the antro-pyloric region. The second route is the posterior-lower region of the pylorus, which is related to the gastro-pancreatic ligament. The nerves ran along the gastroduodenal or right gastroepiploic artery, to the infrapyloric artery, and reached the antro-pyloric region. The third route is the lower antrum region, which is related to the left gastric artery and the nerves of Latarjet. This route involves the branches of Latarjet nerves passing through the lesser curvature, and entering the antro-pyloric region. The animal model, *S murinus,* used in this study, allowed the complete anatomic observation of cadavers, exhibited the important arterial supply routes of the pylorus and anterior and posterior gastric divisions for the innervation of the pylorus.

The route of the superior region is the most important for pyloric innervation, in which the right gastric artery shows a core mission (main rule). The nerves of this route contain both sympathetic and parasympathetic fibers, originating from either the anterior hepatic plexus which arise from the celiac plexus, or the hepatic division of the anterior vagal trunk which joins the proper hepatic artery to the right gastric artery. The route was observed in all 10 cadavers. There were 4 cases of hepatic division distal to the hepatic edge, the division passing through the hepatogastric ligament, terminating in the right gastric artery in 10 cadavers. But only one case showed the pattern illustrated by McCrea[24], whereby a single descending branch of the hepatic division directly reached the pylorus. We agree with Skandalakis *et al*[27] that this pattern is typical but not universal. Namely, pyloric branches of the hepatic division do not, usually, directly reach the pyloric region, but pass via the arterial supply of the region, as the right hepatic artery and its branches.

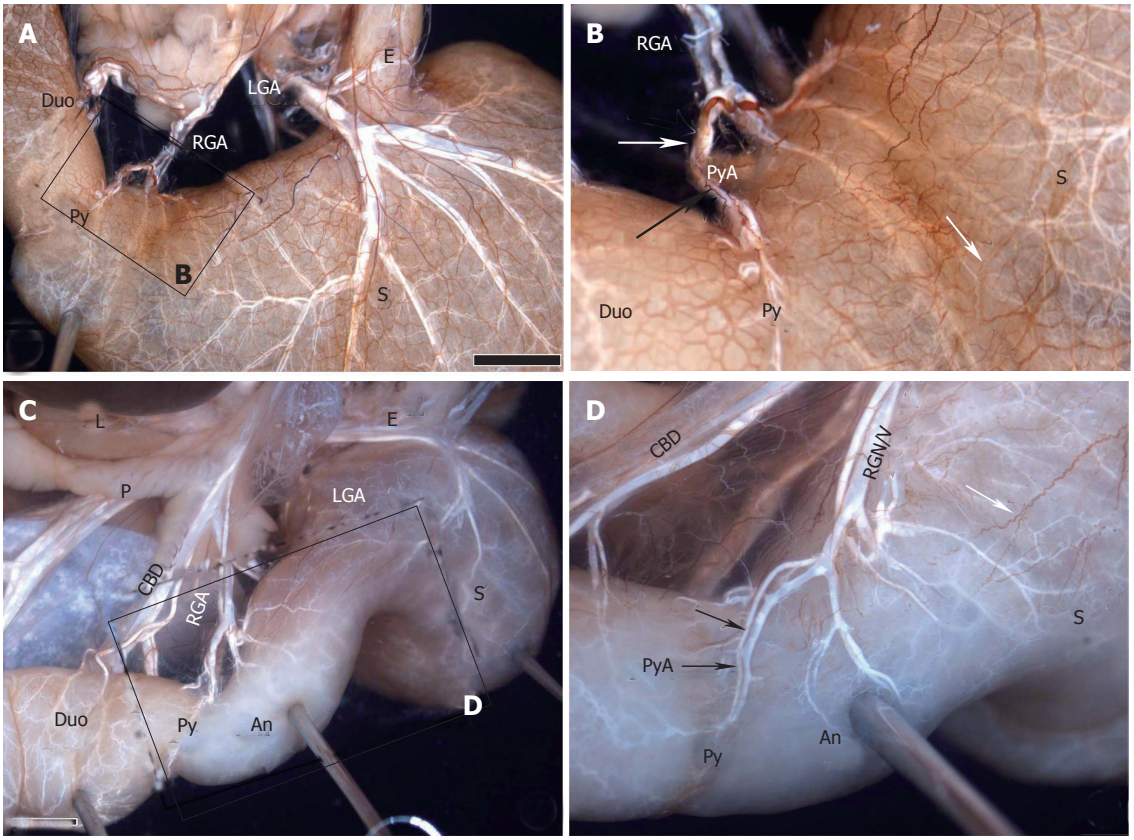


Figure 5 Two cases showing innervation of the pyloric region (Py) in *Suncus murinus* by whole-mount immunostaining. High magnifications of the boxed areas in **A** and **C** are shown in **B** and **D.** White arrows show the nerves of Latarjet intended for the antro-pyloric region. Black arrows indicate the nerves arising from the right gastric artery (RGA), running along the pyloric artery (PyA), and reaching the pyloric region. An, pyloric antrum; CBD, common bile duct; Duo, duodenum; E, esophagus; L, liver; LGA, left gastric artery; P, pancreas; RGA/V, right gastric artery/vein; S, stomach. Scale bar = 2 mm in A and B.

The right gastric artery arises from the hepatic artery and proceeds to the first portion of the duodenum (the supraduodenal artery), the pylorus and the antrum (the suprapyloric branch) along the lesser curvature. These routes via these arteries are important for pyloric innervation via the superior region of the pylorus. The preservation of the right gastric artery and its branches is potentially of great importance in providing earlier gastric emptying after operation. The drawback of pyloric preservation is a prolonged period of gastric suction. This time can be shortened by attention to the preservation of the supraduodenal artery and vagal innervation of the antrum, which is essential for gastric emptying[7]. In any event, as there was a reliable route of the right gastric artery, it is not necessary to preserve the hepatogastric ligament in order to preserve the route in clinic practice. In principle, it is only important to preserve the right gastric artery in order to preserve the innervation and function of the pylorus in PPPD.

For the route of the nerves of Latarjet, innervation originating from the anterior and posterior gastric branches of the nerves of Latarjet passed through the lesser omentum close to the lesser curvature, or extended to and anastomosed with the offshoots along the right gastric artery, or lay beneath the serosa of the gastric wall, and entered the antro-pyloric region. Skandalakis *et al* [26] reported that the nerves of Latarjet could be traced distally to about the level of the incisura in most specimens, but in many cases it reached the pylorus, and in six cases it was visible as far as the first part of the duodenum. It is possible that the nerves of Latarjet innervate the antro-pyloric region according to our observations in humans and *S. murinus*. Great care must be taken to preserve the blood supply from the left gastric artery to the lesser curvature of the stomach and pylorus in PPPD.

For the innervation of the posterior-lower region of the pylorus, it was disregard ordinarily. The route is via the arterial supply of the posterior-lower region of the pylorus. The branches are sent off by the gastroduodenal or the right gastroepiploic artery, termed by the infrapylorus branch or artery, or the retroduodenal branches. These branches come from the gastroduodenal artery in 30% of cases, from the right gastroepiploic artery in 44% of cases or from the ventral pancreaticoduodenal arch in 20% of cases[32]. The gastroduodenal artery should probably be severed distally to its first branch and should be preserved in cases of chronic pancreatitis where dissection could proceed by taking the pancreaticoduodenal artery at its origin from the gastroduodenal artery. Equal attention must also be given during the procedure to preserving innervations to the antrum and pylorus[9]. The gastroepiploic artery, with its large ascending pyloric branch, should probably be taken close to its origin from the gastroduodenal artery[9]. The origin of the innervation in

this route is similar to that of the superior region, coming from the anterior hepatic plexus. Hence, the innervation also contained both the sympathetic and parasympathetic fibers. If the right gastric artery is damaged, the route is able to compensate by its function in innervation. Grace *et al*[33] emphasized that an intact neurovascular supply to the pylorus and the first part of the duodenum is essential for the success of the pylorus preserving operation. By not ligating the gastroduodenal artery and right gastric artery at their origins and not freeing the tissues along the lesser curvature of the antrum and the gastrohepatic ligament-all these tissues remaining intact-the surgeon cannot rotate the proximal duodenum and antrum anteriorly and to the patient's left in PPPD. Thereby, it is advantageous to preserve all blood supply and innervation to antrum, pylorus, and proximal duodenum[16]. In fact, innervation of the antro-pyloric region takes several routes. When dissecting the lymph nodes in the hepatoduodenal ligament, the nerve branches originating from the anterior hepatic plexus to the right gastric artery could be damaged, however, as there are other routes of the posterior-lower region of the pylorus and the nerves of Latarjet, their influence on pyloric function may often be unclear.

In conclusion, we demonstrated the detailed description of the innervation of the antro-pyloric region from clinico-anatomical and morphologic perspectives in this study. The useful methods of whole mount immunostaining with a peripheral neuron marker for *S murinus*, and the alizarin red S staining technique for humans are effective for peripheral nerve labeling, as shown in our studies. There are three routes of pyloric innervation in humans, wherein the route of the right gastric artery is the most important for preserving pyloric region innervation. The function is preserved by more than 80% by preserving the artery in PPPD. However, the route of the infrapyloric artery should not be disregarded. This route is related to several arteries (the right gastroepiploic and gastroduodenal arteries), and the preservation of these arteries is advantageous for preserving pyloric innervation in PPPD. Concurrently, the nerves of Latarjet also perform an important role in maintaining innervation of the antro-pyloric region in PPPD. This is why the pyloric function is not damaged in some patients if the right gastric artery is dissected or damaged in PPPD.

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CLINICAL RESEARCH

Estimating liver weight of adults by body weight and gender

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Abstract

AIM: To estimate the standard liver weight for assessing adequacies of graft size in live donor liver transplantation and remnant liver in major hepatectomy for cancer.

METHODS: In this study, anthropometric data of body weight and body height were tested for a correlation with liver weight in 159 live liver donors who underwent donor right hepatectomy including the middle hepatic vein. Liver weights were calculated from the right lobe graft weight obtained at the back table, divided by the proportion of the right lobe on the computed tomography.

RESULTS: The subjects, all Chinese, had a mean age of 35.8 ± 10.5 years, and a female to male ratio of 118:41. The mean volume of the right lobe was 710.14 ± 131.46 mL and occupied 64.55% ± 4.47% of the whole liver on computed tomography. Right lobe weighed 598.90 ± 117.39 g and the estimated liver weight was 927.54 ± 168.78 g. When body weight and body height were subjected to multiple stepwise linear regression analysis, body height was found to be insignificant. Females of the same body weight had a slightly lower liver weight. A formula based on body weight and gender was derived: Estimated standard liver weight (g) = 218 + BW (kg) x 12.3 + gender x 51 (R^2 = 0.48) (female = 0, male = 1). Based on the anthropometric data of these 159 subjects, liver weights were calculated using previously published formulae derived from studies on Caucasian, Japanese, Korean, and Chinese. All formulae overestimated liver weights compared to this formula. The Japanese formula overestimated the estimated standard liver weight (ESLW) for adults less than 60 kg.

CONCLUSION: A formula applicable to Chinese males and females is available. A formula for individual races appears necessary.

Key words: Estimated standard liver weight; Liver tranplantation

Chan SC, Liu CL, Lo CM, Lam BK, Lee EW, Wong Y, Fan ST. Estimating liver weight of adults by body weight and gender. *World J Gastroenterol* 2006; 12(14): 2217-2222

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INTRODUCTION

Small-for-size graft is a common problem in live donor liver transplantation[1]. In major hepatectomy, a small remnant liver is a key factor attributing to hospital mortality[2], even in the non-cirrhotic liver[3]. Size of the partial liver graft or the remnant liver is often expressed as a percentage of the estimated standard liver weight (ESLW) of the patient. Very often, the native liver of a transplant recipient is small and cirrhotic, and that of a patient undergoing major hepatectomy houses a large tumor. The size of the patient's liver, therefore, has little bearing on the preoperative assessment. Under both circumstances, reference to ESLW has a clinical relevance. The Shinshu group of Japan deduced a formula by drawing a relationship between the estimated standard liver volume (ESLV) and body surface area (BSA) from 96 patients who underwent abdominal computed tomography (CT), yet without liver diseases: ESLV (mL) = 706.2 × BSA (m^2) + 2.4[4]. BSA was derived from the clinical parameters of body weight (BW) and body height (BH) as described by DuBois and DuBois[5]. In view of the low mean age (11.1 years) of the patients in the study, the Shinshu group subsequently validated the formula by the same methodology in an independent sample of 96 adult live liver donors[6]. Nonetheless, this formula when applied to Caucasians underestimates the liver volume from studies based on autopsy data[7] and CT of patients without liver diseases[8].

Underestimation of liver volume was even found in another group of Asian population, the Koreans. In this Korean group the increase in liver volume in relation to BSA showed a nonlinear relationship. A piecewise linear model and a nonlinear model have thus been developed

in Korea[9]. In another study, a sample of 33 Chinese in-patients admitted for abdominal ailments apart from liver diseases were evaluated by CT. The main purpose of the study was to use them as control in comparison with cirrhotic livers and not intended for a broader application of liver size estimation[10]. As early as the sixties, it was pointed out that females of the same body size as males have a smaller liver[11]. A study of a wider scale recently has affirmed this concept[12]. Our study was to clarify the above issues by analyzing the right lobe graft weight which was measured directly on the back-table during donor right hepatectomy in healthy live liver donors. The weight of the whole liver was calculated by the measurements of the liver volumes from CT.

MATERIALS AND METHODS

Study design

From May 10, 1996 to October 25, 2004, 182 consecutive right lobe live donor liver transplants were performed at the University of Hong Kong, Queen Mary Hospital. These 182 live liver donors being evaluated and operated were healthy with normal liver biochemistry. None of them was hepatitis B or C carriers, or had the habit of alcohol consumption. Donor anthropometric data including age, gender, BW (measured to the nearest 0.5 kg), and BH (measured to the nearest 1 cm) were recorded prospectively and entered into a liver transplantation database by 2 designated liver transplant research assistants. BSA was calculated by the formula by DuBois and DuBois: BSA (m^2) = BW (kg)$^{0.425}$ × BH (cm)$^{0.725}$ × 0.007184[5]. Body mass index = BW/BH × BH. CT of the donors was performed before the donor operation. Estimation of the liver volumes on CT was done by 3 dedicated radiologists using the Heymsfield method[13]. They had no information of the body size of the live liver donors or the liver transplant recipients. All subjects underwent single slice spiral CT (HiSpeed Advantage System; General Electric Health Care, Milwaukee, WI) and multi-slice CT study (LightSpeed QX/i 4-MDCT or LightSpeed 16-MDCT, General Electric Health Care, Milwaukee, WI) from 2000. Cuts were made at 5 to 7.5 mm intervals and continuously. Demarcation of the right and left portions of the liver was made by tracing along the middle hepatic vein, corresponding to the Cantlie's line. Volumes of the right lobe plus right caudate lobe (segment 1r) and the left lobe plus left caudate lobe (segment 1l)[14,15], were measured independently. All but 1 donor underwent donor right hepatectomy including the middle hepatic vein. This donor's right lobe graft did not include the middle hepatic vein for anatomical reason (case no. 85). The donor was a non-Chinese, and was therefore excluded. Details of the donor right hepatectomy have been described elsewhere[16]. In short, the transection line was determined by temporary inflow control. Transection by Cavitron ultrasonic surgical aspirator (CUSA; Valleylab, Boulder, CO), was just onto the left of the middle hepatic vein, including the latter into the right lobe liver graft. The right lobe liver grafts were perfused with University of Wisconsin Solution (NPBI, Emmer-Compascuum, the Netherlands), and from case no. 110 of the series, with histidine-tryptophan-ketoglutarate solution (Dr. Franz Köhler, Chemie GmbH, Alsbach-Hähnlein, Germany). The gallbladder was removed in the early phase of the hepatectomy during hilar dissection. No parts of the inferior vena cava, triangular ligament, or coronary ligament were included. The right lobe liver grafts were then weighed at the back-table. The weight of the whole donor liver was calculated by the right lobe graft weight (GW) divided by the volume fraction of the right lobe in relation to the entire liver as measured on the CT.

Exclusion criteria

We excluded 23 donors over this entire period of 8 years from this study. These included fatty change of the liver over 10% as documented by biopsy of the liver graft intraoperatively (n = 9). Non-Chinese donors (n = 6) were also excluded. The above 2 conditions occurred in 1 of the donors (n = 1). Donors with missing data of height were also excluded (n = 4). Donors with BW (n = 2) or BH (n = 1) lying beyond 97.5% were excluded. The number of subjects for analysis thus became 159.

Statistical analysis

Following testing for normal distribution (Kurtosis and Skewness tests), data were expressed as mean ± SD. Simple linear regression analysis by the least-squares fit method was used to plot the relation between calculated liver weight against BSA. This was then done with BW, and also BH as independent variables. Male and female subjects were analyzed separately. By stepwise multiple linear regression analysis, the correlation between calculated liver weight as a dependent variable and with BW and BH as independent variables, and gender as a binary factor, was tested. Goodness of fit of the formula was tested by analysis of residuals. Formulae deduced from other studies and this study were used to calculate the ESLW using BW, BH, BSA, and gender of these 159 subjects as appropriate[4,7-9,11,12,17,18]. ESLV derived from the respective formulae[4,7-10,18] was converted to ESLW by a factor of 1.19 mL/g as derived from analysis of data from this study. This was obtained by plotting liver volume on CT against calculated liver weight of these 159 subjects. The ESLW so derived was compared with the calculated liver weight of this series by 2-sided paired-samples t test. $P < 0.05$ was considered statistically significant. Regression lines of representative series were drawn and compared with the regression line of this study[4,8,9,12]. All statistical analyses were performed by SPSS Version11.0 program (SPSS, Chicago, IL).

RESULTS

Baseline characteristics

Characteristics of the 159 subjects and their livers are listed in Table 1. They were young. Females outnumbered males by 2 fold. Such a female preponderance was attributed to a higher proportion of male recipients (n=118), and their wives volunteered as the donor (n=58). Donors who were healthy had normal built as reflected from the BMI.

Liver weights and volumes

From the measurements made on the preoperative CT,

Table 1 Characteristics of subjects (mean ± SD, *n* = 159)

Age (yr)	**35.8 ± 10.5 (18-57)**
Gender (M : F)	53 : 106
Body weight (kg)	56.3 ± 8.4 (41.0-78.5)
Body height (cm)	161.7 ± 7.5 (144.5-181.3)
Body mass index (kg/m^2)	21.5 ± 2.6 (16.5-29.1)
Body surface area (m^2)	1.59 ± 0.14 (1.30-2.13)
Computed tomography liver volume (mL)	1,099.10 ± 181.51
Right lobe graft volume on CT (mL)	710.14 ± 131.46
Right lobe to total liver volume on CT (%)	64.55 ± 4.47 %
Right lobe graft weight (g)	598.90 ± 117.39
Estimated whole liver weight (g)	927.54 ± 168.78

CT: computed tomography.

the mean volume of the right lobe was 710.14 ± 131.46 mL and the whole liver was 1099.10 ± 181.51 mL. On the back-table, the right lobe grafts after being perfused with preservation solution, weighed 598.90 ± 117.39 g. The right lobe accounted for an average of 64.55% ± 4.47% of the entire liver on the CT. The total liver weight calculated was 927.54 ± 168.78 g.

Regression models

When the calculated liver weight was plotted against BSA for males ($R^2 = 0.37$) and females ($R^2 = 0.26$), the linear relationships were distinctly different (Figure 1A). Those females with the same BSA as males had a lower calculated liver weight. When BW was used instead of BSA, a similar pattern occurred for males ($R^2 = 0.38$) and females ($R^2 = 0.34$) (Figure 1B). The correlation of calculated liver weight with BH was much weaker for males ($R^2 = 0.13$) and in particular for females ($R^2 = 0.02$) (Figure 1C).

By stepwise multiple linear regression analysis, the relation of calculated liver weight with BW, BH, and gender (male = 1, female = 0) was tested. BH was excluded by the collinearity statistics of tolerance from the following formula so derived:

ESLW (g) = 218.32 + BW (kg) × 12.29 + gender × 50.74 (R^2 = 0.48)

or more conveniently:

ESLW (g) = 218 + BW (kg) × 12.3 + gender × 51

The ESLW is predictably correlated with ESLV. Based on linear regression analysis, the relation between ESLW and ESLV is as follows: ESLV (mL) = 302.34 + ESLW (g) × 0.859 (R^2 = 0.64), ESLW (g) = 111.25 + ESLV (mL) × 0.743 ($R^2 = 0.64$). For simplicity, the conversion factor is 1.19 mL/g (Figure 1D).

Comparison of different formulae to estimated liver size

Using the formulae for ESLV and ESLW from other studies[4,7-9,11,12,17,18], and the anthropometric data of the 159 subjects of this study, the ESLV and ESLW were calculated. The ESLW was converted from ESLV by factor of 1.19 mL/g. The ESLWs calculated from each formula were compared with the calculated liver weight of these 159 subjects by two-sided paired-samples *t* test. All except that calculated from the Urata formula ($P = 0.098$) were found to deduce a higher ESLW, with statistically significant differences ($P < 0.000$, Table 2). Amongst these, 4 regression models were selected for comparison using the regression line of best-fit method. In one German study using autopsy data, the ESLW was markedly higher[12], whereas the other series had fairly close ESLWs[4,8,9]. The regression line of the model from Urata correlated nicely in subjects of the middle range of body weights. Nonetheless, divergence was seen in subjects with body weight less than 60 kg, resulting in a higher ESLW.

DISCUSSION

The results of this study demonstrate that in healthy Chinese adults, ESLW was positively related to BW, and such correlation was also gender-dependent. The liver of the male was slightly heavier than that of the female of the same body weight. Furthermore, BH was found to be not required in the estimation of the standard liver weight. Statistically, by the test of collinearity for tolerance, BH was eliminated as an independent factor. Though many studies have used BSA as the independent factor for liver size estimation, in which BH is a key component in calculating BSA, our findings here corroborate with those studies which evaluated BW, BH , and BSA independently[8,12]. This is also in concordance with the use of only body weight as reference in determining the adequacy of the size of the liver graft from studies of North America[19] and Europe[20]. In such convention, the graft weight to recipient body weight ratio is expressed as graft recipient weight ratio (GRWR). However, the intercept of the regression line in this study did not meet at zero, using body weight as denominator as in GRWR, accuracy may be compromised in subjects who deviate much from the mean body weight of the population.

The DuBois formula for calculating BSA[5] has been used for estimating liver size in 4 formulae[4,7,11,18]. The deficiency of the BSA formula is that it is published in 1916 and deduced from planometric measurements of the body castings made on 9 subjects spanning a wide age range[5]. Larger series of BSA estimation by geometric methods revealed that the DuBois equation can predict lower BSA values[21]. The Mosteller equation which illustrates that BSA (m^2) = square root BH (cm) × BW (kg) /3600[22] has been used in 2 series for the sake of easier calculation[8,17]. On the whole, convenience of using BSA is in using the simple formula from linear regression. The easy access to personal computer nowadays may make this point relatively invalid. Using BW and BH from which BSA is derived also evades all possibilities of using data not applicable to the local population from which BSA formulae are deduced. One study showed that liver volume had a curvilinear relation with BSA[9]. The formula for calculating BSA itself has a curvilinear nature with BW and BH. Thus, a pure relation with BW and BH should rather be addressed.

The gender factor in relation to liver weight is alluded to as early as in the sixties[11]. This has been recently validated by another study which included gender in the formula deduced in estimating the ESLW[12]. Such phenomenon of gender difference can be anticipated as the female has a smaller fat-free mass given the same BW and BH[23-25]. A slightly small liver is therefore required to

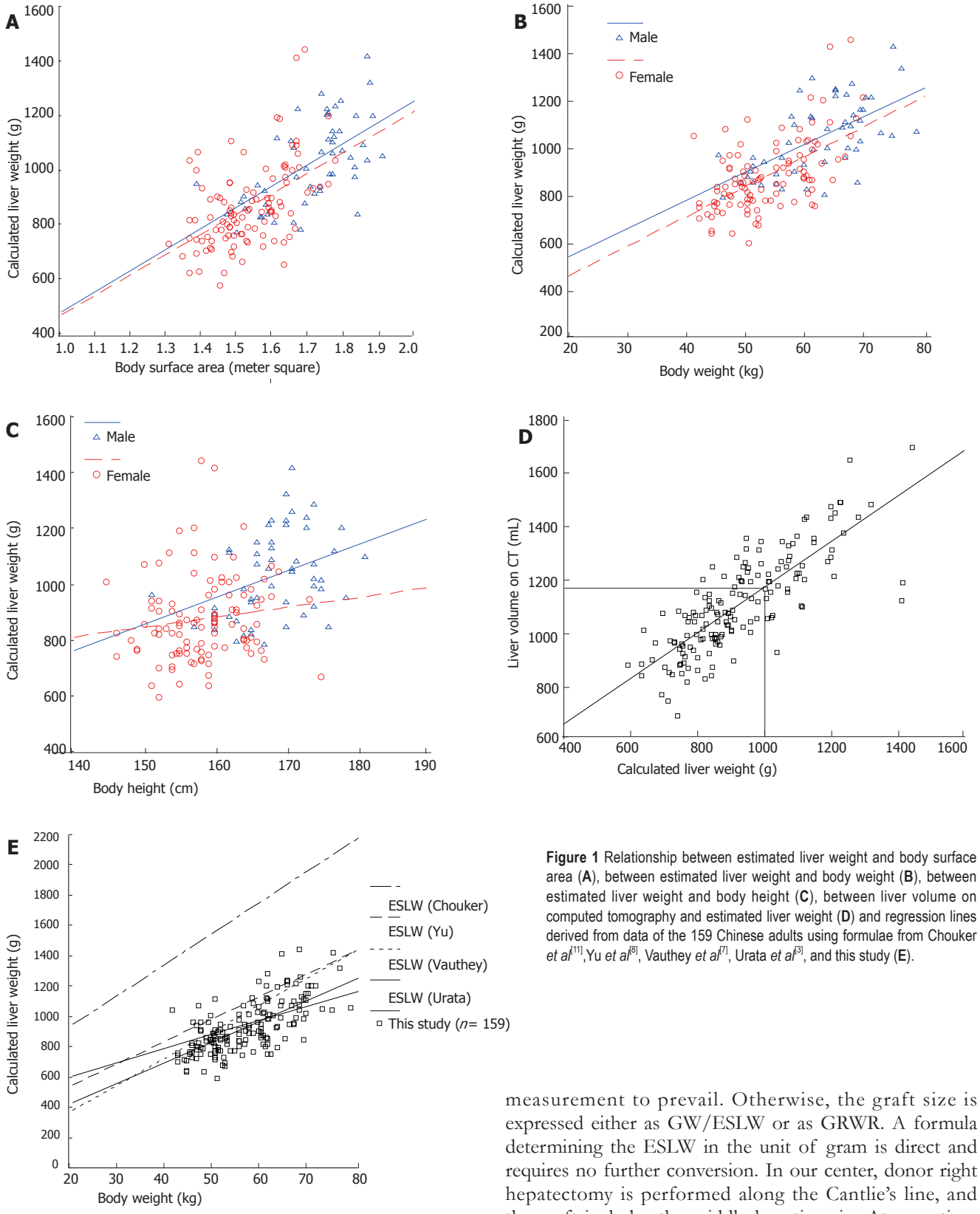

Figure 1 Relationship between estimated liver weight and body surface area (**A**), between estimated liver weight and body weight (**B**), between estimated liver weight and body height (**C**), between liver volume on computed tomography and estimated liver weight (**D**) and regression lines derived from data of the 159 Chinese adults using formulae from Chouker *et al*[11],Yu *et al*[8], Vauthey *et al*[7], Urata *et al*[3], and this study (**E**).

meet her metabolic need. The gender factor is also age-dependent, and becomes less important as the subjects become older[12]. This helps to explain why the gender factor was significant in our series in which the mean age of the subjects was 35.8 years.

Weight in gram is the common unit used in liver transplantation for quantifying the size of the liver graft. Handiness at the back-table enables this unit of measurement to prevail. Otherwise, the graft size is expressed either as GW/ESLW or as GRWR. A formula determining the ESLW in the unit of gram is direct and requires no further conversion. In our center, donor right hepatectomy is performed along the Cantlie's line, and the graft includes the middle hepatic vein. At operation, the line of demarcation between the right and left lobes is determined by temporary inflow control and marked by diathermy. It is our experience that the middle hepatic vein is predictably encountered during the course of liver transection employing the CUSA from the demarcation line to the mid portion of the inferior vena cava. Intraoperative ultrasonography certainly helps to define this plane and navigates the liver transection[26]. Weighing of the right lobe graft obviates the reliance on volume

Table 2 Comparison of regression models

Study	Formula	Mean ESLW (ESLV)	Difference in ESLW	*P*
Deland and North[11]	ESLW = 1020 × BSA^1 – 220	1400.41 g	- 472.86 g	0.000[a]
Heinemann[7]	ESLV = 1072.8 × BSA^1 - 345.7	1141.67 g (1358.59 mL)	- 214.12 g	0.000[a]
Yoshizumi[17]	ESLW = 772 × BSA^2	1225.50 g	- 297.95 g	0.000[a]
Yu[9]	ESLV = 21.585× $BW(kg)^{0.732}$ × $BH(cm)^{0.225}$	1087.32 g (1293.91 mL)	- 159.77 g	0.000[a]
Choukér[12]	ESLW = 452 + 16.434 × BW(kg) + 11.85 × age – 166 × gender (F=1, M=0)	1690.90 g	- 763.35 g	0.000[a]
Urata[4]	ESLV = 706.2 × BSA^1 + 2.4	944.79 g (1124.30 mL)	- 17.24 g	0.098
Vauthey[8]	ESLV = 1267.28 × BSA^2 -794.41	1022.95 g (1217.30 mL)	- 95.40 g	0.000[a]
Lee[18]	ESLV = 691 × BSA^1 + 95	1002.31 g (1192.75 mL)	- 74.76 g	0.000[a]
Lin[10]	ESLV = BH × 13 + BW × 12 - 1530	1048.85 g (1248.13 mL)	- 121.30 g	0.000[a]
This study	ESLW = 218.32 + BW × 12.29 + gender × 50.74 (M=1, F=0)	927.47 g	7.60E-2	0.994
Calculated liver weight		927.54 g		
Conversion factor 1.19 mL/g			paired samples *t* test	[a]$P < 0.05$

ESLV: estimated standard liver volume; ESLW: estimated standard liver weight; BSA: body surface area; BW: body weight; BH: body height; F, female; M, male.
[1]DuBois and DuBois[5]
[2]Mosteller[22]

determination of the liver by CT. Instead, it is the volume ratio of the right versus the left lobe that calculates the weight of the entire liver.

There is no perfect way of measuring the liver volume as this cannot be done with the organ *in-situ* in a healthy human subject. Many attempts have been made to get closest to the actual liver volume. ESLV can be obtained from measurement of the cadaveric internal organs. By definition, these measurements are acquired from non-healthy subjects with age bias, and influenced by sequelae of either the major illness leading to the demise, or the treatments like fluid resuscitation. Autopsy data excluding those with severe postmortem changes, extensive burns, blood transfusion, fluid infusion, and injury and pathological changes of the organ can minimize such errors[27]. By the principle of Archimedes, the liver volume is measured. An average tissue density of 1.04 kg/L[9] to 1.08 kg/L[7] is derived from correlation with the liver weight. Heinemann *et al*[7] have not pointed out the findings of the increase in liver volume in those with longer periods of survival before death. Furthermore, a gradual increase in liver volume has also been observed during the time interval between death and postmortem[7]. Deceased donor livers might be weighed with deduction of 2.3% for the weight of the gallbladder. The time from brain death to organ harvesting is usually short. Nonetheless, changes of volume status as a result of diabetes insipidus and fluid resuscitation, which are common in donors with intracerebral pathologies and perfusion with preservation solution, can still affect the liver size.

The accuracy of CT in assessing the liver volume has been evaluated. Inflow and outflow vessels of the liver from cadavers can be retrieved and dissected free of fascia and fat. They are weighed and underwent CT scan *ex-vivo*. A high correlation between the actual liver volume and the volume assessed by CT has been identified by Heymsfield *et al*[13]. Urata *et al*[4], reported that 19 children with end-stage liver disease undergoing liver transplantation also have their liver volume assessed preoperatively by CT. The liver volume obtained from CT is accurate. Another study reported the livers of fresh sheep can be scanned *in-vitro* and the volume can be measured by water displacement method[28]. All these 3 studies showed that the error of the CT is less than 5%. Nevertheless, it is worth noting that these are measurements of organs excised from deceased or diseased subjects. Misregistration errors made in tracing the peripheries of the liver on CT occur particularly in the thin portions such as the left lateral segment. Partial volume effect and respiratory movements are also possible sources of error. Overestimation for small livers and underestimation for large livers have also been observed[28]. Spiral CT which requires a shorter breath-holding period theoretically should minimize artifacts from breathing movements. This has, however, shown to be of no significance[29].

A potential major source of error in this study might come from the measurement of BW and BH in the ward. As donors were weighed wearing clothes, BW could be expected to have a systematic error of overestimation due to the weight of the clothes. As the BW and BH were measured in different wards over a long period, potential systematic errors from the equipments could be expected to be random instead of systematic. With continuous data collection, accuracy of the formula could be improved by more meticulous recording of BW and BH.

In comparison with ESLW/ESLV derived from other formulae, except that from Urata, the ESLW of the subjects in the current study was significantly smaller. Though statistically not significant, the Urata formula did overestimate liver weight in our adults less than 60 kg. The discrepancy between our formula and the others might be attributed to the fact that our liver grafts were weighed blood-free on the back-table. The liver already flushed with perfusion solution and devoid of back-perfusion via hepatic vein as *in-situ* is smaller and lighter. This nonetheless is the state at which the liver graft harvested is weighed. The conversion factor of 1.19 mL/g could be used for converstion of weight to volume. It is also possible that a smaller liver may be required to meet the metabolic need of the adult Chinese. The racial difference in the amount of visceral adipose tissue has been demonstrated by whole-body magnetic resonance imaging.

Asian Americans have a higher amount of visceral adipose tissue, thus less lean mass for the same body size[30]. A word of caution ought to be made in the application of data obtained from healthy and relatively young subjects to patients with various morbidities like wasting, edema, ascites, and perhaps to those of an older age.

In conclusion, these data demonstrate that ESLW can be derived from simple clinical parameters of weight and gender. The formulae for Caucasians may not be applicable to Chinese adults. Additional studies of more age- and weight-diverse subjects can provide additional information to this subject of interest.

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CLINICAL RESEARCH

Gastrointestinal stromal tumors in a cohort of Chinese patients in Hong Kong

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Abstract

AIM: To investigate the prevalence and clinical pattern of gastrointestinal stromal tumors (GISTs) in Hong Kong Chinese, and to assess the impact of introduction of CD117 on the disease incidence.

METHODS: From the database of the Department of Pathology of Yan Chai Hospital, 47 patients, with GISTs from September 1995 to December 2003 were included in this study. Ten GISTs were diagnosed before the introduction of CD117. The clinical features, tumor characteristics, and treatment were analyzed. Factors predicting tumor related death or recurrence were studied with Cox proportional hazard model.

RESULTS: The patients included 26 males and 21 females, with a mean age of 66.6 years (SD 13.1, range 29-87 years). The estimated prevalence of GISTs was 13.4-15.6 per 100 000 people, with an annual incidence of 1.68-1.96 per 100 000 people. The annual incidence of GISTs before and after the introduction of CD117 was 1.1 per 100 000 people and 2.1 per 100 000 people respectively. Stomach (34 patients, 72.3%) was the most common location for the tumor, followed by the small intestine (8 patients, 17.0%), esophagus (2 patients, 4.3%), omentum (2 patients, 4.3%) and colon (1 patient, 2.1%). Thirty-one patients (66%) had complete tumor resection. Eleven out of 16 deaths (23%) were tumor-related. The median survival time was 26 mo. Five-year survival rate was 61.3%. The significant factors associated with tumor-related death or recurrence were incomplete resection, tumor size 5 cm or above, invasion to the adjacent organ or presence of metastasis.

CONCLUSION: The incidence of GIST in Hong Kong is comparable to that in the United States but lower than that in Finland. The true incidence of GISTs could be underestimated before the introduction of CD117. Incomplete resection, tumor size 5 cm or above, invasion to the adjacent organ or presence of metastasis are factors predicting tumor-related death or recurrence.

Key words: Gastrointestinal tumor; GIST; Prognostic factors; Clinical features; Incidence

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are uncommon mesenchymal tumors that have been a controversial topic since their first description by Golden and Stoot in 1941[1]. In the past three decades, there has been considerable debate regarding its nomenclature, cellular origin, diagnosis, and prognosis[2]. Before the discovery of gain-of-function mutations in the *c-KIT* proto-oncogene in GISTs by Hirota and colleagues in 1998[3], most GISTs were thought to be smooth muscle neoplasm, and were classified as leiomyosarcoma due to their similar appearance by light microscopy. At present, GISTs are defined as spindle-cell, epithelioid, or occasionally pleiomorphic mesenchymal tumours of the gastrointestinal tract that express the *c-KIT* protein[4]. The precise cellular origin of GISTs recently has been proposed to be the interstitial cell of Cajal, an intestinal pacemaker cell[5]. The definition of *c-KIT*-negative GISTs remains a focus of research. In this study, we analyzed 47 Chinese patients with GISTs in Yan Chai Hospital to study their clinical, pathological characteristics, survival pattern and recurrence. The impact of introduction of CD117 on the incidence of this tumor was assessed, as well.

MATERIALS AND METHODS

Patient selection

From the database of the Department of Pathology of Yan Chai Hospital, we selected patients with the diagnoses of gastrointestinal stromal tumors (GISTs), leiomyoma, leiomyoblastoma and leiomyosarcoma from September 1995 to December 2003. Their histology slides were reviewed by a separate pathologist of the Department of Pathology. Patients with the diagnosis of GISTs were recruited to our study.

Clinical information including age, sex, comorbidity of the patients and presentation status of the tumor were recorded. The presentation status of the tumor reflected the extent of disease and the history of prior treatment when the patient was first seen in our hospital. The tumor was categorized as primary, metastatic or locally recurrent. Acute gastrointestinal haemorrhage was defined as significant blood loss requiring transfusion or haemodynamically significant blood loss. The criteria for chronic gastrointestinal haemorrhage were intermittent melena, guaiac-positive stool or iron deficiency anaemia. Ranges of diagnostic tests were used in these patients. They involved evaluating the gastrointestinal tract with contrast such as barium studies, endoscopies or ultrasound of abdomen. Computerized tomography scan and mesenteric angiography were used in some patients. Histology with or without adjacent organ involvement was also recorded. Histological subtype was determined by examination of light microscopy applying different staining methods. Immunohistochemistry stain included vimentin, alpha-smooth muscle actin, desmin, neuron-specific enolase (NSE), S100 protein and CD 34. CD117 was introduced to our Department of Pathology since October 1998. Electronic microscopy was also performed.

Tumor size was tabulated based on actual measurement of the gross surgical specimen or imaging when the tumor was inoperable. It was recorded as the largest diameter in any dimension of the primary tumor and was classified into < 5 cm, 5 to 10 cm, or >10 cm. Resection margins were checked closely for presence of microscopic disease. Tumors were also grouped according to their mitotic numbers as no mitosis (0/10 HPF), low mitotic index (1-4/10 HPF) and high mitotic index (≥5/10 HPF). Tumor cellularity, presence or absence of invasive growth, tumor necrosis and the presence or absence of haemorrhage were also examined.

Complete resection was defined as the excision of all gross disease regardless of microscopic margins. Resections were classified as incomplete when gross residual disease was present after resection. Inoperable was defined as when the tumor was unresectable at surgical exploration. Patients with incompletely resected tumor, metastatic or unresectable tumor were referred to oncology units for chemotherapy and radiation therapy as indicated. In total, 47 patients satisfied the diagnosis of GISTs.

Survival analysis

All times were calculated from the first presentation to the date of last follow-up or death. Time of last follow-up of dead patient was recorded as the time of certification of death. Others were censored at the time of last follow-up in specialist outpatient clinic or admission (clinical or emergency) whichever was later. Clinical variables, tumor characteristics, as well as modality of treatment were analyzed against tumor recurrence or death.

Statistic analysis

Statistical analyses were performed using SPSS 10.0(SPSS Inc., Chicago, USA). Influence of the factors in predicting tumor recurrence or death was assessed by univariate analyses. Multivariate analysis was performed with the Cox proportional hazards model to identify significant prognostic factors. $P<0.05$ was considered statistically significant.

RESULTS

Patient characteristics

Between September 1995 to December 2003, 47 patients (26 men and 21 women) were diagnosed with GIST. Mean follow up time in this cohort was 31 mo (range 0-99 mo). Mean age was 66.6 years (SD 13.1, range 29 to 87). Stomach (34 patients, 72.3%) was the most common site for the tumor, followed by the small intestine (8 patients, 17.0%), esophagus (2 patients, 4.3%), omentum (2 patients, 4.3%) and colon (1 patient, 2.1%). Gastrointestinal bleeding was the most common presenting symptom (28 patients, 60%), followed by epigastric pain (21 patients, 45%), and the presence of an abdominal mass (10 patients, 21%). Three patients (6%) with GIST were diagnosed incidentally. Endoscopically, the most common finding was a round tumor mass, either pedunculated or sessile, resembling a leiomyoma. Huge tumor usually presented as submucosal mass bulging into the lumen. Active bleeding ulcer or ulcer with stigmata of recent haemorrhage was present in 5 cases (71%) (Table 1). Radiological imaging including computerized tomography (CT) scan of the abdomen and barium studies were employed in most of our patients to identify the site and to assess the size of the lesions as well as the presence of any local invasion or distant metastases (Figure 1). Two patients had metastases delineated by CT scan, one in liver and one in omentum respectively, which precluded any curative surgical intervention.

Tumor characteristics

Tumor size ranged from 0.3 to 24 cm (median 4.5 cm, inter-quartile range 3-7.5 cm). Twenty-seven (57%) patients had tumor size less than or equal to 5 cm, 14 (30%) patients with tumor size between 5 cm to 10 cm, whereas 6 (13%) patients presented with tumor size greater than 10 cm. Seven (15%) patients had high mitotic index (≥ 5/10 HPF), 18 (38%) patients low (1-4/10 HPF) and 22 (47%) no mitotic index (0/10 HPF). Necrosis inside the tumor was found in 15 patients (32%) with GISTs. Seven out of 47 patients (15%) had presence of tumor metastases or invasion to adjacent organs. Immunohistochemical analyses were performed in all cases. Eleven (23%) GISTs had markers for neuronal and neuroendocrine tumors,

Table 1 Characteristics of patients with GISTs

Characteristics		*n* (%)
Number of patients		47
Male/Female (ratio)		26:21 (1.23:1)
Mean age at the time of diagnosis(SD)		66.60 (SD 19.1)
Median follow up time in months (IQR)		26.00 (10-43)
Presenting symptoms		
	GI bleeding	28 (60%)
	Epigastric pain	21 (45%)
	Abdominal mass	10 (21%)
	Incidental finding	3 (6.3%)
Locations		
	Stomach	34 (72.3%)
	Small bowel	8 (17.0%)
	Esophagus	2 (4.3%)
	Colon	1 (2.1%)
	Omentum	2 (4.3%)
Resection[1]		
	Complete	31 (66%)
	Incomplete	7 (14.9%)
	Inoperable	5 (10.6%)
Recurrence (after initial surgery)	Total	4 (8.5%)
	Local recurrence	1 (2.1%)
	Distal metastasis	3 (6.4%)
Death	Total	16 (34%)
	Tumor related	11 (23.4%)
	Tumor unrelated	5 (10.6%)

[1]Two patients refused resection of tumor. Two GISTs were discovered incidentally during autopsy. IQR: Inter-quartile range

Table 2 Tumor characteristics of 47 patients with GISTs

		n (%)
Number of patients		47
Median tumor size in cm (IRQ)		6.05 (3-7.5)
	≤5 cm	24 (51)
	>5-10 cm	13 (28)
	>10 cm	4 (9)
Mitotic index		
	High	7 (15)
	Low	18(38)
	No	22(47)
Presence of distal metastasis or local invasion		7 (15)

8(17%) demonstrated immunohistology markers for myoid series. Twenty-eight (60%) GISTs were undifferentiated tumors (Table 2).

Treatment

All patients were assessed for tumor resection either endoscopically or surgically. Thirty-one patients (66%) had complete resection of the tumor, and 7 patients (15%) underwent incomplete resection. In 5 patients (10%), resection of tumor was impossible because of extensive tumor metastases or poor general condition at the time of diagnosis. Endoscopic resection was possible only in 7 patients (15%) (5 gastric, 1 oesophageal and 1 colonic GISTs). All small bowel GISTs required surgical resection. Adjuvant chemotherapy by Imatinib (Glivec) was given in 2 patients post-operatively.

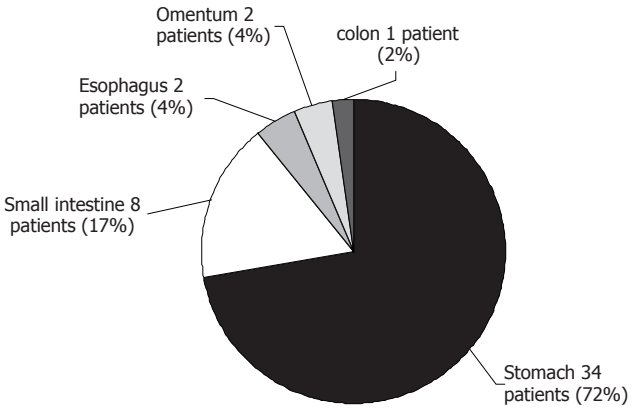

Figure 1 Tumor Locations of 47 GISTs

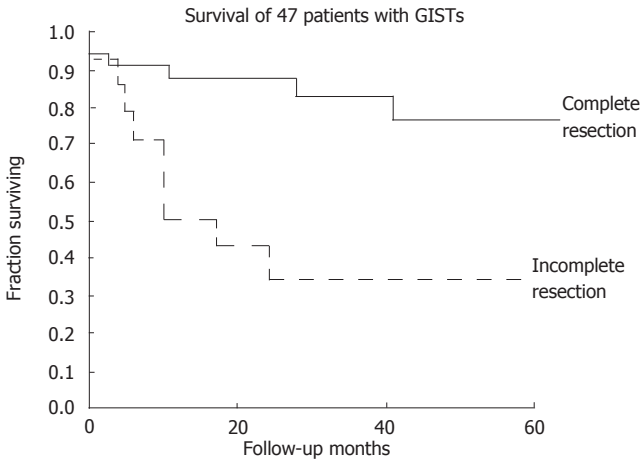

Figure 2 Kaplan-Meier survival curve of GIST patients with complete and incomplete tumor resection.

Survival

Totally 16 deaths were recorded in the study period, and 11 deaths were tumor related. The overall median survival was 26 mo. Five-year survival probability was 61.3% (SE 8.0%). The median survival time for completely resected GISTs was longer than that for incompletely resected or inoperable patients, 37 mo versus 10 mo respectively (log rank test, $P=0.0013$) (Figure 2). Two patients receiving imatinib were still alive at the end of the study, with liver metastatsis. Their survival time censored at the end of the study was 26 mo and 37 mo respectively.

Prognostic factors

By univariate analyses, the significant factors predicting tumor recurrence or tumor related death included tumor size 5 cm or above ($P=0.003$), presence of significant mitotic figures (1/10 HPF or above) ($P=0.021$), presence of necrosis ($P=0.005$), invasion to adjacent organ and/or presence of metastases at the time of diagnosis ($P<0.001$),

Table 3 Univariate analyses of factors predicting GISTs recurrence or tumor related death

Prognostic factors	Hazard ratios	*P* value
Tumor size 5 cm or above	20.5	0.003
Presence of significant mitotic figures (1/10 HPF or above)	3.8	0.021
Presence of necrosis	4.4	0.005
Invasion to adjacent organ and/or presence of metastases at the time of diagnosis	16.5	<0.001
Incomplete tumor resection	4.6	0.002

Table 4 Multivariate analyses of factors predicting GISTs recurrence or tumor-related death

Prognostic factors	Hazard ratios (CI)	*P* value
Tumor size 5 cm or above	9.6 (1.1-86.0)	0.042
Invasion to adjacent organ and/or presence of metastases at the time of diagnosis	14.0 (2.5-78.6)	0.003
Incomplete tumor resection	2.1 (1.1-86.0)	0.032

incomplete tumor resection ($P = 0.002$) (Table 3). By multivariate analyses, tumor size 5 cm or above (hazard ratio = 9.6, $P = 0.042$), invasion to adjacent organ and/or presence of metastases at the time of diagnosis (hazard ratio 14.0, $P = 0.003$), and incomplete tumor resection (hazard ratio = 2.1, $P = 0.032$) remained the significant factors predicting tumor recurrence or tumor related death (Table 4).

DISCUSSION

In this study, we summarized our 8 years' experience in the diagnosis and management of GISTs in a regional hospital in Hong Kong. Stromal tumors of the gastrointestinal tract are rare, accounting for less than 1% of all gastrointestinal tract malignancies. Their presumed origin from smooth muscle cells has led to the use of terms such as "leiomyoma", "leiomyoblastoma" and "epithelioid leiomyosarcoma". However, the exact origin of the tumor was difficult to determine in the early days until discovery of gain-of-function mutations in the *c-KIT* proto-oncogene in GISTs by Hirota and colleagues in 1998[3]. Therefore, the incidence of GISTs was rarely reported in the literature and was not reported in Chinese population.

Our hospital serves a local population of 300 000-350 000. The estimated prevalence of GISTs was 13.4-15.6 per 100 000 people, with an annual incidence of 1.68-1.96 per 100 000 people. This is comparable to that of the United States where the estimated annual incidence is 1-2 cases per 100 000 people, of which 20%-30% are malignant[4]. In contrast, Finland has higher annual incidence of malignant GISTs, 0.4 per 100 000 people[6], i.e. estimated 4 GISTs per 100 000 people.

The true incidence of GISTs could be underestimated before the introduction of CD117. In our hospital, all gastrointestinal tract tumors suspected to be GISTs were routinely tested for CD 117 marker since October 1998.

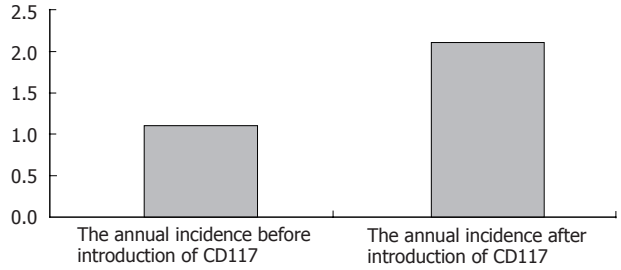

Figure 3 The annual incidence of GISTs before and after the introduction of CD117 was 1.1 per 100 000 people and 2.1 per 100 000 people respectively.

Interestingly, only 10 patients out of 47 (21%) were diagnosed as GISTs before the introduction of CD117. The annual incidence of GISTs after the introduction of CD117 increased from 1.1 per 100 000 people to 2.1 per 100 000 people (Figure 3). Similar trend was also noted in the United States. The estimated incidence of GISTs in the United States was around 0.6 per 100 000 people in the 1980s. The incidence increased to approximately 1-2 patients per 100 000 people after 2 000[4].

There existed some conflicting evidence on male predominance, however, most studies indicated no sex predilection[18, 21, 24, 31]. In our study, the occurrences of GISTs were similar in both sexes (1.2 male to 1 female). The age of presentation had a unimodal distribution, similar to the published data where the majority of patients presents in the fifth to seventh decade of life[4], the mean age of presentation of our patients was 66 years. GISTs are occasionally found in young adults, but they are very rare in children[7].

The vast majority of GISTs arise as a result of somatic mutation, but rare familial cases associated with mutated *c-KIT* have been identified[8, 10-12]. In our study, we could not identify any patient with familial relation. All the patients with GISTs were sporadic, and the predisposing factors were unknown.

Similar to other published data[7,13,14], the stomach was the most frequent site of involvement in our patients (72%), followed by small intestine (17%). The large bowel, esophagus and omentum were least likely involved. Symptomatic lesions have manifestations that depend on tumor size, location and growth pattern[7,13,14]. Ludwig and Traverso reported that GISTs with intraluminal nodule less than 2 cm was generally asymptomatic while tumors >4 cm were associated with symptoms[15]. In the literature approximately 50% of patients presented with acute or subacute gastrointestinal bleeding as the major symptom leading to diagnosis[25]. In our local patients, the most common presenting symptoms were gastrointestinal bleeding (60%), as results of overlying mucosal ulceration, followed by epigastric pain and abdominal mass. Other symptoms included fever, anorexia, dysphagia, obstruction and perforation of bowel were seldom seen in our series. Clinically palpable mass usually implies invasion to adjacent organs or distant metastasis, thus in turn predicts poor outcome of the patients. Surgical resection is the treatment of choice and should be performed with the intention to performing complete en bloc resection of the tumor, as it

is difficult or impossible to differentiate between benign or malignant lesions before or during operation[16]. Tumor size and frozen section during operation are not conclusive [17,18].

Prognostic factors

Gastrointestinal stromal tumors are unique in that their malignant potential is not always predictable. Most GISTs appear relatively low grade histologically, and it has been difficult to distinguish benign from malignant GISTs, especially at the low-grade end of the histological spectrum. Many previous studies suggest a single factor or even combinations of two factors are not sufficient to reliably predict the outcome of GISTs[19]. Currently no accepted staging system exists. Most pathologists use a multiparametric approach to predict the biological behavior of GISTs. The most reproducible predictor of malignancy has been mitotic rate >1-5 mitosis per 10 HPF[20-22]. Other poor prognostic factors include tumor size > 5 cm[23-27], mutation in the c-kit gene[28-30], necrosis[18, 20, 24, 31], infiltration and metastasis to other sites[13]. In our study, significant prognostic factors were tumor size 5 cm or above, invasion to adjacent organ and/or presence of metastases at the time of diagnosis as well as incomplete tumor resection. In contrast to previous studies[20-22], mitotic index did not appear as significant prognostic factor in our cohort. The different approaches to sample tissue from large tumors with considerable heterogeneity for microscopic assessment may explain such discrepancy between different studies. At present, the best method to adequately sample tumor tissue and report mitotic index is still controversial. In our hospital, our pathologists choose random sampling approach and report the highest mitotic index among different tissue samples. From the literatures, approximately 10% to 30% of all GISTs display malignant behavior[4, 14]. Our patients with GIST demonstrated similar ratio regarding malignant GISTs.

Survival

The overall survival appears to reflect the completeness of resection[7, 18, 32]. In Roswell Park Cancer Institute (RPCI), the United States, the median survival rate was significantly higher in those who underwent complete resection (33 mo) as compared to those who underwent palliative surgery (15 mo)[32]. Similarly in our center, the median survival time for completely resected GISTs was significantly longer than incompletely resected or inoperable patients, 37 mo versus 10 mo. The adjuvant therapy appeared to improve the survival and the quality of life in patients with incomplete tumor resections. Despite extensive metastasis, the 2 patients receiving imatinib were still alive at the end of the study. The survival times of 26 mo and 37 mo were much longer than the median 10 mo of other patients with incomplete tumor resection, although the number of patient in this group was too small for statistical analysis.

Recurrence is commonly local and peritoneal, often associated with liver metastasis. Peritoneal metastases are most probably a result of tumor cells seeding from the primary tumor directly into the peritoneal cavity. Liver metastases most probably result from haematogenous seeding into the portal vein[33]. Extra-abdominal disease in the absence of peritoneal involvement is rare[13]. There exist few reports in the literature describing a survival advantage after the resection of abdominal recurrence. In our hospital, local recurrence of GIST was not routinely operated again.

In conclusion, the incidence of GIST in Hong Kong is comparable to that of United States but lower than that in Finland. The true incidence of GISTs could be underestimated before the introduction of CD117. Incomplete resection, tumor size 5 cm or above, invasion to the adjacent organ or presence of metastasis are factors predicting tumor-related death or recurrence. The survival of the completely resected tumor is good. The introduction of medical adjuvant therapy appears to improve the survival and the quality of life in the unresectable and incompletely resected GISTs patients.

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CLINICAL RESEARCH

Establishment of risk model for pancreatic cancer in Chinese Han population

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Abstract

AIM: To investigate risk factors for pancreatic cancer and establish a risk model for Han population.

METHODS: This population-based case-control study was carried out from January 2002 to April 2004. One hundred and nineteen pancreatic cancer patients and 238 healthy people completed the questionnaire which was used for risk factor analysis. Logistic regression analysis was used to calculate odds ratio (ORs), 95% confidence intervals (Cls) and β value, which were further used to establish the risk model.

RESULTS: According to the study, people who have smoked more than 17 pack-years had a higher risk to develop pancreatic cancer compared to non-smokers or light smokers (not more than 17 pack-years) (OR 1.98; 95% CI 1.11-3.49, $P=0.017$). More importantly, heavy smokers in men had increased risk for developing pancreatic cancer (OR 2.11; 95%CI 1.18-3.78, $P=0.012$) than women. Heavy alcohol drinkers (>20 cup-years) had increased risk for pancreatic cancer (OR 3.68; 95%CI 1.60-8.44). Daily diet with high meat intake was also linked to pancreatic cancer. Moreover, 18.5% of the pancreatic cancer patients had diabetes mellitus compared to the control group of 5.8% ($P=0.0003$). Typical symptoms of pancreatic cancer were anorexia, upper abdominal pain, bloating, jaundice and weight loss. Each risk factor was assigned a value to represent its importance associated with pancreatic cancer. Subsequently by adding all the points together, a risk scoring model was established with a value higher than 45 as being at risk to develop pancreatic cancer.

CONCLUSION: Smoking, drinking, high meat diet and diabetes are major risk factors for pancreatic cancer. A risk model for pancreatic cancer in Chinese Han population has been established with an 88.9% sensitivity and a 97.6% specificity.

Key words: Pancreatic cancer; Risk factor; High-risk model

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INTRODUCTION

Pancreatic cancer is one of the most deadly cancers in the world. The mortality over morbidity ratio is 0.99:1. Only 10% patients have cancer cells confined in pancreas at the time of diagnosis, 40% have local invasion and 50% have distal metastasis. Although surgery, radiotherapy and chemotherapy have improved the life quality and survival rate in the cancer patients, only 10% patients show significant improvement. Most patients die from the cancer after 4 to 6 mo from diagnosis. Niederhuber *et al*[1] reported that one-year survival rate was less than 20%, decreasing to 7% for three-year and 3% for five-year. It is proposed that the best way to reduce the mortality is to improve early detection of this deadly cancer. In this paper we analyzed the possible risk factors and symptoms that correlated to pancreatic cancer based on a questionnaire survey among pancreatic patients and control subjects, and established a risk model for cancer estimation with weighted scores of risk factors and symptoms in an attempt to help early detection of pancreatic cancer in Chinese Han population.

MATERIALS AND METHODS

Subjects

A total of 119 cases of pancreatic cancer included in this study were diagnosed by Peking Union Medical College Hospital (PUMCH) between January 2002 and April

Table 1 Diagnostic standards for pancreatic cancer

A. Confirmed diagnosis by pathology
B. A valid confirmation requires at least 2 of the following listed items of imaging tests
(1) Ultrasound indicates there are low-density area in pancreas, as well as pancreatic duct dilation and common bile duct and gall bladder swelling
(2) CT indicates local enlargement and mass occupying lesion in pancreas
(3) ERCP indicates discontinuity of pancreatic duct, having mouse-tail ending, stiff and irregular duct wall, or any pull sign and double-duct sign
(4) MREP indicates there are stenosis and dilation in pancreatic duct and/or bile duct and space-occupying lesion in pancreas
(5) EUS indicates there is low-density, occupying lesion in pancreas
(6) IDUS
(7) Angiography
(8) PET
C. Palpable mass in surgery and at least an item of radiology evidence

ERCP: Endoscopic retrograde cholangiopancreatography
MREP: Magnetic resonance cholangiopancreatography
EUS: Endoscopic ultrasound sonography
IDUS: Intraductal ultrasound sonography
PET: Positron Emission Tomography

2004. The diagnosis was based on pathological, clinical and surgical evidence (Table 1). Among the 119 cases, 42 (35.3%) were confirmed by pathology; 17 (14.3%) by surgical findings; the rest (50.4%) by clinical findings. Those patients with a past history of chronic pancreatitis were diagnosed based on evidence of pancreatic calcification and pancreatic duct dilatation on imaging tests. All patients came from Beijing and its peripheral areas and were Han nationalities. The normal control group was randomly selected from normal general population in the same geographic area. The two groups were matched in gender and age and marriage status (Tables 2 and 3).

Methods

This is a case-controlled epidemiological study. We interviewed each subject in both groups based on a pre-determined questionnaire, which included questions about demographic data, smoking and drinking habits and family history of related diseases with pancreatic cancer. According to World Health Organization's definition of smoking, we defined the smokers as those who have smoked for 12 mo or more continuously or cumulatively in their lives. We measured the smoking history by the unit of pack-year which was defined as smoking of a package (20 pieces) of cigarette per day for one year. The number of pack-year = (the number of cigarette per day/20) × the number of years smoked. Because some persons used the pipe, we converted 50 g of pipe tobacco to 10 pieces of cigarettes. We defined the alcohol drinker as drinking at least twice a week and continuously for at least one year. Regarding the drinking volume, we adopted the unit of "drink", which was commonly used in North America. One drink contains 14 mL or 10.9 g of pure alcohol, which equals to 280 mL of beer (50 mL/L alcohol), 112 mL of wine (125 mL/L alcohol), 70 mL of rice wine (200 mL/L alcohol) or 35 mL of hard liquor (400 mL/L alcohol). In China, one bottle of beer is 640 mL, which is converted to 2.29 drinks. A glass of wine has 50 mL in volume, which is 0.45 drink unit. One cup of hard liquor is 55 mL, which equals to 1.57 drink units. One cup of rice wine having 50 mL in volume is converted to 0.71 drink unit. We assumed the subject drank the same amount of alcohol per day per year. The total drinking volume is calculated by the following formula: Total volume (drink-years) = (Numbers of bottle of beer × 2.29 + Numbers of glass of wine × 0.45 + Numbers of cup of hard liquor × 1.57 + Numbers of cup of rice wine × 0.71) × (Numbers of drinking years). Because a large number of subjects in this study did not drink or only drank once in several weeks or months, we did not adopt the calculating method of gr (alcohol)/per day, which is often used to define heavy drinking.

According to the relative amount of meat and vegetable intake during most time of one's life, diet habit was divided into high meat consumption, high vegetable consumption and equal meat and vegetable consumption.

The high-risk scoring model was established on the basis of multivariate logistic regression analysis. The variables included risk factors and symptoms of pancreatic cancer. Possible risk factors and symptoms described in the questionnaire were compared between two groups with the t test or χ^2 test. The OR value of each factor was also calculated and those with statistical significance were listed. Combined with our clinical findings, significant and borderline risk factors and symptoms were selected as variables. With multivariate logistic regression analysis, we obtained regression coefficient β of each independent variable. Selecting the minimal β value as radix, and through division of every β value by the radix, the weighted numerical scores of each variable were derived. We made small adjustments to some scores based on the suggestions of some experts of epidemiology and gastroenterology and established the risk scoring model. The risk score of each patient could be calculated through simple addition of weighted score of each variable. The risk scores were compared between two groups by the t test. Receiver operating characteristic (ROC) curve was used to set a cutoff value for high cancer risk.

RESULTS

Analysis of risk factors for pancreatic cancer

The body mass index of pancreatic cancer patient was distinctively higher than that of control group. After correction by age and gender, the average weight index of patient group was 24.89 (17.99-36.73), while that of control group was 23.99 (14.53-32.74), with significant difference ($P = 0.033$) between these two groups. A clinical manifestation of pancreatic cancer patient was weight loss in a short term. Among the 109 patients who had weight index, the average weight loss was 6.70 (-5-31) kg during the one-year period before diagnosis. However, among the 197 control subjects who had weight index, their body weight increased by an average of 0.33 (-10.5-5) kg during the same period. The weight changes between the two groups had significant difference ($P < 0.0001$). Therefore, we used the weight index of the subjects one year before they were recruited into the study group.

Heavy smoking was shown as a risk factor for pancreatic cancer. There was no significant difference

Table 2 Inclusion and exclusion criteria for patients and controls

	Inclusion criteria	Exclusion criteria
Patients	(1) The patient voluntarily took part in this research; agreed to take necessary clinical examinations and answer epidemiological questionnaire survey; and give consent to the publication of research data.	(1) The patient did not agree upon the conditions to participate in the study or the patient information was not available at the inclusion time.
	(2) Diagnosed by the pancreatic cancer team from PUMCH.	(2) Not diagnosed by the pancreatic cancer team from PUMCH.
	(3)Clinical examination and epidemiological investigation were acceptable for the patient's condition.	(3) The patient's condition did not allow the clinical examination and epidemiological investigation.
	(4) Patient was diagnosed for pancreatic cancer between 2002 and 2004.	(4) The patient was not diagnosed for pancreatic cancer for the first time between 2002 and 2004.
	(5) Patients did not undergo radiotherapy and anticancer therapy before surgery, and did not have other primary tumors.	(5) Patients whose pancreatic tumor was a metastatic carcinoma or who had other tumors.
	(6) Patients belonged to Han nationality.	(6) Patients were not from Han nationality.
Controls	(1) The person voluntarily took part in this research; agreeed to take necessary clinical examinations and answer epidemiological questionnaire survey; and give consent to the publication of research data.	(1) The person did not agree upon the conditions to participate in the study or the person's information was not available at the inclusion time.
	(2) The person did not have any kind of tumor.	(2) Person had any kind of malignant tumor.
	(3) The person matched with the patients for gender and age (±5 years).	(3) Person suffered from severe coronary heart disease or stroke.
	(4) Being the resident of Beijing or its peripheral area for at least 5 years.	(4) Person did not match with the patient group for gender and age (±5 years).
	(5) Han nationality.	(5) Not from Han nationality.

Table 3 Demographic characters of patients and controls

	Patients		Controls		χ^2	*P*
	n	(%)	*n*	(%)		
Age (yr)						
Male ≤50	16	(20.8)	36	(31.9)		
50-59	17	(22.1)	27	(23.9)	3.6583	0.1606
≥60	44	(57.1)	50	(44.3)		
Female ≤50	9	(21.4	28	(29.5)		
50-59	8	(19.1)	26	(27.4)	3.1297	0.2091
≥60	25	(59.5)	41	(43.2)		
Gender Male	77	(64.7)	113	(54.3)		
Female	42	(35.3)	95	(45.7)	3.3496	0.0672
Marriage status						
Married	115	(96.6)	183	(87.9)		
Divorced	0	(0)	7	(3.4)		
Widowed	4	(3.4)	16	(7.7)	8.093	0.082
Separated	0	(0)	1	(0.5)		
Spinsterhood	0	(0)	1	(0.5)		

between patient group (39.5%) and control group (35.92%) in terms of the percentage of smokers. After we divided each group into three subgroups (0, ≤17 package years and ≥17 package years) in terms of smoking history, we found significant differences between the patient group and control group. For those who smoked more than 17 package years (heavy smoker), the percentage of smokers accounted for 30.7% in patient group, which was 10% higher than that of control group. The risk of developing pancreatic cancer in the heavy smoker subgroup was 1.51 times that of the non-smokers (no statistic significance). When we combined the non-smoker and intermediate smoker subgroups (≤17 package years) together, it showed that the heavy smoker had increased risk of pancreatic cancer (OR, 1.98; 95% CI, 1.11-3.49; $P=0.017$). If divided by gender, there were more male heavy smokers in patient group (47.7%) than control group (30.3%). The risk of pancreatic cancer was increased for male heavy smokers (OR, 2.11; 95% CI, 1.18-3.87, $P=0.012$). However, no conclusion for females was made due to the low percentage of smokers within the patient group and control group.

Heavy drinking was shown as a risk factor for pancreatic cancer. According to the median total drinking volume of control group, we divided the drinkers into three subgroups: non-drinking, low-drinking (≤20 drink-years) and heavy-drinking (>20 drink-years). The result indicated that the distribution frequencies of three subgroups in patient group were 74.0% (88/109), 5.9% (7/109) and 20.2% (24/109) respectively, while in control group were 84.5% (175/207), 8.2% (17/207), 7.3% (15/207) respectively. The distribution in patient group had significant difference from control group. After correction of age, gender and smoking factors, the OR value of low-drinking compared to the non-drinking was 1.003, while for the heavy-drinking subgroup, it increased to 3.681. Therefore, heavy-drinking had higher risk to develop pancreatic cancer.

Diet with high meat consumption was shown as a high risk for pancreatic cancer. In this study, we only explored the relationship between dietary habit and pancreatic cancer. The results showed that those who claimed meat as their major daily diet accounted for 31.0% of patient group, while the number dropped significantly to 7.6% in control group. For those who claimed vegetable was their major daily diet accounted for 39.7% (46/116) in patient group and 28.9% (57/197) in control group. The diet containing half meat and half vegetable was reported by 25.9% (30/116) of patient group and 57.9% (114/197)

Table 4 Benign digestive diseases in patients and controls

Diseases	Patients				Controls				*P*
	n	Yes	No	Not stated	*n*	Yes	No	Not stated	
Chronic pancreatitis	119	2.5	95.0	2.5	205	0.5	99.5	0	0.012
Acute pancreatitis	119	0	98.3	1.7	205	0	100	0	0.06
Cholelithiasis	119	10.01	89.1	0.8	205	4.9	95.1	0	0.08
Cholecystitis	118	9.3	87.3	3.4	205	0.5	99.5	0	<0.0001
Cholecystectomy	119	3.4	95.8	0.8	205	2.9	97.1	0	0.41

Table 5 Clinical symptoms of patients and controls

Symptom	Symptom duration (mo)[a]	Patients				Controls				*P*
		n	Yes	No	Not stated	*n*	Yes	No	Not stated	
Anorexia	2	118	45.8	54.2	0	203	0.5	99.5	0	<0.0001
Epigastric pain	3	119	60.5	38.7	0.8	181	2.2	97.8	0	<0.0001
Backache	2	119	32.8	66.4	0.8	205	2.0	98.1	0	<0.0001
Hypogastralgia	3	119	16.0	82.4	1.7	205	3.4	96.6	0	<0.0001
Abdominal pain	2	119	38.7	61.3	0	205	4.9	95.1	0	<0.0001
Jaundice	1	119	42.0	58.0	0	205	0.5	99.5	0	<0.0001
Skin itch	1	119	13.5	86.6	0	205	8.7	91.3	0	0.0032
Weight loss	3	119	76.5	22.7	0.8	205	2.4	97.6	0	<0.0001

of control group. Therefore, people who had meat as their major diet faced higher risk of developing pancreatic cancer.

Also we found that 18.5% patients with pancreatic cancer had a history of diabetic mellitus; however this ratio dropped to 5.8% in the control group. There was significant difference between patient group and control group in having chronic pancreatitis, cholelithiasis and cholecystitis (Table 4). Moreover, we asked each subject for any cancer family history among the primary and secondary generation relatives, but no significant differences were found between the two groups. In patient group, those who had a family history of cancer accounted for 26.9%, while in the control group was 31.3% ($P=0.4064$). For those who lost weight in patient group, 48.9% patients lost weight within 3 mo, 33.3% in 4-6 mo, 16.7% in 7-12 mo, and 1.1% in more than 1 year. In addition, 45. 6% of patients had weight lost less than 5 kg, 30.0% between 6 to 10 kg, and 24.4% more than 10 kg (Table 5).

Although most factors had significant difference between two groups, their OR value did not show significance except for smoking and drinking due to relatively small sample of this study.

Establishment of the high-risk scoring model of pancreatic cancer

With multivariable logistic regression and some adjustments, we established the high-risk scoring model (as shown in Table 6).

The score of pancreatic cancer group was 80.6 ± 30.0 (95% CI 74.9-86.3), and the score of normal control group was 7.4 ± 11.9(95% CI 6.0-8.7), ($P<0.001$, Mann-Whitney test). According to the scores of pancreatic group and normal control group, we protracted ROC curve and the area below the curve was 0.981. When selecting $\geqslant 45$ as the differential cutoff between pancreatic cancer and normal control, the sensitivity and specificity of diagnosis were quite ideal, which were 88.9% and 97.6% respectively.

DISCUSSION

Because the two groups in our study were matched according to age and gender, there were no significant differences of the two factors. Based on previous studies on risk factors we included the two factors into high risk model. We have reported earlier that mortality of pancreatic cancer rises with age[2]. In a study involved 1619 cases of pancreatic cancer, 3.95% of the patients died before 40, but the mortality rate between 65 to 80 years was more than 5 times that of the average[2], which is consistent with other reports[3-6]. From 1991 to 2000, the epidemiological data of pancreatic cancer in China showed that the average male's mortality rate, revised mortality and standardized mortality grew 4.23%, 5.1% and 3.1% per year respectively, but the average growth rates for female were 7.7%, 8.6% and 6.74% respectively[2]. Although the gender disparity had been decreasing during this period, the increasing mortality rate in female has outpaced male. The age standardized mortality rate of male and female decreased from 1.97 in 1999 to 1.41 in 2000, which was consistent with report by Zheng *et al*[7].

Smoking is the only widely recognized risk factor for pancreatic cancer. Compared to non-smokers, the mortality rate of pancreatic cancer in smokers increased by 1.2-3.1 fold, and showed a dose-effect relationship. We found that the risk of pancreatic cancer occurrence increased in the heavy-smoking group (OR, 1.98; 95% CI, 1.11-3.49; $P=0.017$), which is consistent with previous literatures[4,8,9]. There was no consistent conclusion drawn for the risk of drinking associated with pancreatic cancer in literatures[4,10-12]. The OR value in the heavy-drinking

Table 6 Risk scoring model for pancreatic cancer

Risk factor	Criteria	Points
Gender	Male	2
Age (yr)	>60	7
Alcohol drinking	>20 drink-yr	4
Smoking	>17 pack-yr	5
Diabetic mellitus history		17
High meat consumption		7
Family history of pancreatic cancer		15
Chronic pancreatitis		12
Cholelithiasis history		8
Cholecystitis history		1
Anorexia		25
Epigastric pain		25
Weight loss		37
Jaundice		30

subgroup was 3.70 (95% CI, 1.604-8.443) compared to non-drinking subgroup. Therefore, we considered the risk of drinking associated with pancreatic cancer mainly existed in the heavy-drinking patients. Immigration epidemiological research showed that the mortality rate of pancreatic cancer among Asian and African populations in their homeland was low. However, for Asians and Africans who lived in western countries and adopted western diet habits, the risk of having pancreatic cancer was similar to or even higher than the Caucasians[13]. From our study, the percentage of people who had high meat consumption in their daily diet was much higher in the patient group than in control group ($P < 0.0001$). Currently, 35% of pancreatic cancers were attributed to the dietary factor. It was reported earlier that the vegetable abundant diet could prevent 33%-50% of pancreatic cancer cases[14].

Whether diabetes is the etiologic factor or the early manifestation of pancreatic cancer is still controversial. When probing into the relationship between them, we cannot draw a conclusion because it is impossible to carry out a randomized prospective research. At the same time, it is difficult to obtain evidence from animal experiments because of lacking credible animal model for type II diabetes. Currently, most investigations have used case-control study and prospective cohort study in general population. In our case-control study, 18.5% of pancreatic cancer patients had diabetes history, while only 5.8% of the control group did. This finding is in accordance with other researches[15-18].

The relationship between chronic pancreatitis and pancreatic cancer is still under debate. Our study showed that 22.5% of pancreatic cancer patients had chronic pancreatitis while only 0.49% of control group did. Karlsen reported that the risk of pancreatic cancer increased 13 times from a follow-up investigation of 715 chronic pancreatitis patients for an average of 10 years during 1971 to 1995[19]. At the same time, the author noticed that the incidence of pancreatic cancer was much higher than non-pancreatic tumors among those patients.

A population based case-control study showed that performing cholecystectomy a year before the diagnosis of pancreatic cancer was related to the occurrence of pancreatic cancer. However, the risk gradually decreased along with the delay of the surgery, but still positively related [11]. Because pancreas and bile duct tumors are difficult to distinguish, some researchers believed that patients with concealed pancreatic tumor were likely to have cholecystectomy performed because of presumed cholelithiasis. However, we did not discover any relation between pancreatic cancer and cholecystectomy in our study. Therefore, it still awaits further clarification.

We established the high-risk scoring model on the basis of the results of case-control study and clinical experience. The risk factors in the high-risk model including smoking, weight loss and diabetes are basically the same as that of overseas studies. For instance, in the cancer risk index established by Harvard University[20], the risk factors for pancreatic cancer are smoking (moderate to high dose), family history of pancreatic cancer, diabetes, chronic pancreatitis and carbohydrate ingestion. In that index, they mainly do allotment of fraction according to the OR value of each risk factor. However, our model is to confirm the fraction of each factor according to the result of logistic multivariate regression, and it reveals the relative contribution of each risk factor to pancreatic cancer. Our risk model is to help clinical diagnosis, which is different from the cancer risk index of Harvard University whose purpose is cancer prevention. So we added the associated symptoms of pancreatic cancer to the risk model. Although the main symptoms of pancreatic cancer such as abdominal pain and jaundice usually appear late when the tumor is already in advanced stage, there are some non-specific symptoms such as anorexia and weight loss which are very obscure and easy to be overlooked by patients and doctors or mistakenly diagnosed as other diseases and functional abnormity[21,22]. So tackling the symptom clues might be helpful for screening and early diagnosis of pancreatic cancer. Our research showed that weight loss, epigastric pain and diabetes all had significant differences in the two groups, but weight loss in mo offers the best indication for diagnosis, so its score is the highest.

In conclusion, we have established a risk factor model for early screening of pancreatic cancer in Chinese Han population through case-control study. In future, we will apply this model in areas that have high incidence of pancreatic cancer in Han population to further improve our model.

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RAPID COMMUNICATION

Prevalence of hepatitis B virus precore stop codon mutations in chronically infected children

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Abstract

AIM: To find out whether there is a significant difference in the prevalence of the precore stop codon mutation between HBeAg positive and anti-HBe positive children.

METHODS: We investigated a large pediatric population of 155 European children (mean age 10.9 years) with chronic hepatitis B by PCR and direct sequencing. Ninety were HBeAg positive and 65 had seroconversion to anti-HBe. Additionally genotyping was performed.

RESULTS: Seventy-four (48%) of the sequenced HBV strains were attributed to genotype D and 81 (52%) to genotype A. In the group of 90 HBeAg positive patients, 2 (2.2%) 1896-G-to-A transitions leading to precore stop codon mutation were found, and in the group of 65 anti-HBe positive children, 5 (7.7%) were identified harbouring HBeAg-minus mutants. The difference was not statistically significant ($P = 0.13$).

CONCLUSIONS: HBeAg minus variants as predominant viral HB strains play a minor role in the course of chronic hepatitis B in European children.

Key words: Hepatitis B; Precore mutants; Children; HBeAg minus variants

Wintermeyer P, Gerner P, Gehring S, Karimi A, Wirth S. Prevalence of hepatitis B virus precore stop codon mutations in chronically infected children. *World J Gastroenterol* 2006; 12(14): 2235-2238

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INTRODUCTION

Chronic hepatitis B (HB)-infection is a serious health problem worldwide and the leading cause of liver cirrhosis and hepatocellular carcinoma (HCC). In Germany 0.3%-0.5% of the population are HBsAg carriers and it is estimated that approximately 5% of them are children. Mutations preventing the expression of HBeAg, so-called precore stop codon mutations, have been reported to aggravate liver disease and to cause fulminant hepatitis in children as well as in adults[1-3]. Currently it is not yet clear whether these mutations influence the response to antiviral treatment. However, a recent study has shown a better outcome after interferon-alpha treatment for chronic hepatitis in individuals without precore mutant strains before treatment[4]. The most common stop-mutation is the G1896A-substitution in the precore region of HB-virus. A guanine (G) to adenine (A) mutation of the HBV precore gene at nucleotide 1896 (numbered from the EcoRI site) leads to a conversion of codon 28 from TGG (tryptophan) to TAG, which is a stop codon, thereby rendering HBV incapable of producing HBeAg[5]. In adults the prevalence of this mutation ranges between 20%-95%, whereas it is more common in HBeAg-negative than in HBeAg-positive patients[6]. Results of a cross-sectional multicenter study of adults with chronic HBV infection in the United States show an association of the presence of precore variants with higher serum HBV DNA levels in HBeAg-negative but not in HBeAg-positive patients[7]. The selection of precore variants is dependent on HBV genotype. It is most common in patients with genotype D and rare in patients with genotype A[8]. This phenomenon is related to base pairing in the stem-loop structure of the pregenome encapsidation sequence[9,10].

Fukuda *et al*[11] have postulated that hepatitis B virus exists mainly as a quasi species. On account of this, a correlation of nucleotide sequences with clinical and serological findings has to be made with caution. Available data about prevalence and significance of precore mutations in childhood are limited. A study of children with chronic HBV infection ($n = 60$) has shown the 1896-G-A-transition in 93% as mixed infection, with a similar prevalence of mixed viral populations in responders and non-responders to interferon treatment[12]. In the course of chronic HBV infection in children the frequency of precore mutant is increased after seroconversion from HBeAg to anti-HBe. It has been postulated that the higher

the aminotransferase levels are, the higher and the earlier the mutant emerges[13].

The aim of this study was to evaluate the prevalence of precore mutations in a large cohort of European children with chronic hepatitis B.

MATERIALS AND METHODS

Patients

A cross sectional testing of sera from 155 randomly assigned HBsAg positive chronic hepatitis B virus carriers was performed. The median age of the children at the time of blood sampling was 10.9 years. Ninety were HBeAg positive and 65 had seroconversion to anti-HBe 1 - 3 years before blood sampling. Fifty-four (35%) were female, 101 (65%) male; 108 (70%) of Caucasian, 42 (27%) of Mediterranean and 5 (3%) of Southeast Asian origin. ALT was elevated in 66 (73%) of HBeAg positive individuals (mean: 1100 nkat/L) and in 18 (28%) of anti-HBe positive individuals (mean: 367 nkat/L; normal <417 nkat/L).

HBV molecular analyses

DNA was extracted from sera with the QiaAmp blood kit (Qiagen, Chatsworth, CA, USA) and eluted with 50 μL distilled water according to the manufacturer's recommendations. The precore region of the HBV genome was amplified and re-amplified with a proof reading of expand-polymerase (Expand High Fidelity PCR System) by nested PCR. The following primers were used for amplification (nucleotide positions are according to the unique EcoRI site[14]: Sense P1: 5' -TGTCAACGACCGACCTTGAG-3' (nt 1683-1702), anti-sense P2: 5'-CAATGCTCAGGAGACTCTAAGGC-3' (nt 2045-2023); nested PCR: sense P3: 5'-GAGGAGT TGGGGTAGGACATT-3' (nt 1736-1756), anti-sense P4: 5'-TAGCTCCAAATTCTTTATA-3' (nt 1936-1918). PCR was performed in a 50-μL mixture with 20 pmol of each primer (Roth, Germany), 100 mmol/L of each dNTP, and 2.5 units of a Taq-Tgo polymerase mixture (Expand High Fidelity PCR System, Roche, Germany) diluted in 10 × expand polymerase buffer (Expand High Fidelity PCR System, Roche, Germany) in a DNA thermal cycler (Eppendorf Mastercycler personal). The amplified products were visualized by 20 g/L agarose electrophoresis and ethidium bromide staining.

Direct sequencing of PCR products

The amplified PCR products were purified by QIA Quick PCR-purification kit (Quiagen, Chatsworth, CA) according to the manufacturer's recommendations and precipitated with isopropanol to remove residual dNTPs and primers and re-suspended in a final volume of 10 μL distilled water. Nucleotide sequences of the PCR products were determined using a dye terminator cycle sequencing kit (big dye vs 3.1) in an automated sequencer (ABI PRISM, Foster City, CA, USA). The sequencing primers were the same as those used for DNA amplification.

Genotyping

The HBV-DNA sequences were assigned to the appropriate genotype based on the restriction fragment length polymorphism (RFLP) created by Ava2 and Dpn2 action on an amplified segment of the pres-S-region according to Lindh *et al*[15]. PCR of certain serum samples was performed under conditions as previously described with the following primers: Sense P1: 5'-CGA GGCAGGTCCCCTAGAAGAAGAA-3' (nt 2356-2380), anti-sense P2: 5'-GTCCTAGGAATCCTGAA-3' (nt 187-171); nested PCR: sense P3: 5'-TCACCATATTCT TGGGAACAAGA-3' (nt 2819-2841), anti-sense P4: 5' -TTCCTGAACTGGAGCCACCA-3' (nt 82-63). The PCR products were incubated with restriction enzymes Ava II and Dpn II (New England Biolabs, Inc., USA) for 3 h at 37 °C in a 15 μL reaction sample according to the manufacturer's recommendations. The products were visualized by 2 g/L agarose electrophoresis and ethidium bromide staining.

Statistical analysis

Fisher's exact test was used for statistical analysis $P < 0.005$ was taken as significant.

RESULTS

A total of 155 patients were enrolled. Seventy-four (48%) of the sequenced HBV strains were attributed to genotype D and 81 (52%) to genotype A. In the group of 90 HBeAg positive patients, 2 (2.2%) 1896-G-to-A transitions leading to precore stop codon were found, and in the group of 65 anti-HBe positive children, 5 (7.7%) were identified harbouring HBeAg-minus mutants. The difference was not statistically significant ($P = 0.13$).

First, all sera were randomly selected for analysis without association with patients' identities. We were then able to additionally investigate four HBeAg positive sera of the five patients who showed precore-mutations in anti-HBe positive status of the disease in order to find out if mutations already emerged in the HBeAg positive status. One of them was also positive for the G1896A-substitution in the HBeAg positive phase. All mutations identified were found in HBV DNA sequences of genotype D (Figures 1, 2).

The ALT levels in the two HBeAg positive sera with precore-mutation were 883 and 2983 nkat/L, reflecting a considerable elevation in one patient compared to the wild type sera of HBeAg positive status (mean: 1100 nkat/L). In the five anti-HBe positive individuals with the G1896A-substitution, the ALT levels were mildly elevated (mean: 467 nkat/L) compared to the group of 60 patients with wild type virus infection (mean: 367 nkat/L).

DISCUSSION

In contrast to previous studies with adults in particular, which reported HBeAg stop codon mutants in 20-95% of investigated patients (6, 16), we found a very low prevalence in children, both in the HBeAg (2.2%) and anti-HBe positive (8%) phase of the disease. However, our results demonstrated consistently that precore variants were more common in anti-HBe positive children, but the difference did not reach statistical significance. We were

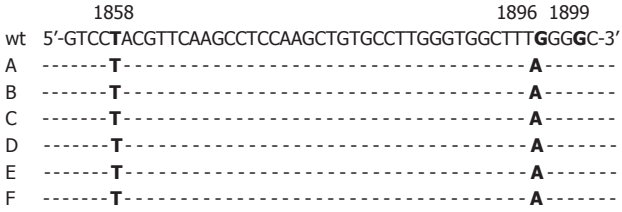

Figure 1 Precore region of HB virus with G1896A-substitution in 6 patients compared to the wild type-sequence (wt). Samples A and B were HBeAg positive. Samples while C-F were anti-HBe positive. All patients belonged to genotype D showing T at nucleotide position 1858.

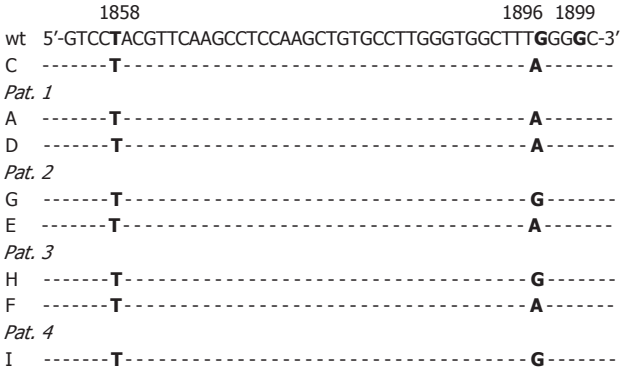

Figure 2 Precore region of HB virus in anti-HBe and HBeAg positive sera of 4 patients compared to the wildtype-sequence (wt). Samples A and C, G and D, H and E, I and F each belonged to one patient respectively. Samples C-F were anti-HBe positive, showing the G1896A-substitution. Samples A, G, H and I were HBeAg positive. All but sample A were negative for the mutant virus strain. Only one patient (No. 1) showed the G1896A-substitution both in anti-HBe and HBeAg positive serum (samples A and C). All patients belonged to genotype D showing T at nucleotide position 1858.

not able to show that mutations in anti-HBe positive sera have necessarily emerged already in the HBeAg positive phase of the disease. Thus, our data suggest that there is at least no strong evidence for the hypothesis that the mutant is selected by host immune pressure[13].

Due to the fact that the occurrence of the G1896A mutation is restricted to HBV genotypes with T at nucleotide position 1858 (genotype D)[17], it was not surprising that no mutations were identified in the group of genotype A virus strains. It has to be conceded that the existence of viral quasi-species including small amounts of precore stop mutations could not be excluded in our survey due to the method of direct sequencing. However, considering the high proportion of genotype D in our survey (48%), the postulation of a high prevalence of the G1896A precore mutation in these HBV strains[16] was not confirmed in chronically infected children.

It was reported that the precore mutation is common in Mediterranean and Asian populations, and two studies have shown a high prevalence of 16%-40% of the HBeAg minus variants in German adult populations as well[18,19]. It seems obvious that the age of the patients is more important than their ethnic origin.

Due to the elevated levels of transaminases in HBeAg positive sera, precore mutations may influence the inflammatory activity of the disease. But indeed, evidence is limited due to the small number of patients. In contrast to former studies, wherein a difference in clinical courses between genotypes B and C was described[20], this seems to be different for the most prevalent genotypes A and D in Europe, because they were equally distributed in our patients.

In summary, HBeAg minus variants as predominant viral HB strains play a minor role in the course of chronic disease in European children. Our results confirm the recent study of Söderström and colleagues who postulated that the most influential factors of HB-infection in childhood are epidemiologic parameters and the route of transmission[21].

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RAPID COMMUNICATION

Copper toxicosis gene *MURR1* is not changed in Wilson disease patients with normal blood ceruloplasmin levels

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Abstract

AIM: To analyze our Wilson disease patient cohort ($n = 106$) for alterations in the gene coding for *MURR1*.

METHODS: Patients with an established diagnosis of Wilson disease but normal ceruloplasmin blood levels were chosen for our study ($n = 14$). Patients with two known disease-causing mutations in the *ATP7B* gene were not included. The three exons of the human *MURR1* gene were sequenced after amplification of the genomic DNA by polymerase chain reaction.

RESULTS: Our study did not reveal any mutations leading to an amino acid change in the *MURR1* sequence of Wilson disease patients. A polymorphism at 472 bp of the coding sequence could be confirmed.

CONCLUSION: The *MURR1* gene plays no role in the pathogenesis of Wilson disease patients with normal serum ceruloplasmin levels.

Key words: Wilson Disease; ATP7B; MURR1; COMMD1

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INTRODUCTION

In humans, Wilson disease (WD) is an autosomal recessively inherited disorder of copper metabolism[1, 2] characterized by the impaired biliary excretion of copper. Wilson disease leads to toxic copper accumulation predominantly in the liver and brain, causing liver cirrhosis and severe neurological defects. Common clinical findings in WD are low serum ceruloplasmin (CP) levels, elevated hepatic copper contents, elevated urine 24-h copper excretion and Kayser-Fleischer rings[3]. Homozygous or compound heterozygous mutations in the copper-transporting P-type ATPase ATP7B lead to Wilson disease[4, 5].

The genetic background is highly variable, with more than 300 mutations reported so far[6]. But not all cases are unambiguous because no mutations in the *ATP7B* gene have been found in some WD patients. It is unclear why no *ATP7B* mutations are detectable in a subgroup of patients presenting with typical features of Wilson disease. It might be due to an incomplete analysis of the *ATP7B* gene or due to other yet unidentified defects of genes involved in copper metabolism.

The clinical presentation is highly variable even among patients with the same mutation. In Wilson disease a low serum ceruloplasmin level is a typical finding and can be observed in 80%-90% of the patients. Ceruloplasmin is a copper binding ferroxidase in blood[1]. Today's understanding of the underlying molecular mechanisms[7] is that ATP7B is predominantly localized to the trans Golgi network and transports copper across the membrane to the lumen of the Golgi apparatus where apoceruloplasmin is loaded with copper. In case of a malfunction of ATP7B, apoceruloplasmin can not be loaded with copper and is degraded more rapidly, resulting in reduced blood levels of ceruloplasmin. Under elevated copper conditions, ATP7B translocates from the trans Golgi network to a vesicular compartment where it may facilitate biliary copper excretion[8, 9].

Recently, the autosomal recessively inherited canine copper toxicosis has been described in Bedlington terriers. Like in Wilson disease these dogs develop copper accumulation in the liver due to impaired biliary copper excretion leading to chronic hepatitis and cirrhosis. Neurological abnormalities have not been reported. The genetic basis of this defect is a deletion of the exon2 of the *Murr1* gene[10-12] mapped to 10q26 in Bedlington terriers. The human orthologous gene has been identified on chromosome 2p13-16 and is distinct from the *ATP7B* gene locus[11]. Furthermore, affected dogs present with normal ceruloplasmin serum levels, suggesting that the defect is beyond the trans-Golgi network. A direct interaction be-

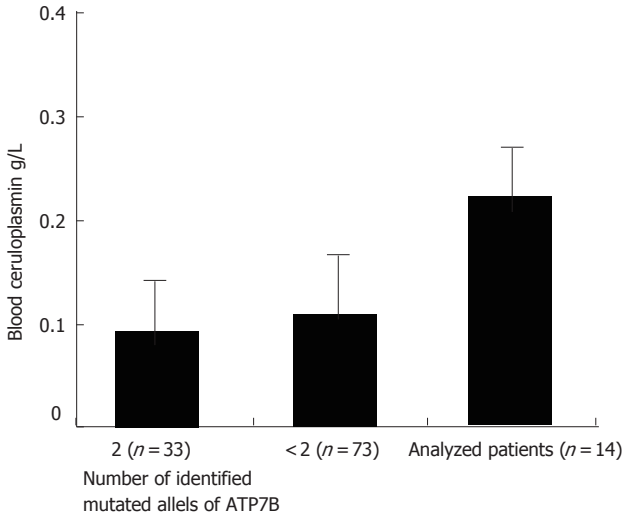

Figure 1 Mean ceruloplasmin blood levels in patients with two or less identified mutated alleles of ATP7B.

tween MURR1 and ATP7B has been reported[13]. There is biochemical evidence that decreased MURR1 levels lead to intracellular copper accumulation[14]. Based on these observations a role of MURR1 in the biliary copper excretion downstream of ATP7B has been suggested[15].

It would be interesting to identify a human disorder caused by defects in the human *MURR1* gene. Recently, a novel protein family (COMMD proteins) of structural and functional homologues of MURR1 (COMMD1) has been identified[16]. Recently, we reported an association between an *MURR1* polymorphism and onset of neurological and hepatic symptoms in WD patients homozygous for the most common *ATP7B* mutation H1069Q. Onset of disease was significantly earlier in patients with a heterozygous state at codon Asn 164 (GAT/GAC) than in patients with wild type (GAT/GAT)[17]. In the former study patients with low ceruloplasmin serum levels were included.

To identify possible disease related mutations in the *MURR1* gene in the current study we focused on patients with Wilson Disease but normal ceruloplasmin serum levels and at least one unknown mutation of *ATP7B*.

MATERIALS AND METHODS

Patients

Data of patients with an established diagnosis of Wilson disease were collected ($n = 106$). The diagnosis of Wilson disease was based on the criteria of the 8th International Conference on Wilson Disease and Menkes disease[19] (Leipzig, Germany April 16-18, 2001). For this study only patients with at least one undetermined mutation of the *ATP7B* gene were selected ($n = 73$), patients with two known disease defining mutations were excluded ($n = 33$). The *ATP7B* gene was sequenced in most patients in cooperation with Professor Ferenci, Department of Gastroenterology and Hepatology, Vienna, including the H1069Q mutation state in all patients. For our investigation out of the subset of WD patients without or with only one disease defining mutation, patients with normal or only slightly reduced ceruloplasmin levels were selected ($n = 14$) at the beginning of the study.

Ceruloplasmin cutoff was defined at a CP level of 0.15 g/L (normal in healthy persons: 0.2-0.6 g/L). Patients were excluded if the finding of a CP level above 0.15 g/L could be explained otherwise (e.g. contraceptive medication, pregnancy, acute phase reaction). A total of 14 patients with a CP level > 0.15 g/L and without any or with only one *ATP7B* mutation could be identified (Figure 1).

Mutation analysis of MURR1

Mutation analysis of *MURR1* was performed as described in detail previously[17]. In short, total DNA from the 14 selected patients was isolated and the three exons of the *MURR1* gene were amplified by polymerase chain reaction. The used primer combinations (exon 1 sense primer 5‘-GGT GGT TTT GCA CAG GCT ATT TAG-3‘, exon 1 anti-sense primer 5‘-GGC TTG TGA GGA CAG GGG AAG G-3‘; exon 2 sense primer 5‘-CAG TGA TTT AAG AGT CAC TC-3‘, exon 2 anti-sense primer 5‘-GCT GAA TAG ACA AGC TAA CAT GTA-3‘; exon 3 sense primer 5‘-GGG TAT TTT GAG TTT GGT CAT GC-3‘, exon 3 anti-sense primer 5’-TGA GAA CCT CTG CAC TGG AAC-3‘) resulted in PCR products covering the exons and parts of the 3` untranslated region and parts of the 5` region upstream of the start codon[17]. Additional putative exons or putative regulatory regions of the *MURR1* gene were not analyzed.

PCR products were purified as described previously[17]. Sequencing reactions were carried out by SEQLAB (Sequence Laboratories, Goettingen, Germany) or by MPI sequencing (Max Planck Institute, Dresden, Germany). For later analysis NM-152516 and AB17881 (NCBI sequence Viewer, http://www.ncbi.nlm.nih.gov/) were used as reference sequence. Nucleotide changes were numbered corresponding to their position in the *MURR1* mRNA beginning with the adenine of the ATG start codon.

RESULTS

The human orthologue of the *Murr1* gene encoded a protein of 190 amino acids. The gene spanned nearly 235 kb. Both introns were about 100 kb each in size (Figure 2). Therefore only the three exonic sequences were analyzed. In this study no mutations changing the amino acid sequence were found in the analyzed patients. A polymorphism at 472 bp of the coding sequence was detected (Table 1). Ten Patients (71%) were homozygous for wild-type GAT, 3 patients (21%) were heterozygous GAT/GAC and 1 patient was homozygous for GAC. The frequency of these variations was in line with previous reports[17]. We already reported a putative association between the GAT/GAC heterozygous state at codon Asn-164 with an earlier onset of disease in H1096Q *ATP7B* homozygous patients [17]. However, in the present study no significant genotype/phenotype correlation could be found, which might be due to the small number of patients.

DISCUSSION

Some patients with Wilson disease show no disease causing mutation in the *ATP7B* gene. Therefore other patho-

Table 1 MURR1 gene analysis in WD patients and associated CP blood values listed by WD gene mutation

Mutation in the *ATP7B* gene	*n*	*MURR1* gene base changes			CP level
		GAT/GAT	GAT/GAC	GAC/GAC	
2299InsC/ m n d.	1	1	-	-	0.15
3400DelC/ m n d.	1	1	-	-	0.18
G1030C/ m n d.	1	1	-	-	0.28
H1069Q/ m n d.	1	-	-	1	0.31
m.n.d./ m n d.	7	7	-	-	0.23 (± 0.09)
m.n.d./ m n d.	3	-	3	-	0.26 (± 0.08)

m n d: mutation not detected.

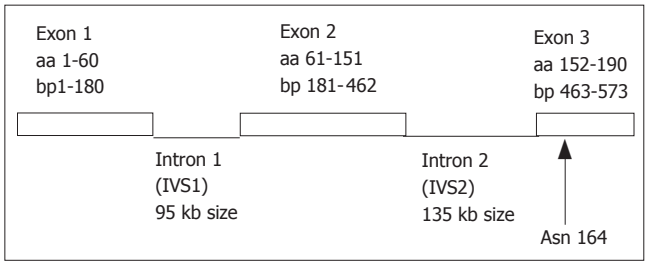

Figure 2 Map of the human *MURR1* gene. Localization of the reported polymorphism (492 T > C) in exon 3 is marked.

genetic factors might be involved. Due to the in part comparable phenotype of canine copper toxicosis and the reported interaction with *ATP7B*, the *MURR1* protein is an interesting candidate.

In this study we focused on Wilson disease patients showing a comparable phenotype to the copper toxicosis in dogs in regard to the normal ceruloplasmin serum level. However, in our group of patients with non-homozygous *ATP7B* mutation, no mutation in the coding sequence of the *MURR1* gene was found. Mutations in other parts of the Murr1 gene were not analyzed but could affect the gene expression and thus affect the phenotype. The finding that copper toxicosis gene *MURR1* is not changed in Wilson disease patients with normal blood ceruloplasmin levels needs to be evaluated in a larger clinical study.

Although our data do not necessarily rule out the possibility that MURR1 is involved in biliary copper excretion under physiological conditions, there is no direct evidence that it is indeed a disease-causing factor in human Wilson disease. This is in agreement with the findings of other investigators[17, 18]. In fact, a homozygous mutation of *ATP7B* resulting in the absence of this protein in the trans-Golgi network may be the only cause of Wilson disease. In our study population with detectable heterozygous or absent mutations of *ATP7B*, the other mutations may have not been identified yet. A low normal ceruloplasmin concentration was also observed in patients with homozygous mutations, suggesting that it may represent an undefined compensatory process by which apoceruloplasmin is loaded with copper by an *ATP7B* independent route, even outside the trans-Golgi network. This route seems to be less efficient but could result in low normal serum coeruloplasmin levels.

Although the possibility that *MURR1* is involved in biliary copper excretion in humans has not been ruled out, *MURR1* does not seem to play a role in the pathogenesis of Wilson disease.

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RAPID COMMUNICATION

Actigraphy: A new diagnostic tool for hepatic encephalopathy

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Abstract

AIM: To assess the actigraphy, an ambulatory and continuous monitoring of wrist motor activity fitted to study sleep/wake patterns in hepatic encephalopathy (HE).

METHODS: Twenty-five cirrhotic patients (17 M, 8 F, mean age 56 ± 11 years, 24/25 alcoholic, Child-Pugh A , B, C: 2, 6, 17) were included. The patients were classified into 3 groups: stage 0 group ($n = 12$), stage 1-2 group ($n = 6$), and stage 3-4 group ($n = 7$) of encephalopathy. Over three consecutive days, patients had clinical evaluation 3 times a day with psychometric test, venous ammoniemia, flash visually evoked potentials (VEP), electroencephalogram and continuous actigraphic monitoring for 3 d, providing 5 parameters: mesor, amplitude, acrophase, mean duration of activity (MDAI) and inactivity (MDII) intervals.

RESULTS: Serum ammonia and VEP did not differ among the 3 groups. Electroencephalography mean dominant frequency (MDF) correlated significantly with clinical stages of HE ($r = 0.65$, $P = 0.003$). The best correlation with HE stage was provided by actigraphy especially with MDAI ($r = 0.7$, $P < 10^{-4}$) and mesor ($r = 0.65$, $P < 10^{-4}$). MDAI correlated significantly with MDF ($r = 0.62$, 0.004) and was significantly shorter in case of HE compared to patients without HE (stage 0: 5.33 ± 1.6 min; stage 1-2: 3.28 ± 1.4 min; stage 3-4: 2.52 ± 1.1 min; $P < 0.05$). Using a threshold of MDAI of less than 4.9 min, sensitivity, specificity, positive predictive value, negative predictive value for HE diagnosis were 85%, 67%, 73% and 80%, respectively.

CONCLUSION: Actigraphy may be an objective method to identify HE, especially for early HE detection. Motor activity at the wrist correlates well with clinical stages of HE. MDAI and mesor are the most relevant parameters.

Key words: Hepatic encephalopathy; Cirrhosis; Actigraphy

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INTRODUCTION

Hepatic encephalopathy (HE) diagnosis is based on clinical criteria. Serum ammonia, evoked potentials and electroencephalogram (EEG) have low sensitivity[1-3]. Actigraphy allows ambulatory and continuous monitoring of motor activity and is fitted to study sleep/wake patterns. The actigraph worn on the non dominant wrist is based on a miniaturized acceleration sensor that translates physical motion to a numeric representation. It has already been successfully used in insomnia and extra pyramidal syndrome[4,5]. We assessed the actigraphy in HE.

MATERIALS AND METHODS

Twenty-five cirrhotic patients (17 M, 8 F, mean age 56 ± 11 years, 24/25 alcoholic, Child-Pugh A ,B, C: 2, 6, 17) were consecutively included. Patients with serum sedative or alcohol detection were excluded. Patients were classified into 3 groups: stage 0 group ($n = 12$), stage 1-2 group ($n = 6$), and stage 3-4 group ($n = 7$) of acute encephalopathy. HE causes were alcoholic hepatitis ($n = 5$), severe liver insufficiency ($n = 3$), digestive bleeding ($n = 1$), bacterial infection ($n = 1$), or undetermined ($n = 3$). Over three consecutive days, patients had clinical evaluation 3 times a day with the psychometric test Trail making test A when permitted by consciousness, venous ammoniemia using da Fonseca-Wollheim method, flash visual evoked potentials (VEP) with measurement of latencies of wavex N2, P2, N3, EEG and continuous actigraphic monitoring for 3 d using a small non dominant wrist-worn piezoelectric accelerometer (Gaehwiler electronic actimeter, CH 8634 Hombrechtiken, Switzerland) with a 0.1 G lower limit of sensitivity. Activity (wrist movements) measured at one-min intervals over a period of 24 h, was stored into the actigraph memory. Data downloaded to a compatible computer, were analysed by MONITOR software. The cosinor method or entire data linear adjustment by least-square method, provided 3 parameters: mesor (rhythm-adjusted mean), amplitude (half the variability between peaks to troughs), acrophase (peak time). Mean duration

of activity (MDAI) and inactivity (MDII) intervals independent of recording time, were measured. An activity interval was defined as a period during which the patient had more than 2 movements per minute, and an inactivity interval as a period during which the patient had less than 2 movements per minute. After 3 d, means of pooled data were calculated. Recording was made in hospitalized patients, so that physical activity was standardized.

Statistical analysis

Quantitative data were compared by ANOVA. Correlations were performed by Pearson test ($P<0.05$).

RESULTS

There was no difference between the 3 groups according to venous ammonia (41 ± 17 *vs* 46 ± 33 *vs* 65 ± 50 μmol/L respectively) or VEP (N3 = 201 ± 31 *vs* 205 ± 38 *vs* 227 ± 70 milliseconds). Electroencephalography mean dominant frequency (MDF) correlated significantly with clinical stages of HE ($r=0.65$, $P=0.003$). The best correlation with HE stage was provided by actigraphy especially with MDAI ($r=0.7$, $P<10^{-4}$) (Figure 1) and mesor ($r=0.65$, $P<10^{-4}$). Moreover MDAI correlated significantly with MDF ($r=0.62$, 0.004) and was significantly shorter in case of HE compared to patients without HE (stage 0: 5.33 ± 1.6 min versus stage 1-2: 3.28 ± 1.4 versus stage 3-4: 2.52 ± 1.1; $P<0.05$). In stages 0 and 1-2 of HE, MDAI correlated significantly with Trail making test A ($r=-0.61$; $P<0.05$). Using a threshold of MDAI of less than 4.9 min, sensitivity, specificity, positive predictive value, negative predictive value for HE diagnosis were 85%, 67%, 73% and 80%, respectively.

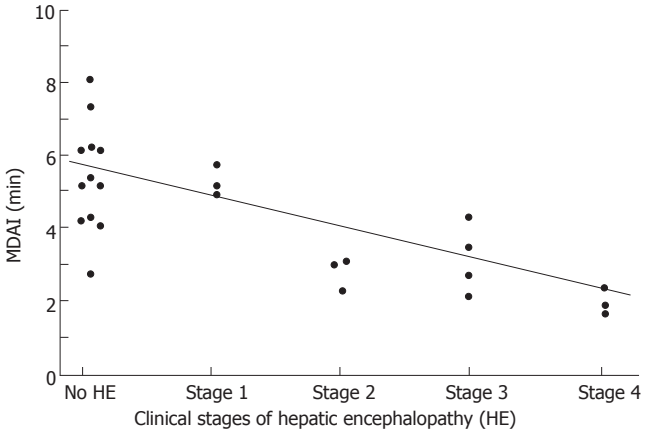

Figure 1 Correlation between clinical stages of hepatic encephalopathy and mean duration of activity intervals (MDAI), $r=0.7$, $P<0.0001$.

DISCUSSION

We suggest for the first time that actigraphy may be an objective method to identify HE. In our study, motor activity at the wrist recorded over three days, correlated well with clinical stages of HE. MDAI and mesor were the most relevant parameters. According to other works, neither serum ammonia[1] nor VEP[2] correlates with HE, contrary to EEG. However, EEG is a hospital procedure which has low specificity[3]. Using actigraphy, Cordoba *et al*[6] have previously shown a decrease of motor activity in cirrhotic patients without clinical evidence of HE. Seven out of 20 cirrhotic patients complained of unsatisfactory sleep, but they did not perform psychometric tests to screen subclinical encephalopathy. Actigraphy[4,5] is non invasive and simple to use. It can provide data under natural conditions over several days, which allows to take into account the oscillations in HE. One limit in our study is the decrease of motor activity due to bed-rest. However, we showed a significant relationship between actigraphy and encephalopathy. Actigraphy exhibits non specific changes in motor activity, but since we standardized physical activity, the observed changes might be due to HE. Our results suggest that actigraphy is especially fitted for early HE detection, since MDAI is significantly shorter in HE stage 1-2 patients compared to HE stage 0 patients. Moreover in cases of HE stages 0 and 1-2, MDAI, mesor, and acrophase correlate significantly with Trail making test A, a psychometric test routinely used to detect sub clinical encephalopathy. Further studies are needed to confirm actigraphy interest in sub clinical encephalopathy, a predictive factor of HE which is reversible by lactulose.

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RAPID COMMUNICATION

Hepatoprotective effect of manual acupuncture at acupoint GB34 against CCl_4-induced chronic liver damage in rats

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Abstract

AIM: To investigate the hepatoprotective effect of manual acupuncture at Yanglingquan (GB34) on CCl_4-induced chronic liver damage in rats.

METHODS: Rats were injected intraperitoneally with CCl_4 (1 mL/kg) and treated with manual acupuncture using reinforcing manipulation techniques at left GB34 (Yanglingquan) 3 times a week for 10 wk. A non-acupoint in left gluteal area was selected as a sham point. To estimate the hepatoprotective effect of manual acupuncture at GB34, measurement of liver index, biochemical assays including serum ALT, AST, ALP and total cholesterol, histological analysis and blood cell counts were conducted.

RESULTS: Manual acupuncture at GB34 reduced the liver index, serum ALT, AST, ALP and total cholesterol levels as compared with the control group and the sham acupuncture group. It also increased and normalized the populations of WBC and lymphocytes.

CONCLUSION: Manual acupuncture with reinforcing manipulation techniques at left GB34 reduces liver toxicity, protects liver function and liver tissue, and normalizes immune activity in CCl_4-intoxicated rats.

Key words: Manual acupuncture; Yanglingquan (GB34); CCl_4-induced liver damage; Hepatoprotective effect

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INTRODUCTION

Herbal medicine and acupuncture are the two main methods to treat disease in oriental medicine. Because of chemical residue contamination, there is recent gaining suspicion that herbs may be harmful to the liver. Accordingly, acupuncture is getting more interest these days for the treatment of liver diseases in oriental medical clinics. In the present study, we tried to investigate the effects of manual acupuncture on long-term liver damage. To investigate the effects of manual acupuncture at GB34 on liver damage, we used CCl_4-intoxicated rat model and chose GB34 as an acupoint to protect liver and treat liver damage induced by CCl_4 administration.

GB34 is an acupoint located on the gall bladder meridian. In oriental medical theory, the liver and gall bladder corresponds to each other and their meridians are also closely related with each other in the 'interior-exterior relationship'[1,2]. Gall bladder meridian pertains to the gall bladder organ and connects with the liver organ. The liver meridian pertains to the liver organ and connects with the gall bladder organ. Therefore, the acupoints on the liver meridian are used to treat gall bladder organ diseases as well as liver organ diseases. Consequently, the acupoints on the gall bladder meridian are used to treat liver organ diseases as well as gall bladder organ diseases. Hence, GB34 is closely related with the liver as well as the gall bladder. It explains why GB34 is used to treat liver disease so often. Moreover, GB34 is the *He* (meaning "sea") point of gall bladder. In oriental medical theory, a "sea *He*" point is considered to be the entrance of the meridian energy to the corresponding organ[3]. Therefore, GB34 influences the liver and gall bladder more strongly than other acupoints. The functions of this point are regulating and tonifying the liver, regulating the gallbladder, spreading liver *Qi* (oriental medical term for "vital energy"), subduing liver *Yang*, draining liver pathogens, *etc*. GB34 has been clinically used for hypochondriac pain, jaundice, hepatitis, acute biliary tract diseases, cirrhosis of the liver and hypertension due to liver *Yang* excess, *etc*[1,4].

We presume that neuronal activity is involved in the transmission of acupuncture stimulation, so the animals were not anesthetized during the acupuncture administra-

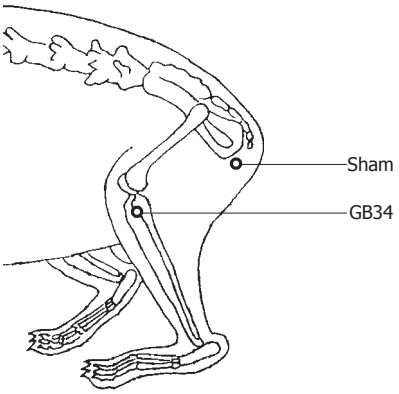

Figure 1 GB34 and sham point for acupuncture.

tion. To keep the animals from moving during the acupuncture administration, they were put in cages with five holes for tail and four limbs. To estimate and exclude the effect of stress from restriction within the cage, the rats in the control group were also kept in the cages in the same manner as the acupuncture group.

The action of acupuncture could be influenced by acupuncture techniques as well as point selection. There are two main categories of acupuncture techniques: reinforcing technique and reducing technique. Clockwise needle rotation, scraping downward of needles, and odd number of manipulating operations are considered as reinforcing techniques. On the other hand, counterclockwise needle rotation, scraping upward of needles, and even number of manipulating operations are considered as reducing techniques. Reinforcing acupuncture manipulation techniques are used for chronic and deficient syndrome, while reducing techniques are used for acute and excess syndrome [1]. Since the animals in the present study were injected with CCl_4 for a long period, we considered it as a chronic and deficient condition, and therefore, we administered acupuncture with reinforcing manipulation techniques.

MATERIALS AND METHODS

Animals

Sprague-Dawley male rats (200-250 g) were purchased from Deahan Biorink Co. The animals were adapted to the environment of 22±2℃ room temperature, 12-h light/dark cycle for 2 wk and had free access to water and food. Our animal experiment has been conducted in accordance with the Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Experimental design

Experimental animals were randomly divided into four groups: normal; control (CCl_4); Sham (CCl_4 + manual acupuncture at sham point); and GB34 (CCl_4 + manual acupuncture at left GB34). Each group consisted of 7 rats. Liver injury was induced by intraperitoneal injections with 500 mL/L CCl_4 (Sigma, USA) solution in olive oil (1 mL/kg), twice a week for 10 wk. Manual acupuncture was administered 3 times a week during the same period[5].

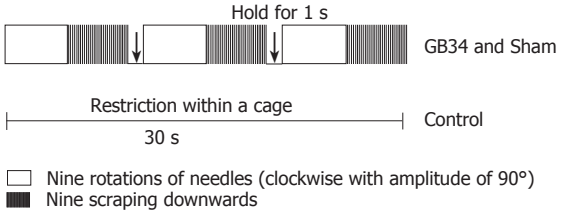

Figure 2 Acupuncture scheme.

Acupuncture

Cages with five holes for the tail and four limbs were manufactured for this study. During acupuncture administration, the animals were kept in the cage with left hind limb fastened to the wall of the cage with tape. Sterilized disposable stainless steel needles (0.25 mm × 30 mm, Dongbang Acupuncture Inc., Korea) were inserted perpendicularly as deep as 2-3 mm at left GB34 or sham point. Rat GB34 was determined according to human GB34 which locates on gall bladder meridian, in the depression anterior and inferior to the head of the fibula[1]. A non-acupoint in the gluteal region was selected as a sham point (Figure 1). After the needles were inserted, they were rotated clockwise with amplitude of 90° nine times, and scraped downward nine times. Nine rotations and nine scrapes constituted one manipulation unit, and three manipulation units constituted one treatment session. After each manipulation unit, there was a hold for about 1 s. The full manipulation took about 30 s. The rats in the control group were kept in a cage for 30 s with the left hind limb tied without acupuncture treatment (Figure 2). All acupuncture was administered by a trained and experienced oriental medical doctor who was unknown about the research protocol except the needle manipulating methods.

Liver index measurement

Rat's body weight was measured before the animals were sacrificed. Rat liver was removed and weighed right after the animal was sacrificed and the liver index (% of liver weight/body weight)[6,7] was estimated.

Biochemical analysis and blood counts

Forty-eight hours after the last administration with CCl_4, the rats were anesthetized with ethyl ether and blood samples were taken from the heart. Blood was centrifuged at 3000 r/min for 15 min and serum was taken. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphotase (ALP), total cholesterol in serum and the populations of RBC, WBC, lymphocytes in plasma were detected.

Histological analysis

The rats were sacrificed and the liver tissues were obtained individually from each group and fixed in 40 g/L formaldehyde. After decalcification in 50 mL/L formic acid, the specimens were processed for paraffin embedding. Tissue sections were obtained and stained with hematoxylin and eosin (HE) or masson's trichrome (MT). Tissue destruction and fatty changes of liver were observed at 400× magnification.

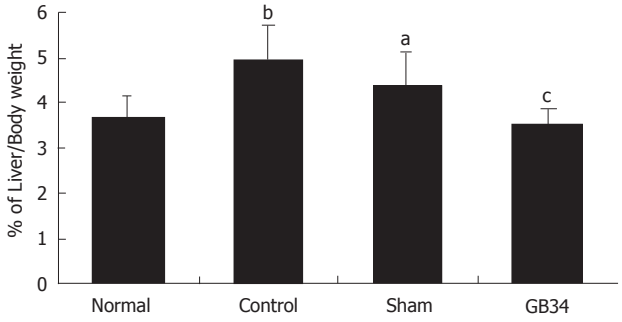

Figure 3 Effect of manual acupuncture at BG34 on liver index of CCl_4-intoxicated rats. [a]$P < 0.05$, [b]$P < 0.01$ *vs* normal group; [c]$P < 0.05$ *vs* control group.

Statistical analysis

Data were obtained from the rats which survived to the end of the experiment. All in normal group, 3 in control group, 3 in sham group and 5 in GB34 group survived to the end of the experiment. Data were expressed as mean ± SD. Statistical significance of difference between groups was determined using ANOVA, followed by *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Liver index

CCl_4 injection induced a significant increase in liver index. On the other hand, manual acupuncture at GB34 lowered it similar to the normal value (Figure 3).

Serum ALT, AST, ALP and total cholesterol

ALT, AST, ALP and total cholesterol in serum were increased remarkably by long-term CCl_4 administration, indicating damage to the liver. Manual acupuncture at GB34 significantly reduced serum ALT, AST and total cholesterol in comparison with the control group. Serum ALP was also reduced by manual acupuncture at GB34 but no statistical significance was found (Figure 4).

Blood cell counts

The number of RBC was slightly reduced by CCl_4 intoxication and restored by manual acupuncture at GB34, though statistical significance was not observed. The number of WBC and the percentage of lymphocytes out of WBC was significantly reduced by CCl_4 intoxication and restored by manual acupuncture at GB34 close to the normal level (Figure 5).

Liver histology

Histological analysis using HE stain showed that necrosis of liver tissue and fatty changes were viciously induced by CCl_4 administration. The group treated with manual acupuncture at GB34 showed reduced feature of hepatocyte necrosis and fatty change compared to the control group and the sham group. In addition, MT staining showed lower accumulation of extracellular matrix in the GB34 group compared to the control group and sham group (Figure 6).

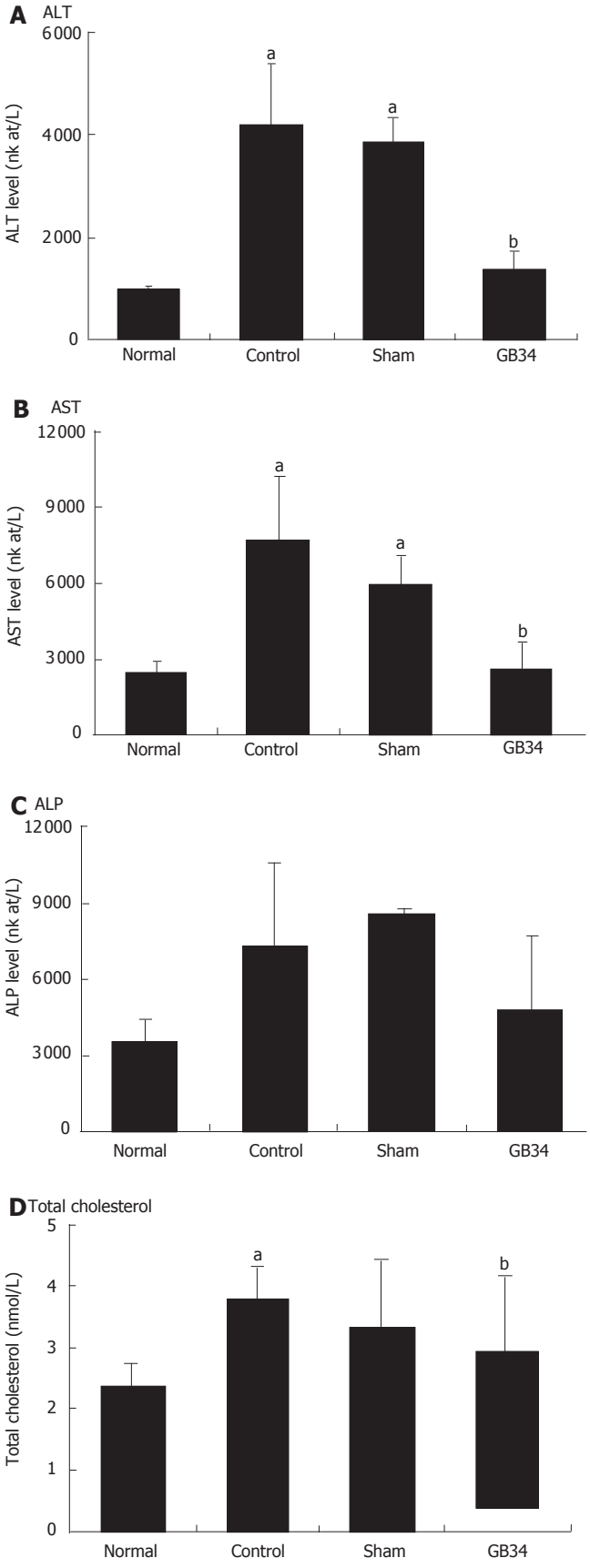

Figure 4 Effect of manual acupuncture at GB34 on serum ALT, AST, ALP and total cholesterol of CCl_4-intoxicated rats. [a]$P < 0.01$ *vs* normal group; [b]$P < 0.05$ *vs* control group

DISCUSSION

CCl_4 has been widely used to induce experimental hepatic

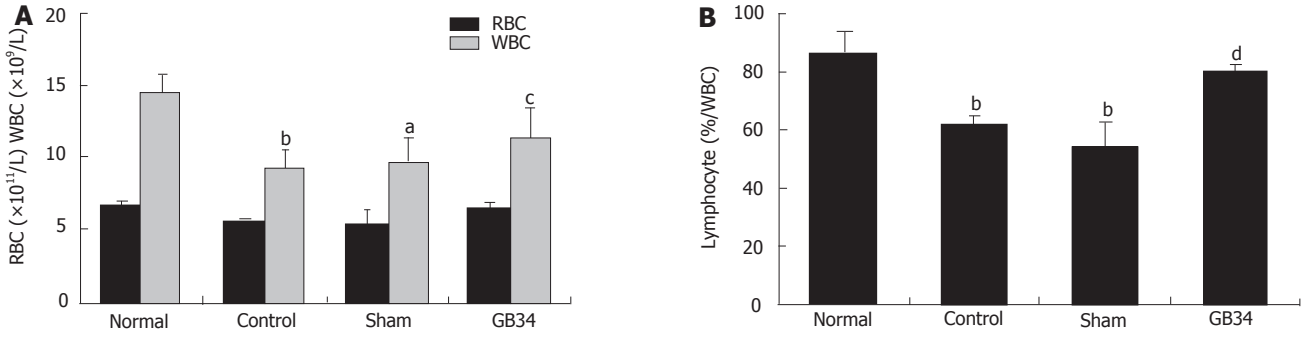

Figure 5 Effect of manual acupuncture at GB34 on RBC, WBC and lymphocytes in blood. $^{a}P<0.05$, $^{b}P<0.01$ *vs* normal group; $^{c}P<0.05$, $^{d}P<0.001$ *vs* control group.

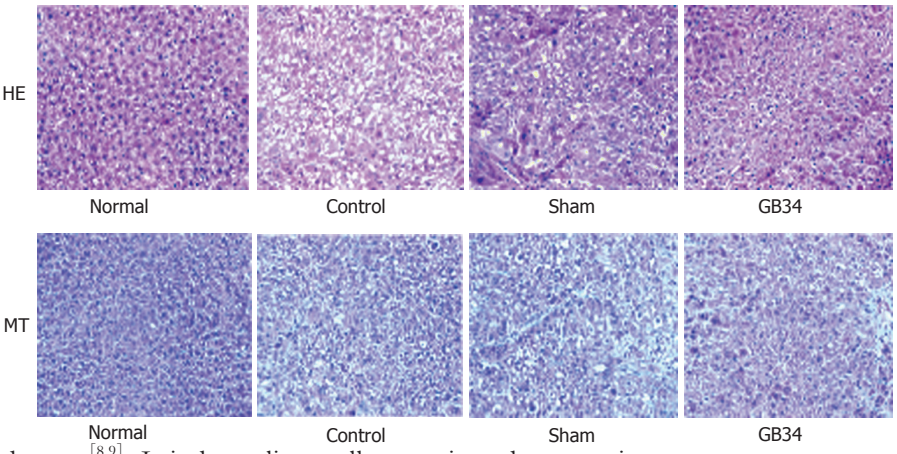

Figure 6 Histological results of liver tissue. Necrosis and fatty change could be found in the control group (top). GB34 group revealed lower accumulation of extracellular matrix compared to the control group and sham group (bottom) (× 400 magnification)

damage[8,9]. It induces liver cell necrosis and apoptosis, and can be used to induce hepatic fibrosis or cirrhosis by repetitive administration[10-12]. Liu *et al*[13] investigated the effect of manual acupuncture at ST36 and LR3 on CCl_4-induced acute liver damage. In the present study, we investigated the effect of manual acupuncture at GB34 on chronic liver damage induced by long-term CCl_4 administration.

ALT and AST are the specific markers to assess hepatocellular damage leading to liver cell necrosis[14]. Slight to moderate increases in ALP (1-2 times normal) occurred in liver disorders[15]. Serum cholesterol is one of the general indications of the synthetic and general metabolic capacity of the liver[16]. In the present study, CCl_4 injection significantly increased serum ALT, AST, ALP and cholesterol levels, indicating induction of hepatic damage. Manual acupuncture at GB34 inhibited the increases of these parameters, indicating that manual acupuncture at GB34 protected liver and reduced liver toxicity. Histological analysis also showed that the acupuncture at GB34 protected liver tissue against CCl_4-intoxication. Our histological analysis in this study was only qualitative. Quantitative assessment of anti-fibrotic effect of acupuncture, such as determination of hepatic hydroxyproline

content, would be meaningful in the next study.

In the present study, long-term CCl_4 administration reduced the population of leucocytes and lymphocytes in blood. We infer that this reduction was due to the decline of immune activity by long-term liver damage. Manual acupuncture at GB34 recovered the population of leucocytes and lymphocytes in blood. Therefore, we presume that manual acupuncture at GB34 restored the immune activity, which is also in agreement with Hau's report on the white cell increase by acupuncture[17]. However, the effect of acupuncture on inflammation in liver is not clear yet. It is known that inflammatory cells are infiltrated in CCl_4-intoxicated liver, and there exists an abnormality of cytokines in liver tissue. For instance, TNF-α and IL-10 are increased, and IL-2 and IFN-γ are reduced in CCl_4-intoxicated liver tissue[18,19]. More profound studies are required on how acupuncture influences the chronic inflammatory conditions of the liver.

Based on the results of the present study, we speculate that manual acupuncture at GB34 is beneficial to protect liver function and tissue, reduce hepatic toxicity and normalize immune activity against CCl_4-intoxication in rats. The hepatoprotective effect of manual acupuncture at GB34 in this study may be related to the immune reinforcing effect of acupuncture or neuro-immune interaction on the pathway of the transmission of acupuncture stimulation.

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RAPID COMMUNICATION

Aberrant expression of krÜppel-like factor 6 protein in colorectal cancers

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Abstract

AIM: To investigate whether krÜppel-like factor 6 (KLF6) plays an important role in the development and/or progression of colorectal cancer.

METHODS: A total of 123 formalin-fixed and paraffin-embedded colorectal cancer specimens were analyzed by immunohistochemistry using tissue microarray for the expression of KLF6 protein. The specimens were collected over a 3-year period in the laboratories at our large teaching hospital in Seoul, Republic of Korea. The correlation of KLF6 expression with clinicopathologic parameters was analyzed by χ^2 test and Bartholomew test.

RESULTS: Normal colonic epithelium showed weak to moderate expression of KLF6, whereas reduced KLF 6 expression or loss of KLF6 expression was seen in 45 (36.6%) of the 123 colorectal carcinoma specimens. Interestingly, aberrant expression of KLF6 was detected in 25 (43.1%) of 58 cases with metastasis to regional lymph node and in 31 (47.0%) of 66 tumors more than 5 cm in size. Statistically, loss of KLF6 expression was significantly associated with tumor size ($P < 0.05$). However, there was no significant correlation between KLF6 expression and Dukes' stage (Bartholomew test, $P > 0.05$), tumor location and lymph node metastasis (χ^2 test, $P > 0.05$).

CONCLUSION: Loss of KLF6 expression may be a common and early event in colorectal carcinogenesis.

Key words: KLF6; Mutation; Immunohistochemistry; Aggressiveness; Tumor stage; Colorectal cancer

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INTRODUCTION

Colorectal cancer is the second leading cause of cancer-related death in the Western world[1]. In Korea, it accounts for an estimated 11.2% of all malignancies, with 11.6% in the male population and 10.7% in the female population[2]. Thus, colorectal cancer remains a significant contributor to the world's health burden. At a molecular level, great progress has been made in the last two decades in identification and characterization of the genetic changes involved in the malignant colorectal transformation process. Thus, the concept of multistage carcinogenesis is now widely accepted as a consequence of multiple genetic alterations accumulated in cancer cells [3]. However, the molecular mechanisms underlying dysregulated cell growth in colorectal cancer remain the subject of intensive investigation.

Krüppel-like factor 6 (KLF6) is a ubiquitously expressed zinc finger transcription factor that is part of a growing KLF family. The KLF family is broadly involved in growth-related signal transduction, cell proliferation, development and apoptosis, as well as angiogenesis[4, 5]. In functional analysis, wild-type KLF6 up-regulates the cell cycle inhibitor p21 in a TP53-independent manner and suppresses growth, whereas tumor-derived KLF6 mutants fail to upregulate p21 or suppress proliferation in prostatic and non-small cell lung cancer cells[6, 7]. In addition, introduction of KLF6 disrupts cyclin D1-dependent kinase 4 complexes and forces the redistribution of p21, which promotes G1 cell cycle arrest[8]. Furthermore, KLF6 plays

Table 1 Relationship between KLF6 expression and clinicopathologic parameters of colorectal carcinoma

Clinicopathologic parameters	KLF6 +	KLF6 -	Positive (%)	*P*
Stage				0.2969[1]
A	11	2	84.6	
B	29	18	61.7	
C	32	23	58.2	
D	6	2	75.0	
Lymph node metastasis				0.1754[2]
+	33	25	56.9	
-	45	20	69.2	
Site				0.0730[2]
Right	12	13	48.0	
Left	66	32	67.3	
Tumor size				0.0100[2]
> 5 cm	35	31	53.0	
≤ 5 cm	43	14	75.4	
Total	78	45	63.4	

[1]Bartholomew test; [2]χ^2 test.

a role as an inhibitor of cell proliferation by counteracting the function of the c-Jun protooncoprotein[9].

Recently, genetic alterations of *KLF6* gene have been identified in several human cancers, including colorectal cancer, hepatocellular carcinoma, prostate and gastric cancer[10-13]. Reduced expression of KLF6 protein has been found in non-small cell lung cancers and glioblastomas[7, 14]. Interestingly, the survey of gene expression in a panel of 60 NCI cancer cell lines showed that expression of KLF6 mRNA was reduced in colon cancer cell lines[15]. However, there is no report describing the expression pattern of KLF6 protein in colorectal cancers.

In the present study, to determine whether altered expression of the KLF6 protein is involved in colorectal cancer development, we analyzed the expression pattern of KLF6 on a series of 123 colorectal cancer specimens.

MATERIALS AND METHODS

Tissue samples

One hundred and twenty-three formalin-fixed and paraffin-embedded colorectal cancer specimens collected between 2002 and 2003 were enrolled in this study. No patient had a family history of colorectal cancer. Tumor stage was classified according to Dukes' criteria. Thirteen patients were classified as Dukes' A, 47 as Dukes' B, 55 as Dukes' C and 8 as Dukes' D[16]. Two pathologists screened histological sections and selected areas of the representative tumor cells. Three tissue cores (0.6 mm in diameter) were taken from each tumor sample and placed in a new recipient paraffin block using a commercially available microarray instrument (Beecher Instruments, Micro-Array Technologies, Silver Spring, MD) as previously described[17]. One cylinder of normal colonic mucosa adjacent to each tumor was also transferred to the recipient block.

Immunohistochemistry for KLF6

The primary polyclonal rabbit anti-KLF6 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used. Immunostaining was performed on microarray tissue sections with a tyramide signal amplification kit (NEN Life Science, Boston, MA) for signal intensification. Antigen retrieval was performed by microwave heating in a citrate buffer (pH 6.0). Other procedures were performed as previously described[18]. Reaction products were developed with diaminobenzidine (Sigma, St Louis, MO) and counterstained with hematoxylin. Because all the corresponding normal colonic mucosae showed KLF6 expression, tumors were considered as negative when nuclear and cytoplasmic staining was seen in less than 20% of cancer cells. We calculated the KLF6 positive and negative cancer cells in three representative areas at X200 magnification. We only considered the percentage of stained nuclei in each tumor, independent of the intensity. Three pathologists independently reviewed the results and those cases with discrepant results were discussed until agreement was achieved. As a negative control, we used non-immune rabbit serum instead of the KLF6 antibody.

Statistical analysis

The correlation of KLF6 expression with clinicopathologic parameters, such as tumor site, size, lymph node metastasis and clinical stage, was analyzed by χ^2 test and Bartholomew test. $P < 0.05$ was considered statistically significant.

RESULTS

Expression of KLF6

We assessed the pattern of KLF6 protein expression in 123 colorectal carcinoma specimens by immunohistochemistry using tissue microarray. The KLF6 expression was positive in cytoplasm and nuclei of normal colonic mucosal epithelial cells, but weak to moderate in lymphocytes and fibroblasts. The expression of KLF6 in tumor cells was compared to the expression in their corresponding normal colonic mucosae. Interestingly, reduced KLF6 expression or loss of KLF6 expression was found in 45 (36.6%) of the 123 colorectal carcinoma specimens, in which immunostaining on cytoplasm and nuclei of tumor cells was predominantly (Figure 1). Unexpectedly, overexpression of the KLF6 in cancer was also found in 12 cases. Negative immunoreactivity was seen in 15.4% (2 of 13 cases) of stage A, 38.3% (18 of 47) of stage B, 41.1% (23 of 56) of stage C, and 25.0% (2 of 8) of stage D, respectively (Table 1). Loss of expression was found in 25 (43.1%) of 58 cases with metastasis to regional lymph nodes and 32 (32.7%) of 98 left-side colorectal cancers. In addition, immunostaining was negative in 31 (47.0%) of 66 cases with tumors >5 cm. Statistically, loss of KLF6 expression was closely associated with tumor size (χ^2 test, $P < 0.05$). However, there was no significant correlation between KLF6 expression and Dukes' stage (Bartholomew test, $P > 0.05$), tumor location and lymph node metastasis (χ^2 test, $P > 0.05$).

DISCUSSION

It has been reported that several KLFs play an essential role in maintaining the homeostasis of mammalian cells through the regulation of proliferation and

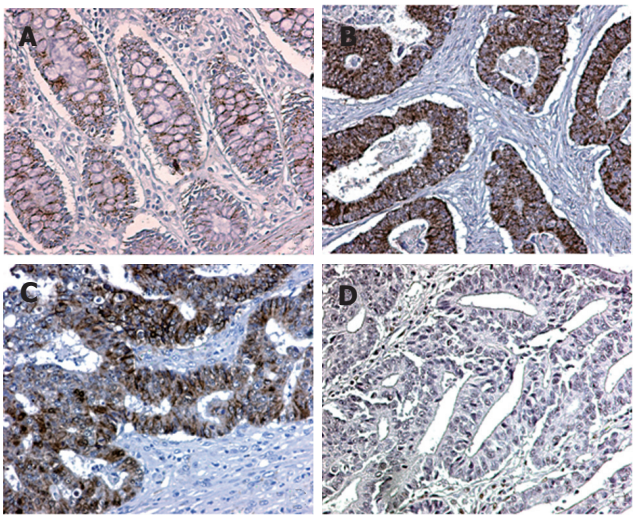

Figure 1 Expression of KLF6 protein in colonic mucosa and cancer. **A:** Positive reaction mainly in colonic glandular epithelial cells; **B:** Predominantly strong immunopositivity in cytoplasm of colon cancer cells; **C**: Predominantly strong immunopositivity in nuclei of colon cancer cells; **D**: Negative staining of KLF6 in cancer cells (original magnifications: X 200).

differentiation[4, 5]. The KLF6 is regulated during mammalian development and tissue regeneration, while its aberrant expression is associated with tumor formation. Recently, KLF6 has been identified as a tumor suppressor gene in several kinds of cancer because of reduced expression and its frequent genetic alterations, such as loss of heterozygosity and mutation, as well as functional suppression of cell proliferation. To further clarify the role of KLF6 in colorectal cancer, we examined loss of expression in 123 colorectal cancer samples.

In this study, reduced KLF6 expression or loss of KLF6 expression was found in 45 (36.6%) of the 123 colorectal adenocarcinoma specimens. Statistically, loss of KLF6 expression was closely associated with tumor size (χ^2 test, $P<0.05$). However, there was no significant correlation between reduced KLF6 expression and other clinicopathologic parameters, including Dukes' stage (Bartholomew test, $P>0.05$), tumor location and lymph node metastasis (χ^2 test, $P>0.05$). It has been reported that the expression of KLF6 protein is frequently down-regulated in non-small cell lung cancer and glioblastoma [7, 14]. Interestingly, down-regulation of KLF6 expression is not associated with the histology or surgical-pathological stages of lung tumors[7]. Taken together, these results further support that down-regulation of KLF6 may contribute to the development of human solid cancers and that inactivation of KLF6 may occur in the development of colorectal cancer as an early event.

Unexpectedly, upregulation of KLF6 was also seen in 14 colorectal cancer cases. Since KLF6 is broadly involved in growth-related signal transduction, cell proliferation, differentiation and apoptosis, upregulation of KLF6 might reflect compensatory mechanisms directed at reestablishing the equilibrium in proliferation, differentiation, and survival characteristics of colonic epithelial cells disrupted during carcinogenesis. Because mutation of the KLF6 is frequently found in colorectal cancer [13], over-expression of KLF6 might be caused by the genetic alteration of the *KLF6* gene. Further studies are necessary to investigate the precise molecular mechanisms underlying its over-expression.

Tissue microarray (TMA) consists of numerous tissues and tumor types. The use of TMA can stain a large number of cases at minimal cost and allow a rapid assessment of the staining profile. However, whether cores of tumor are representative of whole sections remains unclear. It was reported that immunostaining of one to four 0.6-mm cores is correlated with whole sections staining [19], suggesting that the use of small cores is equivalent to whole section immunohistochemistry. Another issue for consideration is that the scoring of TMA immunohistochemistry experiments remains subjective and not easily quantifiable. Significant efforts have been made to quantify protein expression levels on TMA sections [20], but most TMAs are subjectively scored by eyes. In this study, expression of KLF6 in three cores of tumor tissues was compared to their corresponding normal colonic mucosae by three pathologists. Although we did not perform immunohistochemistry on whole section, our results on TMA may be consistent with those of whole section immunohistochemistry.

Generally, the inactivation of a tumor suppressor associated with loss of expression results from genetic or epigenetic alterations, such as mutation, allelic loss or hypermethylation of the promoter region of the genes. Recently, genetic alterations of *KLF6* gene have been identified in several human cancers, including hepatocellular carcinoma, prostate, gastric and colon cancers[10-13]. However, no mutation of KLF6 has been found in human cancer[21-23]. Further studies on a large number of patients are necessary to find out the discrepancy in incidence of *KLF6* mutation. Interestingly, the *KLF6* mRNA is also frequently down-regulated in non-small cell lung cancer, glioblastoma, and colon cancer cell lines[7, 14, http://smd.stanford.edu/cgi-bin]. No mutation has been found in these cancer tissues and KLF6 is down-regulated in all samples with allelic loss[7]. Additionally, the *KLF6* is suggested to be a methylation-silenced gene in esophageal squamous cell carcinoma[24]. In the present study, we found that KLF6 expression was reduced or lost in 36.6% of colorectal cancers (Table 1). Although we did not perform the genetic or epigenetic analysis of the *KLF6* gene, our results suggest that reduced KLF6 expression or loss of KLF6 expression may be a common and early event in colorectal carcinogenesis.

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RAPID COMMUNICATION

Acute myopathy associated with liver cirrhosis

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Abstract

AIM: Many cirrhotic patients have muscular symptoms and rhabdomyolysis. However, myopathy associated with liver cirrhosis has not been established as a disease entity. We evaluated the clinical significance of acute myopathy associated with liver cirrhosis.

METHODS: We retrospectively reviewed the medical records of 5440 cirrhotic patients who had been admitted to Gyeongsang National University Hospital from August 1997 to January 2003. Among these, 99 developed acute myopathies, and they were analyzed with respect to clinical and laboratory parameters, and outcomes.

RESULTS: The Child-Pugh classification at the time of myopathy onset was A in 3(3.1%) cases, B in 33(33.3%), and C in 63 (63.6%). Infection was identified as the most predisposing factor to myopathy. Fifty percent of 18 idiopathic cases who were tested for influenza antibody were positive. Forty-two of the 99 cases were complicated by acute renal failure, and 25 (59.5%) of these expired. Apart from 6 cases lost to follow-up, 64 of 93 recovered, giving a mortality rate of 31.2%. Mortality was higher in Child-Pugh class C than in B or A.

CONCLUSION: Acute myopathy can develop as a serious complication in liver cirrhosis. Its frequency, severity and mortality depend on underlying liver function, and are higher in decompensated liver cirrhosis. Influenza should be considered as an etiologic factor in idiopathic cases. It is proposed that acute myopathy associated with liver cirrhosis be called 'hepatic myopathy', and that careful monitoring for hepatic myopathy is necessary in the patients with advanced liver cirrhosis.

Key words: Myopathy; Rhabdomyolysis; Liver cirrhosis

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INTRODUCTION

Since many investigators[1-3] had been interested in a causal relationship between alcohol ingestion and acute myonecrosis, Martin *et al*[4] systematically described the clinicopathological features of acute and chronic myopathy associated with alcoholism, and defined this as alcoholic myopathy. Now, it is well known that alcoholism and alcoholic liver diseases can be accompanied by alcoholic skeletal myopathy[5,6]. Moreover, a higher prevalence of muscle cramps was reported in patients with liver cirrhosis than in a matched population without cirrhosis, and it was suggested that muscle cramps be included as a recognized symptom of cirrhosis[7]. Muscular symptoms such as muscle cramps, weakness, aching and tenderness are also common in patients with liver cirrhosis, and even rhabdomyolysis can occur[8-10]. However, myopathy associated with liver cirrhosis has not been established as a disease entity. Here, we investigated the clinical characteristics of acute myopathy, which developed in patients with liver cirrhosis, and evaluated its clinical significance.

MATERIALS AND METHODS

Inclusion criteria

Of the 5440 patients with liver cirrhosis who had been admitted because of various problems related to cirrhosis to Gyeongsang National University Hospital from August 1997 to January 2003, 99 patients who developed acute myopathy were included. We reviewed and analyzed their medical records retrospectively so to find out predisposing or etiologic factors, and possible factors influencing the frequency, severity, and prognosis of acute myopathy associated with liver cirrhosis.

A diagnosis of liver cirrhosis was made based on liver biopsy, or clinical, laboratory and radiological evidence of chronic hepatocellular dysfunction and portal hypertension. Underlying liver function was assessed by Child-Pugh's classification based on laboratory data and physical signs at the time of most recent visit.

Acute myopathy was defined as an elevated serum muscle enzymes, i.e., creatine kinase (CK, 45-390 IU/L), lactate dehydrogenase (LDH, 30-170 IU/L), and aspartate

Table 1 Clinical characteristics of patients with liver cirrhosis who developed acute myopathy

Clinical parameters	Number of cases ($n=99$)
Sex (M:F)	74:25 (2.96:1)
Age (yr) range	27 - 76
mean	51 ± 10
Etiology of LC	
Alcohol	47
CVH (B)	41
CVH (C)	5
Wilson's disease	1
Idiopathic	5
Child-Pugh Class	
A:B:C	3:33:63

LC, Liver cirrhosis; CVH, Chronic viral hepatitis.

Table 2 Predisposing factors of acute myopathy in patients with liver cirrhosis ($n=99$)

Predisposing factors	Number of cases (%)
Infection	46 (46.5)
Respiratory infection	17
Gastrointestinal infection	12
Genitourinary infection	8
Septic shock	5
Alcohol	8 (8.1)
Exercise or trauma	8 (8.1)
Transarterial embolization	4 (4.0)
Herb medicine	3 (3.0)
Dehydration	2 (2.0)
Gastrointestinal bleeding	1 (1.0)
Idiopathic	27 (27.3)

transaminase (AST, 5-45 IU/L), and/or accompanied by acute muscular symptoms, such as muscle cramps, generalized or localized muscle weakness, muscle edema, muscle pain, or tenderness.

Possible predisposing factors were defined as the events occurring within one week prior to the onset time of myopathy.

Acute renal failure was diagnosed when the serum creatinine level increased by more than 50% of the baseline value, to above 1.5 mg/dL[11]. If there was no improvement in serum creatinine despite optimization of intravascular volume in azotemia, hepatorenal syndrome was diagnosed[12].

Exclusion criteria

Exclusion criteria were a history of myocardiac infarction, cardiomyopathy, cerebrovascular accident and other cardiovascular diseases, thyroid disease, peripheral neuropathy, phlebitis, primary muscle disease, heat stroke, and underlying kidney disease, and the ingestion of agents known as triggering agents of myopathy such as digitalis, cimetidine, clofibrate, lithium, opiate, nifedipine, beta 2-agonist, beta-blockers, penicillamine (except for Wilson's disease), cyclosporine, quinidine, antispastic drugs, verapamil, amphetamine, cocaine, vitamin E, or taurine[9,13].

RESULTS

General characterisitics

Ninety-nine (1.8%) of 5440 patients with liver cirrhosis developed acute myopathy. With exception of 4 patients who developed myopathy during hospitalization after transarterial embolization, 95 patients were admitted due to acute myopathy. Seventy-four patients were male, giving a male to female ratio of 2.96. Mean age of patients was 51 ± 10 years (range; 27-76 years), and most were in the 6th decade. The etiology of the liver cirrhosis was alcohol in 47, hepatitis B virus in 41, hepatitis C virus in 5, Wilson's disease in 1, and cryptogenic in 5 (Table 1).

Underlying liver function

Child-Pugh classification at the time of myopathy onset was A in 3 (3.0%), B in 33 (33.3%), and C in 63 patients (63.6%) (Table 1).

Etiologic factors

The most predisposing factor to myopathy was infection (46.5%); respiratory tract infections including common cold in 17, gastrointestinal infections in 12, urinary infection in 8, and septic shock in 5. Alcohol (8.1%), exercise or trauma (8.1%), transarterial embolization (4.0%), herb medicine (3.0%), dehydration (2.0%), and gastrointestinal bleeding (1.0%) were also possible predisposing factors of acute myopathy, and 27 cases (27.3%) were idiopathic (Table 2). In cases of transarterial embolization, acute myopathy developed after hepatic arterial embolization for primary liver cancer in two, and after splenic arterial embolization for splenomegaly with severe pancytopenia in two. Influenza antibody was checked in 18 of the 27 idiopathic cases and 9 (50%) had positive results.

Symptoms and signs

Patients presented with muscle pain (57.6%) and/or generalized muscle weakness (23.2%). However, in 19 patients (19.2%) muscular symptoms were masked with concomitant complications, namely, hepatic encephalopathy (15.2%) or spontaneous bacterial peritonitis (4%).

Muscle enzyme changes

Peak levels of AST, CK, and LDH in serum were 1264.8 IU/L, 20693.2 IU/L, and 1926.7 IU/L, respectively. The intervals from symptom onset to peak levels of these muscle enzymes were 5.1, 5.4, and 6.2 days. According to Child-Pugh class, AST, CK, LDH levels were 925, 6820.7, 1013 IU/L in A, 1359.8, 21083.3, 1991.7 IU/L in B, and 1231.2, 21147.9, 1936.1 IU/L in C, respectively. No statistically significant difference was found in muscle enzymes according to Child-Pugh class (Table 3).

Myoglobin

Sixty cases received a urine orthotolidine test for myoglobin; 38 (63.3%) were negative, and 22 (37.6%) positive. The incidence of myoglobinuria was not different significantly between patients with or without renal failure (41.2% *vs* 33.4%). Serum myoglobin concentrations were high in all patients, but did not differ in myoglobinuria-

Table 3 Muscle enzymes in patients with liver cirrhosis and acute myopathy

	AST (IU/L)	CPK (IU/L)	LDH (IU/L)
Class A	925	6820.7	1013
Class B	1359.8	21083.3	1991.7
Class C	1231.2	21147.9	1936.1
Total	1264.8	20693.2	1926.7
Peak day[a]	5.1	5.4	6.2

[a]The intervals from symptom onset to peak levels.

negative and -positive patients (565 ± 267 *vs* 575 ± 183 mg/dL, $P > 0.05$), or between patients with or without renal failure (616.3 ± 332.1 *vs* 629.9 ± 269.8 mg/dL, $P > 0.05$).

Radionuclide bone scan

A ^{99m}Tc-HDP (hydroxymethylene diphosphonate) radionuclide bone scan was performed in 28 patients, and radionuclide muscular uptake increased in 18 (64.3%) who received the test within a few days of onset. However, it should be noted that all 10 patients who did not show increased muscular uptake received the scan when they showed signs of symptomatic improvement rather immediately after onset.

Hospital course

Guidelines of discharge from hospital were the complete disappearance of muscular symptoms and a normalization or maintenance of muscle enzymes at < 1.5 times the normal value. Mean length of hospital stay was 20.8 ± 14.9 d, and this was longer for Child-Pugh B or C patients than for Child-Pugh A patients (21.6 ± 13.1 d, 20.4 ± 16 d *vs* 12.2 ± 3.1 d). Acute renal failure was a complication in 42 of the 99 (42.4%) acute myopathy patients.

Clinical outcomes

Of the 93 patients followed, i.e., except the 6 lost to follow-up, 64 (68.8%) recovered, and 29 (31.2%) expired. In-hospital mortality was higher in Child-Pugh class C (24/59, 40.7%) than in Child-Pugh class B (5/31, 16.1%) or A (0/3, 0%)(Table 4, Figure 1). Forty of the 93 cases (43.0%) were complicated by acute renal failure, and 25 (62.5%) of these expired. The incidences of renal failure were 0%, 30.3%, and 49.2% in Child-Pugh A, B, and C, respectively. The most common causes of death were hepatic failure (14, 48.3%), renal failure (6, 20.7%), septic shock (6, 20.7%), and upper gastrointestinal bleeding (3, 10.3%).

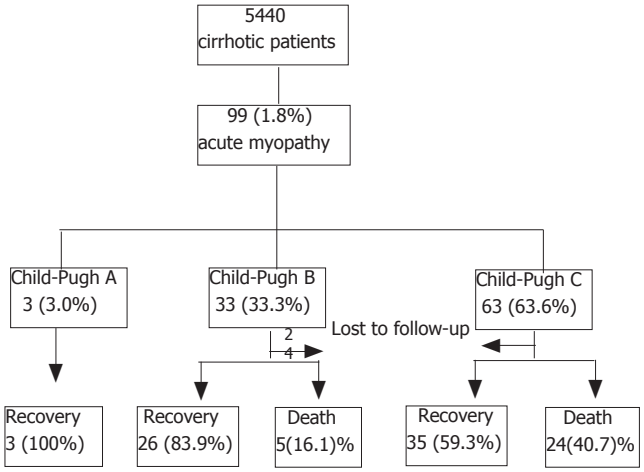

Figure 1 Clinical course of the patients with liver cirrhosis and acute myopathy.

DISCUSSION

Many investigators[7-9] have reported on the high prevalence of muscle cramps in patients with liver cirrhosis. Konikoff *et al*[7] found an 88% incidence of painful cramps in 33 cirrhosis patients, as compared to 21% in a matched population without liver disease. They concluded that the strikingly high incidence and uniformity of the phenomenon might justify the inclusion of painful muscle cramps among the recognized symptoms of cirrhosis. Abrams *et al*[8] suggested that cramps in cirrhotic patients are specifically related to the development of cirrhosis, and that a worsening liver function may be a risk factor for the development of cramps. Angeli *et al*[9] found that the prevalence of cramps was higher in cirrhotic patients than in controls, and that it was related to the duration of recognized cirrhosis and to the severity of liver function impairment. Moreover, they concluded that the pathophysiological link between cirrhosis and cramps may associate with a reduced effective circulating volume, and also indicated that weekly human albumin infusion may be an effective treatment for cramps in cirrhosis. However, these reports focused upon muscle cramps as a symptom observed in liver cirrhosis patients, and not on the clinical and laboratory findings associated with muscular symptoms. Moreover, in addition to muscular symptoms such as muscle cramps, weakness, aching and tenderness, rhabdomyolysis can also occur in liver cirrhosis patients[8-10]. However, no systematic investigation has been conducted on rhabdomyolysis development in cirrhotic patients.

Rhabdomyolysis may be defined as a clinical and laboratory syndrome resulting from skeletal muscle injury with the release of muscle cell contents into the plasma[14]. Increased concentrations of these released substances, such as CK, permit clinicians to diagnose this syndrome[14]. We found an incidence of acute myopathy of 1.8% among hospitalized liver cirrhosis patients. We investigated here cirrhotic patients with elevated muscle enzyme concentrations and/or muscular symptoms, i.e., rhabdomyolysis. And, we described this syndrome as acute myopathy rather than rhabdomyolysis, to better characterize the syndrome and to highlight its significance in liver cirrhosis patients, much like that of the myopathies of alcoholism and alcoholic liver diseases.

We observed that almost all cirrhotic patients who developed acute myopathy had a poor liver function, i.e., Child-Pugh B or C at the time of onset, with exception of 3 cases with Child-Pugh A. Therefore, patients with advanced liver cirrhosis and a poor liver function should be monitored carefully for acute myopathy, in addition to the better known complications of hepatic encephalopathy,

Table 4 Clinical outcome of patients with liver cirrhosis and acute myopathy by Child-Pugh's classification ($n=93$)

Outcome	Child-Pugh class (%)			
	A	B	C	Total
Recovery	3(100)	26(83.9)	35(59.3)	64(68.8)
Death	0(0)	5(16.1)	24(40.7)	29(31.2)
Total	3	31	59	93[a]

[a]Six patients (2 with Child-Pugh B and 4 with C) were lost to follow-up.

gastrointestinal bleeding, or hepatorenal syndrome.

Compared with rhabdomyolysis in the general population, in which drug overdose and septicemia are the most common etiologic factors[15], we found that the most predisposing factor to myopathy development in cirrhotic patients was infection (46.5%), including respiratory, spontaneous bacterial peritonitis (*E. coli*), urinary tract infection, and *Vibrio vulnificus* sepsis. Furthermore, 50% of idiopathic cases were positive for anti-influenza antibody, suggesting that an infectious etiology may account for over 50% of acute myopathy associated with liver cirrhosis, and that influenza should be considered an etiologic factor of acute myopathy developing in cirrhotic patients without a definite etiology. In addition to influenza, various viral and bacterial infections have been reported to be etiologic factors of rhabdomyolysis in the general population[16-20]. Although the precise pathophysiology underlying virus or bacteria-induced rhabdomyolysis is unknown, proposed pathophysiological mechanisms include direct viral or bacterial invasion of skeletal muscle and toxin generation[17]. Muscle biopsies performed on rhabdomyolysis patients found lymphocytic infiltrate or viral inclusions, which support the hypothesis of direct viral invasion[21-23]. Viral DNA PCR analysis of muscle specimens and the identification of viral particles and bacteria in the muscle biopsy specimens, and the isolation of virus by culture from muscle specimens provide more compelling evidence of direct viral or bacterial invasion[22-24].

As 19.2% of our subjects presented with symptoms of concurrent complications other than muscular symptoms, acute myopathy may be difficult to identify in some cirrhotic patients by symptoms alone, i.e., without testing muscle enzymes. Because cirrhotic patients can develop various complications, muscle enzymes investigations are recommended for the detection of acute myopathy. On this point, previous investigations[7-9] were limited because they evaluated only muscular symptoms.

Increased levels of muscle enzymes are the result of skeletal muscle injury and the consequent release of muscle cell contents into the plasma[14]. AST, CK, and LDH peaked 5-6 d after symptom onset, and symptom alleviation was followed by a reduction in muscle enzyme levels (data not shown). The patients with decompensated liver cirrhosis had higher plasma levels of muscle enzymes than cirrhotic patients with a well preserved liver function. These findings represent the natural course of acute myopathy development in cirrhotic patients.

Rhabdomyolysis may or may not result in myoglobinuria, depending on the amount of myoglobin released into plasma, the glomerular filtration rate, and the urine concentration[14]. In the present study, myoglobinuria was present in only 37.6% of subjects who took the urine orthotolidine test; however, serum myoglobin was elevated in all patients. Moreover, myoglobinuria and serum myoglobin levels were unrelated to each other or to the development of renal failure. Different urine sampling times may also have influenced the low incidence of myoglobinuria. Though myoglobinuria is an important clue for the diagnosis of rhabdomyolysis, it cannot be used alone to identify all myopathies. Increased levels of muscle enzymes and serum myoglobin are more sensitive for the diagnosis of rhabdomyolysis, and in particular, CK is a highly sensitive marker of muscle injury[25].

Many case reports have mentioned the usefulness of Tecnetium-99 m bone scintigraphy for the early diagnosis, and for determining the location and extent of the muscle damage in rhabdomyolysis[26-28]. In the present study, a ^{99m}Tc-HDP radionuclide bone scan was diagnostic in 64.3% of the patients evaluated. Patients evaluated within a few days after onset showed increased muscular uptake, but not all patients showing symptomatic improvement who received this test showed increased muscular uptake. Thus, ^{99m}Tc bone agent scintigraphy may be useful for the evaluation of the degree and extent of muscle injury, if it is timely performed.

The length of hospital stay was about 12 d in patients with Child-Pugh A and about 3 wk in the patients with Child-Pugh B or C. Over 40% of cirrhotic cases with acute myopathy were accompanied by acute renal failure. The incidence of renal failure depended on Child-Pugh class, and was highest in patients with Child-Pugh C. Although the pathophysiologic mechanisms of rhabdomyolysis-related acute renal failure are unknown, several mechanisms have been proposed, for example, tubular obstruction by myoglobin plugs and/or urate, renal vasoconstriction caused by the inhibitory effect of myoglobin on endothelial vasodilator production, and toxic free radical produced by ferrous compound, a metabolite of myoglobin[29, 30]. The frequency of acute renal failure in cases of rhabdomyolysis was reported to be 16%-33% in the general population[14,31], and 40.4% in acute myopathy associated with liver cirrhosis. In the present study, among rhabdomyolysis cases complicated by acute renal failure, 62.5% expired, and acute renal failure accounted for 20.7% of these mortalities. Thus, acute renal failure seems to have a prognostic role and its frequency was found to depend on Child-Pugh class. These findings suggest that the status of underlying liver function may be the most important prognostic factor in cases of acute myopathy associated with liver cirrhosis.

The mortality rate reported in the general population was 10%-12%[14, 15, 31], and the overall mortality rate in the present study was 31.2%, which is higher than those reported in cases of rhabdomyolysis in the general population. The mortality rate also depended on the status of underlying liver function. In particular, in-hospital mortality of Child-Pugh C patients was 40.7%. The most common cause of death in cirrhotic patients with acute myopathy was hepatic failure (48.3%).

There are some limitations in this study. First, since the present study was based on retrospective review of medi-

cal records of only symptomatic hospitalized patients, the incidence of acute myopathy was underestimated. In addition, all 5440 cirrhotic patients were hospitalized because of various problems related to cirrhosis so that a majority of them had advanced diseases and the patients with well compensated diseases were not included. Therefore, for all that an analysis of all cirrhotic patients including those without any complication is necessary for defining the predisposing factors for acute myopathy, we could not compare underlying disease function between the patients with myopathy and those without. Thirdly, the diagnosis of myopathy was based on the laboratory and clinical findings, not electromyography or muscle biopsy. However, the decompensated cirrhotic patients can hardly receive electromyography or especially muscle biopsy. Furthermore, the term 'myopathy' was used as rhabdomyolysis in this study.

We found that acute myopathy development is a serious complication in liver cirrhosis patients and that this has several predisposing factors. Moreover, its frequency, morbidity, and mortality were found to depend on underlying liver function and to be higher in decompensated liver cirrhosis. The authors propose that acute myopathy associated with liver cirrhosis be called 'hepatic myopathy' and that careful monitoring is required for its early recognition in the advanced liver cirrhosis patients. The avoidance of predisposing factors by identifying disease mechanisms is certain to reduce the occurrence and mortality of hepatic myopathy. Further studies on the pathophysiologic mechanism and treatment of hepatic myopathy are warranted.

Footnote: Abstract of this paper was presented at Digestive Disease Week 2004, New Orleans, USA

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RAPID COMMUNICATION

Signal transduction of bombesin-induced circular smooth muscle cell contraction in cat esophagus

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Abstract

AIM: To investigate the mechanism of bombesin-induced circular smooth muscle cell contraction in cat esophagus.

METHODS: Specific G protein or phospholipase C involved in cat esophagus contraction was identified, muscle cells were permeabilized with saponin. After permeabilization of muscle cells, the Gi3 antibody inhibited bombesin-induced smooth muscle cell contraction.

RESULTS: Incubation of permeabilized circular muscle cells with PLC-β3 antibody could inhibit bombesin-induced contraction. H-7, chelerythrine (PKC inhibitor) and genistein (protein tyrosine kinase inhibitor) inhibited bombesin-induced contraction, but DAG kinase inhibitor, R59949, could not inhibit it. To examine which mitogen-activated protein kinase (MAPK) was involved in bombesin-induced contraction, the specific MAPK inhibitors (MEK inhibitor, PD98059 and p38 MAPK inhibitor, SB202190) were used. Preincubation of PD98059 blocked the contraction induced by bombesin in a concentration-dependent manner. However, SB202190 had no effects on contraction.

CONCLUSION: Bombesin-induced circular muscle cell contraction in cat esophagus is madiated *via* a PKC or a PTK-dependent pathway or p44/p42 MAPK pathway.

Key words: Bombesin; G protein; Phospholipase C; Protein kinase C; Protein tyrosine kinase; MAP Kinase; Cell contraction

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INTRODUCTION

Bombesin is originally isolated from *Bombina bombina*, a European frog. Utilizing antibodies to amphibian bombesin, bombesin-like immunoreactivity has been identified in mammalian brain, lung, and gut[1]. Bombesin has a wide range of biological effects that include release of hormones, stimulation of pancreatic enzyme secretion, inhibition of gastric emptying, modulation of gastric acid secretion and contractions of various smooth muscle preparations from isolated gut[2]. Until now, four different subtypes of bombesin receptors have been discovered, which can lead to the activation of multiple cell signaling pathways[3]. The primary structures of all these receptor subtypes as deduced from corresponding cDNAs, display seven transmembrane domains coupled to signaling pathways via heterotrimeric G proteins[4-6].

Protein kinase C (PKC) is a family of homologous serine/threonine kinases and presents in cytoplasm. Upon agonist stimulation, it rapidly translocates to the particulate or membrane fraction[7,8]. PKC plays a role in the regulation of sustained agonist-induced contraction of various vascular smooth muscle preparations[9].

Increased protein tyrosine phosphorylation occurs rapidly (within seconds) in response to both polypeptide growth factors and vasoconstrictor hormones[10,11]. Furthermore, epidermal growth factor and platelet-derived growth factor increase vascular tone[12], and are blocked by genistein and typhostins, both of which are inhibitors of tyrosine kinases, indicating that tyrosine phosphorylation is involved in the contractile response[12].

The p44/42 MAP Kinase pathway consists of a protein kinase cascade linking growth and differentiation signals

with transcription in nuclei. Activated p44/p42 MAP kinase translocates to the nuclei and activates transcription by phosphorylation of transcription factors such as Elk-1 and Stat3. A selective and potent inhibitor of the p44/42 MAP kinase cascade, PD98059, has been identified[13]. This compound binds to inactive MEK and prevents phosphorylation and activation by Raf.

To test whether bombesin-induced contraction is mediated via a PKC- or PTK- or MAPK- dependent pathway and which G protein and phospholipase C isozyme is coupled to bombesin, we investigated the signals in mediating contraction induced by bombesin in cat esophageal circular muscle cells.

MATERIALS AND METHODS

Materials

R59949, PD98059 and SB202190 were purchased from Calbiochem (La jolla, CA). G protein antibodies (Gi1, Gi2, Gi3, Gq, and Go), and PLC isozyme antibodies (β1, β3, γ1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HEPES, collagenase type F, and other reagents were purchased from Sigma (St Louis, MO).

Preparation of dispersed muscle cells

Single muscle cells were isolated as previously described[14,15]. Muscle strip was incubated overnight in normal potassium HEPES buffer containing 1 g/L papain, 1 mmol/L DTT, 1 g/L bovine serum albumin (BSA) and 0.5 g/L collagenase (type F, Sigma) and equilibrated with 950 mL/L O2 and 5 mL/L CO_2 to maintain a pH of 7.0 at 31 ℃. The normal potassium-HEPES buffer contained 1 mmol/L CaCl2, 250 μmol/L EDTA, 10 mmol/L glucose, 10 mmol/L HEPES, 4 mmol/L KCl, 131 mmol/L NaCl, 1 mmol/L $MgCl_2$ and 10 mmol/L taurine. Next day we warmed up the tissue at room temperature for 30 min and incubated the tissue in water bath at 31 ℃ for 30 min. After incubation, the digested tissue was washed with 50 mL enzyme-free solution on 360 μm Nitex filter and reincubated in enzyme-free solution at 31 ℃ and gassed with 950 mL/L O_2 and 5 mL/L CO_2 and the cells were allowed to disperse spontaneously for 10-20 min. Suspensions of single muscle cells were harvested by filtration through 500 μm Nitex meshes. Before beginning the experiment, the cells were heated at 31 ℃ for at least 10 min to relax the cells. Throughout the entire procedure, care was taken not to agitate the fluid to avoid cell contraction in response to mechanical stress.

Preparation of permeabilized smooth muscle cells

Cells were permeabilized to diffuse the agents such as G protein antibodies or PLC isozyme antibodies. After completion of the enzymatic phase of the digestion process, the partly digested muscle tissue was washed with an enzyme-free cytosolic buffer containing 20 mmol/L NaCl, 100 mmol/L KCl, 5.0 mmol/L $MgSO_4$, 0.96 mmol/L NaH_2PO_4, 1.0 mmol/L EGTA, 0.48 mmol/L $CaCl_2$, and 2% bovine serum albumin. The cytosolic buffer was equilibrated with 950 mL/L O_2 and 5mL/L CO_2 to maintain a pH of 7.2 at 31 ℃. Muscle cells dispersed spontaneously in this medium. The cytosolic buffer contained 0.48 mmol/L $CaCl_2$ and 1 mmol/L EGTA, yielding 0.18 μmol/L free Ca^{2+} calculated as previously described[16]. After dispersion, the cells were permeabilized by incubation for 5 min in cytosolic buffer containing saponin (75 mg/L). After exposure to saponin, the cell suspension was centrifuged at 350 r/min and the resulting pellet was washed with saponin-free modified cytosolic buffer containing antimycin A (10 μmol/L), ATP (1.5 mmol/L) and an ATP-regenerating system consisting of creatine phosphate (5 mmol/L) and creatine phosphokinase (166.7μkat /L). After the cells were washed free of saponin, they were resuspended in modified cytosolic buffer.

Measurement of contraction by scanning micrometry

Contraction of isolated muscle cells was measured by scanning micrometry[17]. An aliquot of cell suspension containing 10^7 muscle cells/L was added to HEPES medium containing the test agents. The reaction was terminated by addition of formalin (10% final concentration). The length of 40-50 muscle cells treated with a contractile agent was measured at random by scanning micrometry, phase contrast microscope (model ULWCD 0.30 Olympus, Japan) and digital closed-circuit video camera (CCD color camera, Toshiba, Japan) connected to a Macintosh computer (Apple, Cupertino, CA) with a software program, NIH Image 1.57 (National Institutes of Health, Bethesda, MD) and compared with the length of untreated cells. Contraction was expressed as the percentage decrease in mean cell length from control.

Statistical analysis

Data were expressed as mean±SE. Data differences between means were determined by Student's *t* test.

RESULTS

Characterization of G protein subtype-coupled receptor of bombesin

Freshly isolated smooth muscle cells were stimulated for 30 s with bombesin (10^{-6}mol/L) and bombesin induced the contraction of smooth muscle cell (24.3%±2.2% decrease in cell length from control). G proteins, Gi1, Gi2, Gi3, G (40 ku), Gq (42 ku), Gs (46 ku) in cat esophagus cells were established as previously described[17,18]. To identify the specific G protein involved in cat esophagus contraction, muscle cells were permeabilized with saponin preincubated in cytosolic medium containing G protein antibody to allow the antibodies to diffuse into the cytosolic region of the cell membrane. These antibodies could block receptor-induced activation of G protein by binding to the terminal peptide region of G protein that could interact with the receptor. After permeabilization, the Gi3 inhibited contraction, but Gi1, Gi2, Go, Gq did not (Figure 1).

PLC-β3 mediated bombesin-induced contraction

We have previously shown that PC-PLC inhibitor D609 could block the bombesin-induced contraction[17]

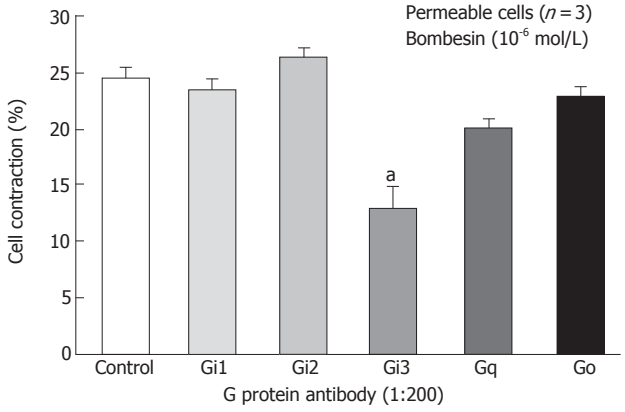

Figure 1 Inhibition of bombesin induced-contraction in permeabilized esophageal circular muscle cells by antibodies to G protein isoforms (Mean ± SE, Student's *t test*, $^aP < 0.05$ *vs* Control).

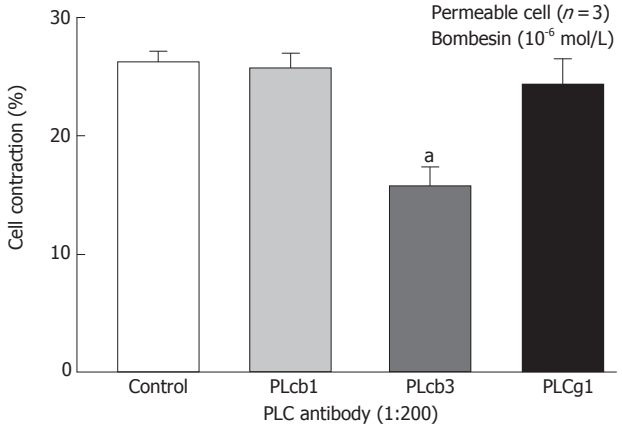

Figure 2 Inhibition of bombesin-induced contraction in permeabilized esophageal circular muscle cells by antibodies to PLC isoforms (Mean ± SE, Student's *t test*, $^aP < 0.05$ *vs* Control).

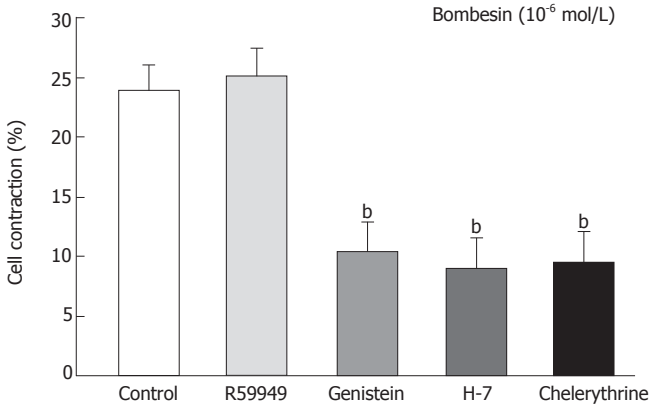

Figure 3 Contractile response of smooth muscle cells from cat esophagus to bombesin in presence of protein kinase C inhibitors (mean ± SE, $n = 4$, Student's *t test*, $^bP < 0.01$ *vs* Control).

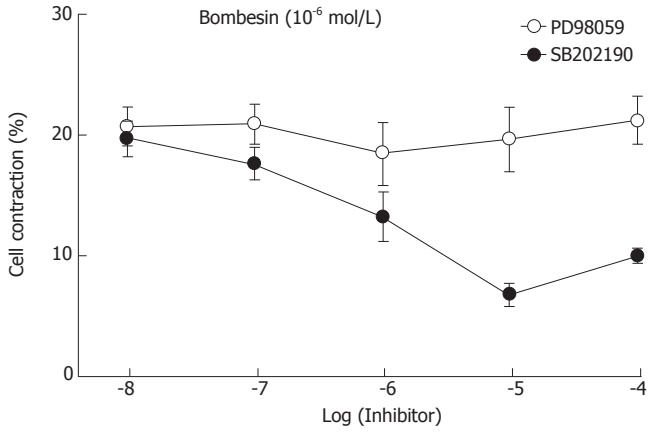

Figure 4 Effect of MEK inhibitor, PD98059 and p38 MAP kinase inhibitor, SB202190 on the bombesin-induced cat esophageal smooth muscle cell contraction (mean ± SE, $n = 4$).

and Western blot analysis of homogenates obtained from dispersed smooth muscle cells using monoclonal antibodies to PLC isozymes could demonstrate the presence of immunoreactive protein bands corresponding to 150 Ku PLC-β1, and PLC-β3 antibody, and 145 Ku PLC-β1 antibody[18]. Incubation of permeabilized circular muscle cells for 1 h with PLC- 3-specific antibody inhibited bombesin- (10^{-6} mol/L) induced contraction ($P < 0.05$). No other PLC-β3-specific antibodies had any significant effect on the contraction (Figure 2).

Role of protein kinase C and tyrosine kinase in bombesin-induced contraction

Cells were preincubated with either the tyrosine kinase inhibitor genistein (10^{-5} mol/L) for 20 min or with the protein kinase C inhibitor H-7 for 15 min (10^{-5} mol/L) or chelerythrine (10^{-5} mol/L) and diacylglycerol (DAG) kinase inhibitor R59949 (10^{-5} mol/L) for 1 min respectively, before the addition of bombesin (10^{-6}mol/L). Bombesin-induced contraction was inhibited by preincubation with genistein as follows: percent age decrease in cell length was 24.3% ± 2.2% *vs* 10.4% ± 2.5% and 9.1% ± 2.5% *vs* 9.5% ± 2.5% in the cells preincubated with H-7 and chelerythrine, respectively (Figure 3).

Role of MAPK in bombesin-induced smooth muscle cells contraction

To examine which MAPK was involved in bombesin-induced contraction, specific MAPK inhibitors were used. Preincubation of PD98059 (the MEK inhibitor) blocked the contraction induced by bombesin in a concentration-dependent manner. The maximal inhibition was observed in 10^{-5}mol/L (Figure 4). However, preincubation of SB202190 (the p38MAPK inhibitor) did not inhibit bombesin-induced concentration, suggesting that bombesin-induced concentration might be mediated via the p44/p42 MAPK pathway.

DISCUSSION

Bombesin is an amidated tetradecapeptide originally purified from the skin of the European frog *Bombina bombina*[1]. Exogenous introduction of this peptide into various organ systems elicits a wide range of responses including secretion of gastrointestinal, adrenal and

pituitary hormones and gastric acid, pancreatic enzyme and mucous, as well as regulation of smooth muscle contraction and modulation of neuronal firing rate[19]. Bombesin receptor is a member of the rhodopsin family of receptors containing seven transmembrane domains coupled to a G protein[20]. Molecular cloning studies have revealed the identity of three mammalian bombesin receptors: gastrin-releasing peptide (GRP receptor)[6], neuromedin B (NMB receptor)[21] and bombesin receptor subtype 3 (BRS-3)[4]. These three receptors share about 50% amino acid sequence identity. In adult animals, GRP-R and NMB-R are widely expressed in the central nervous system[21] and gastrointestinal tract[22,23], whereas BRS-3 shows limited expression in the hypothalamus but is expressed in secondary spermatocytes[4]. Peripheral administration of bombesin produces a variety of effects directly or indirectly linked to the activation of receptors for bombesin-like peptides in the gastrointestinal tract, including exocrine pancreatic secretion, gastrointestinal peptide hormone release, smooth muscle contraction and reduction of food intake. Bombesin receptor is activated after ligand binding and then catalyzes the exchange of GDP bound to the Gα subunit for GTP. After dissociation from Gβγ subunits, functional GTP binds to Gα subunit and activates β isoform of phospholipase C that catalyzes the hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP2) in the cell membrane[24]. Gq (42 Ku), Gi1 (40 Ku), Gi2 (40 Ku), Gi3 (40 Ku), Go (40 Ku), Gs (46 Ku) in esophageal circular muscle have been detected by western blot[17]. In addition, when the same G protein antibodies are used to examine which G protein could mediate the contractile response of esophageal muscle, bombesin-induced contraction of esophageal muscles is inhibited by antibodies against the *alpha* subunits of Gi3[17].

We have previously shown that the response of esophageal cells to bombesin is blocked by the inhibitor of specific phosphatidylcholine-phospholipase C (PLC), D609[17] and Western blot analysis can demonstrate the presence of immunoreactive protein bands corresponding to 150 Ku PLC-β1, PLC-β3 antibody, and 145 Ku PLC-γ1 antibody[18]. Permeabilization was used to examine the participation of PLC isozymes in bombesin-induced muscle contraction in this study. Antibodies to PLC-β3, similarly to rabbit intestine[25], could inhibit bombesin-induced contraction. PLC-β1 and PLC-γ1 antibodies had no effect by themselves. The result suggests that PLC-β3 plays a role in mediating esophageal muscle contraction.

Many vasoconstrictor agonists increase protein tyrosine phosphorylation and ERK activity in smooth muscle preparations[26,27]. Furthermore, tyrosine kinase inhibitors block agonist-induced contraction[26,28,29], suggesting that this pathway is important for smooth muscle contraction. In this study, we investigated the regulation of tyrosine phosphorylation following bombesin stimulation and the role of this pathway in the contractile response. Genistein, a tyrosine kinase inhibitor, reduced contraction in response to bombesin in cat esophagus cells, suggesting that tyrosine kinases are involved in bombesin-induced contraction pathway.

Protein kinase C (PKC) is an enzyme activated by DAG, a second messenger produced by the PLC-catalyzed hydrolysis of PIP2. PIP2 hydrolysis produces two signaling molecules, DAG and IP3. DAG is the physiological activator of the classical and novel isoforms of PKC[30], whereas IP3 regulates intracellular Ca^{2+} movements[31]. DAG kinase plays an essential role in attenuation of DAG signals in agonist-stimulated cells. DAG kinase, which phosphorylates DAG to phosphatidic acid, is divided into a membrane bound and a soluble form. DAG kinase inhibitor increases PKC activity by blocking the phosphorylation of DAG to phosphatidic acid[32]. In this study, we showed that R59949 (the DAG kinase inhibitor) did not increase the bombesin-induced contraction. We found that PKC inhibitor, H-7 and chelerythrine, blocked the contraction induced by bombesin. This result is similar to those of Bitar *et al*[33].

MAP kinase is a serine/threonine-specific protein kinase, activation and phosphorylation of which are induced by a variety of extracellular factors such as mitogenic growth factors and neuropeptides[34,35]. In colonic smooth muscle cells[36,37], agonist-induced contraction involves a kinase cascade initiated by PKC and activation and redistribution of MAP kinase. The activation of MAP kinase by bombesin is rapid (within 15 s), reaching a maximum within 30 s, followed by a decline[37]. We found that p44/p42 MAP Kinase was involved in the bombesin-induced contraction in cat esophagus, indicating that the bombesin-induced signaling pathway progresses to p44/p42 MAP kinase to induce contraction.

In conclusion, bombesin induces circular muscle cell contraction in cat esophagus, which is mediated by protein kinase C and tyrosine kinase pathway. Bombesin induces contraction via Gi3 and PLC-β3. The contraction is mediated via p44/p42 MAP kinase pathway. Our findings provide the basic and clinical experimental data on bombesin-induced contraction and its signal transduction in esophagus.

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RAPID COMMUNICATION

Molecularly defined adult-type hypolactasia in school-aged children with a previous history of cow's milk allergy

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Abstract

AIM: To assess the role of lactase non-persistence/persistence in school-aged children and their milk-related symptoms.

METHODS: The genotypes for the C/T$_{-13910}$ variant associated with lactase non-persistence/ persistence were determined using PCR-minisequencing in a group of 172 children with a mean age of 8.6 years (SE = 0.02, 93 boys) participating in a follow-up study for cow's milk allergy. The parents were asked to assess their children's milk consumption and abdominal symptoms.

RESULTS: The presence of allergy to cow's milk was not associated with the C/C$_{-13910}$ genotype related with a decline of lactase enzyme activity during childhood (lactase non-persistence). The frequency of the C/C$_{-13910}$ genotype (16%) was similar to published figures for the prevalence of adult-type hypolactasia in Finland. The majority of the children (90%) in this series consumed milk but 26% of their families suspected that their children had milk-related symptoms. Forty-eight percent of the children with the C/C$_{-13910}$ genotype did not drink milk at all or consumed a low lactose containing diet prior to the genotyping ($P < 0.004$ when compared to the other genotypes).

CONCLUSION: Analysis of the C/T$_{-13910}$ polymorphism is an easy and reliable method for excluding adult-type hypolactasia in children with milk-related symptoms. Genotyping for this variant can be used to advise diets for children with a previous history of cow's milk allergy.

Key words: Adult-type hypolactasia; Primary lactose malabsorption; Genetic testing; Cow's milk allergy

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INTRODUCTION

Lactase deficiency (LD, lactase non-persistence) is the most common cause of milk intolerance in children and adolescents world-wide[1]. It plays a significant role in recurrent abdominal pain in populations with common consumption dairy products[2]. Immunologically mediated adverse reaction to ingested proteins of cow's milk is referred to as cow's milk allergy (CMA), the symptoms of which usually appear under one year of age[3]. CMA affects 1.9%-3.2% of infants[4] though recent studies suggest that allergy to cow's milk protein may have an impact on abdominal symptoms at schoolage also[5]. CMA is easily differentiated from LD if it manifests at an early age with skin and/or respiratory symptoms[6]. In cases of CMA that present with gastrointestinal symptoms, the clinical picture may overlap with symptoms caused by low lactase activity especially at schoolage, when the downregulation of lactase enzyme activity occurs[7].

The differential diagnosis of milk-related symptoms in children is difficult to establish because of the variability of clinical symptoms and inaccurate diagnostic laboratory tests[8]. The diagnosis of lactose malabsorption is based on the measurement of disaccharidase activities in intestinal biopsy specimens, a method which is not suitable for every day clinical practice. The indirect commonly used lactose-tolerance test (LTT) is not reliable in children and results in up to 30% of false positive results, thus reducing its value in clinical use[9]. In addition, children with normal lactose digestion complain of symptoms during LTT[10].

Recently, a C to T single nucleotide polymorphism residing 13 910 base pairs upstream of the lactase-phlorizin

hydrolase (LCT) gene has been shown to associate with lactase persistence/non-persistence, the C/C-13910 genotype defining lactase non-persistence as well as C/T-13910 and T/T-13910 genotype lactase persistence[11]. The association of the C/T-13910 variant with disaccharidase activities and lactase/sucrase ratio (L/S) has been verified in a total of >600 intestinal biopsy specimens[7, 11, 12]. In Finnish children (8-20 years of age) the mean level of lactase activity among subjects with the C/C-13910 genotype is 6.5 U/g protein. The C/T-13910 genotype is 29.9 U/g protein and 50.0 U/g protein respectively, showing a trimodal distribution of the lactase activity[7]. Functional evidence for the C/T-13910 variant in regulation of lactase activity has been obtained in several studies[12-15] and a greater increase in LCT promoter activity was reported for the T-13910 variant[13, 14]. The down-regulation of intestinal lactase varies according to ethnicity but the differences at the timing of down-regulation are not marked and most commonly start to appear around 5-6 years of age[7]. At the age of 12 years, all children with the C/C-13910 genotype have low lactase activity in their intestines[7].

The aim of the present study was to evaluate the role of lactase non-persistence in milk consumption and milk-related clinical symptoms by analysing the C/T-13910 genotypes of lactase persistence/non-persistence in a group of 172 school-aged children from eight to nine years of age with or without a previous history of CMA.

MATERIALS AND METHODS

Subjects

This study was part of a prospective follow-up study of children with a history of CMA diagnosed at a mean age of seven months [16]. The present study group comprised 172 school-aged children (mean age 8.6 years; SE = 0.02, 93 boys, 79 girls) who were clinically examined during August 2003-March 2004 at the Helsinki University Central Hospital, Helsinki, Finland. Ninety-three children (54%) had a previous diagnosis of CMA and 79 children comprised the control group. All these children were subjects in the study by Saarinen and collaborators[17], in which 6209 unselected infants born between August 1994 and November 1995 in the Helsinki region were followed up from birth for the development of CMA. The presence of CMA was confirmed by a challenge test[17]. At the time of the present visit, the families were asked about the children's milk consumption and possible milk-related symptoms and those agreeing to participate in genetic testing of adult-type hypolactasia were included in the study. IgE-mediated hypersensitivity to cow's milk was measured by skin prick test. A diameter >3 mm exceeding the negative control was considered as a positive response[18]. Those children who were still avoiding milk due to previous CMA were re-challenged by cow's milk[16]. A supplementary questionnaire on the amount of milk consumed and possible abdominal symptoms during the preceding week was mailed later. Celiac disease was screened as previously described[19].

Ethics

The study was approved by the Ethical Committee of the Hospital for Children and Adolescents, University of Helsinki. The families/children gave their informed consent.

Genotyping

DNA was isolated from blood by phenol-chloroform extraction as previously described[20] and DNA fragments spanning the C/T-13910 variant were amplified using one biotinylated primer and one unbiotinylated primer (primer sequences available on request). Briefly, PCR amplifications were carried out in a 50-μL volume with genomic DNA (100 ng), primers (20 ng), dNTPs (200 μmol/L), and 0.5 U of Taq polymerase in a standard buffer (Dynazyme, Finnzymes, Espoo, Finland). The PCR cycle conditions were as follows: an initial round of denaturation at 94 ℃, then 35 cycles at 94 ℃ for 30 s, at 53 ℃ for 30 s, at 72 ℃ for 1.25 min and a final extension at 72 ℃ for 10 min. Ten μl of the PCR product was captured in a streptavidin-coated microtiter well (Thermo Electron, Vantaa, Finland) and two parallel minisequencing reactions were carried out for each PCR-product. The minisequencing reaction contained 10 pmoles of the minisequencing primer (primer sequence available on request), 0.1 μL of tritium-labelled dNTP (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK), and 0.05 U of DNA polymerase (Dynazyme, Finnzymes, Espoo, Finland). The microtiter wells were incubated for 15 min at 56 ℃ and finally the wells were washed. The detection primer was eluted and the eluted radioactivity was measured in a liquid scintillation counter (Rackbeta 1209, Wallac, Finland) as previously described [21].

Statistical analysis

The Mann-Whitney *U* test and Spearman's rank correlation test were used for nonparametric comparisons. Fisher's exact 2-sided test was also used.

RESULTS

Twenty-six percent of the families (45/172) suspected that their children had milk-related symptoms at 8-9 years of age at the time of this study. A significantly greater proportion of these came from those diagnosed to have CMA (76%, 34/45) at a mean age of seven months[17] than those serving as controls ($P < 0.003$).

Skin-prick test with cow's milk was positive (> 3 mm) in 12% of the children undergoing these tests (20/168) and all these children had a previous history of CMA (Table 1). At the time of this study, the challenge test with cow's milk was positive in 11 of the 16-challenged children[16].

The frequency of C/C-13910 genotype defining adult-type hypolactasia was 16% in the total study group of 172 children. There was no correlation with the genotype and positive reaction in the skin prick test or food challenge. Of the 27 children with the C/C-13910 genotype, two were challenged with cow's milk at the time of this study and one of them had a positive challenge for cow's milk. The milk consumption of different genotypes is presented in Table 2. Screening for celiac disease was negative in 170/172 of the children. The two children with a positive screening test consumed milk and reported no abdominal symptoms. The genotypes in these two children were C/

Table 1 Milk consumption and abdominal symptoms in school-aged children with a history of cow's milk allergy[1] and their controls

	Previous allergy[1]	Controls
Questionnaire completed	92% (n=86)	97% (n=77)
Milk product consumption	80% (n=69)	100%[a] (n=77)
Milk consumption < 1 dL/d	36% (23/69)	16%[b] (12/77)
Low lactose content in diet	14% (10/69)	8% (6/77)
Abdominal symptoms: Flatulence	13% (11/86)	8% (6/77)
Loose stools	5% (4/86)	4% (3/77)
Skin prick test >3 mm to cow's milk	22%[2] (20/91)	0 (0/77)

[1]milk challenge positive at a mean age of 7 mo; [a]P < 0.0001 *vs* milk consumption; [b]P < 0.02 *vs* milk product consumption.

Table 2 Milk consumption and abdominal symptoms in school-aged children genotyped for adult-type hypolactasia

	T/T1-13910	C/T[1]-13910	C/C[2]-13910
Total number of children			
n=172	n=68	n=77	n=27
Milk products consumed	95%	95%	93%
(n=163)	(54/62)	(67/74)	(25/27)
Drinks milk < 1 dL/day	22%	18%	44%[a]
(n=146)	(12/54)	(12/67)	(11/25)
Low lactose content in the diet	7%	4%	36%[b]
(n=146)	(4/54)	(3/67)	(9/25)
Skin prick test >3 mm to cow's milk	13%[5]	11%[5]	12%[3]
(n=168)	(9/67)	(8/75)	(3/26)

[1]lactase persistence; [2]defines adult-type hypolactasia; [a]P < 0.02 *vs* genotypes associated with lactase persistence (T/T-13910 plus C/T-13910); [b]P < 0.002 *vs* T/T-13910 genotype or C/T-13910 genotype; [3]each of these prick positive children had a diagnosis of cow's milk allergy at a mean age of seven mo[17].

Table 3 Abdominal symptoms during a one-week period in school-aged children genotyped for adult-type hypolactasia

	Total	T/T[1]-13910	C/T[1]-13910	C/C[2]-13910
Questionnaire completed	n=163	n=62	n=74	n=27
Abdominal symptoms	(n=38)	15% (n=9)	27% (n=20)	33%[a] (n=9)
Flatulence	(n=17)	5% (n=3)	12% (n=9)	19%[b] (n=5)
Periumbilical pain	(n=10)	6.5% (n=4)	5.4% (n=4)	7.4% (n=2)
Loose stools	(n=7)	3.2% (n=2)	5.4% (n=4)	3.7% (n=1)
Upper epigastric pain	(n=7)	5% (n=3)	4.0% (n=3)	3.7% (n=1)
Constipation	(n=5)	0	5.4% (n=4)	3.7% (n=1)
Dyspepsia	(n=1)	0	1.4% (n=1)	0

[1]lactase persistence; [2] defines adult-type hypolactasia; [a]P=0.05, [b]P<0.04 *vs* T/T-13910 genotype.

Table 4 Probable changes in milk consumption of families after receiving the results of genetic testing for adult-type hypolactasia in school-aged children

	T/T[1]-13910	C/T[1]-13910	C/C[2]-13910
Questionnaire completed	94% (62/68)	97% (74/77)	100% (27)
No change in milk consumption	89% (n=54)	81% (n=61)	59%[3] (n=16)
Less lactose containing milk	0	0	33% (n=9)
More lactose containing milk	8% (n=5)	11% (n=8)	0
Not decided	5% (n=3)	7% (n=5)	7% (n=2)

[1]lactase persistence; [2]defines adult-type hypolactasia; [3]33% of the families consumed low lactose containing diet prior to genetic testing.

T-13910 and T/T-13910 (data not shown).

Fifty-two children out of the total study group of 172 children drank less than one dL of milk per day or did not consume milk products. The C/C-13910 genotype defining adult-type hypolactasia was present in 25% (13/52) and CMA in 21% (11/52, confirmed in a challenge test) of these children. Each child with CMA had a previous diagnosis of CMA. Milk consumption did not have any significant effect on body mass index (BMI, kg/m^2) and BMI was similar in children with different genotypes for adult-type hypolactasia (data not shown).

The questionnaire on daily milk consumption and abdominal symptoms was filled in by 95% (163/172) of the families (Table 3). Adult-type hypolactasia was suspected in 9% (15/163) of the children by their parents but was confirmed by genotyping in not more than 20% of these children (3/15 with the C/C-13910 genotype). One of these three children with the C/C-13910 genotype had a diagnosis of lactose malabsorption based on intestinal biopsy at the age of 7 years and the other two were noticed to have lactose-related symptoms at the age of 1.5 and 7 years. Two of the 27 children with the C/C-13910 genotype did not consume milk because of CMA. Eighty percent of the children (20/25) with the C/C-13910 genotype who had no CMA at this age had a low lactose containing diet at home or drank quantities of milk less than one dL/day already prior to the genetic testing (Table 2). A similar diet with milk restriction was practiced more rarely among children with genotypes associated with lactase persistence (31/119 of the children with C/T-13910 or T/T-13910 genotypes who answered the question and did not have allergy to milk, P< 0.004). Children with the C/C-13910 genotype did not report significantly more abdominal pains than children with the C/T-13910 or T/T-13910 genotypes but there was a difference in the presence of flatulence (P< 0.04, Table 3). Among the children with the C/C-13910 genotype, 4/15 children had milk restriction beyond two years of age because of milk allergy. Two of these four children experienced lactose-related symptoms.

The acceptance of genetic testing was good, as only two families did not participate in the study. Based on the interviews of the families among the children with the C/C-13910 genotype, 30% of the families reported to have another family member with symptoms of adult-type hypolactasia. Thirteen percent of the families of the total series and one third of the children with the C/C-13910 genotype reported that the result of the genetic test for adult-type hypolactasia had a probable effect on their milk consumption (Table 4). Of the families with genotypes associating with lactase persistence (C/T-13910 or the T/T-13910 genotype), 10% reported that the result of the genetic test was helpful in avoiding unnecessary restrictions on milk consumption.

DISCUSSION

Our results show that the overlap of IgE-mediated cow's milk allergy, CMA, and lactase non-persistence is unlikely

to occur at schoolage. The prevalence of adult-type hypolactasia in our study was 16% corresponding to the reported frequency in our population[7,11,22,23] and was not associated with CMA but correlated with the consumption of low lactose containing diets and the presence of flatulence. All children with a positive skin prick test for cow's milk at this age (12%) had a previous diagnosis of CMA, settled at a mean age of seven months as previously reported in detail[16]. It is noteworthy that not a single child in the non-CMA group turned IgE-positive for milk at schoolage, further confirming that IgE-mediated reactions with cow's milk develops at an early age. Tolerance to cow's milk develops in the majority of children with CMA (70%) by the age of three and eight to nine years. At the time of the present study, 85% of the children recovered from CMA[16].

It was reported that one third of the Finnish children at the age of eight years with the genotype C/C$_{-13910}$ have a high intestinal lactase activity[7] and are unlikely to develop symptoms caused by adult-type hypolactasia. It should however be borne in mind that in children the decline of intestinal lactase occurs slowly and depends on ethnicity [7]. Based on our previous study about the timing of down-regulation of lactase activity[7], we can estimate that about 10% (0.63 × 16%) of the children being studied have a reduced lactase activity (<10 U/g/protein) and all the others have a high lactase activity (>10 U/g/protein). In the present study, 60% of the children with the C/C$_{-13910}$ genotype consumed a low lactose containing diet or had milk-related symptoms at this age and 40% of those with the C/C$_{-13910}$ genotype were considered milk-tolerant. The avoidance of milk and consumption of low lactose containing diet was much more common among children with the C/C$_{-13910}$ genotype than among those with the genotypes associated with lactase persistence. This confirms our preliminary finding[7] and suggests that the children have experienced milk-related symptoms triggering a reduced lactose intake.

It is common, however, that children with abdominal dysfunction after consuming milk are unaware of its cause[24, 25]. This was also obvious in our series as the suspicion of milk-related symptoms was not increased in the families of the children with the C/C$_{-13910}$ genotype defining adult-type hypolactasia. However, restricted lactose intake was more common in these families. Although this dietary modification, the children with the C/C$_{-13910}$ genotype reported more flatulence when compared to those with the genotype T/T$_{13910}$ associated with lactase persistence ($P < 0.04$). There was no difference in the frequency of abdominal pain between the children with either CMA or lactose malabsorption and the control children.

It is common that only some individuals who self-report them as lactose intolerants are in fact lactose maldigesters when tested objectively[26-30]. The data on children, however, are limited. In our series, every fifth child suspected not to tolerate lactose was confirmed to have the predisposing genotype for adult-type hypolactasia. The lack of awareness of lactose maldigestion may increase abdominal symptoms in children as reported by Webster *et al*[31] who noticed that after a proper diagnosis of lactose malabsorption, the avoidance of milk products is more rigorous and results in a decrease in complaints. It is unclear whether individuals with adult-type hypolactasia and lactose maldigestion but without obvious symptoms of lactose intolerance should avoid milk or not. A recent double-blind study suggested that lactose maldigesters might benefit from lactose avoidance even though the diagnostic criteria for lactose intolerance are not fulfilled[32]. On the other hand, studies in adults reported that as many as 32% of lactose mal-absorbers experience no symptoms from lactose containing milk products[26], but at present there are no means to predict this at an individual level.

In the present study, 10% of the children with the C/T$_{-13910}$ genotype reported symptoms such as flatulence and loose stools suggestive of milk intolerance. One of these children had CMA (milk challenge positive) but in the other cases the causes for these symptoms were unknown. It is possible that some of these children are carriers of a mutation of congenital lactase deficiency (CLD), a rare congenital disorder which may result in low lactase activity but no disease manifestations in heterozygotes[33, 34]. Celiac disease may cause secondary hypolactasia but screening tests for the disease are negative in these children.

Parents easily suspect milk as a causative agent for abdominal symptoms in countries where dairy products are widely used. In the present study, 26% of the families reported a suspicion of milk-related symptoms in their school-aged children. The majority of these children did not drink milk nor had low lactose containing diets. When milk-related symptoms were suspected, the parents recognized CMA easily due to the previous history of CMA but their suspicion of adult-type hypolactasia was seldom confirmed. Accurate diagnosis of possible milk-related symptoms poses a challenge for clinicians as the elimination of milk and a proper challenge test for milk proteins or tolerance test for lactose are both time-consuming and may give unsatisfactory results[8]. The genetic testing of adult-type hypolactasia performed from a drop of blood was accepted well by the families as 99% of the families agreed to test their children. About 10% of the families reported that a negative result for adult-type hypolactasia was helpful as they may now avoid unnecessary restrictions on milk consumption.

In conclusion, a suspicion of milk-related symptoms is common in everyday clinical practice. The genetic test of C/T$_{-13910}$ polymorphism is reliable in excluding adult-type hypolactasia in children with milk-related symptoms. Genotyping for this variant may help in planning the diet for children with a suspicion of milk-related symptoms.

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RAPID COMMUNICATION

Gilbert's syndrome: High frequency of the $(TA)_7$ TAA allele in India and its interaction with a novel CAT insertion in promoter of the gene for bilirubin UDP-glucuronosyltransferase 1 gene

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Abstract

AIM: To identify the variants in UDP-glucuronosyltransferase 1 (UGT1A1) gene in Gilbert's syndrome (GS) and to estimate the association between homozygosity for TA insertion and GS in India, as well as the frequency of TA insertion and its impact among normal controls in India.

METHODS: Ninety-five GS cases and 95 normal controls were selected. Liver function and other tests were done. The promoter and all 5 exons of UGT1A1 gene were resequenced. Functional assessment of a novel trinucleotide insertion was done by *in silico* analysis and by estimating UGT1A1 promoter activity carried out by luciferase reporter assay of appropriate constructs in Hep G2 cell line.

RESULTS: Among the GS patients, 80% were homozygous for the TA insertion, which was several-fold higher than reports from other ethnic groups. The mean UCB level was elevated among individuals with only one copy of this insertion, which was not significantly different from those with two copies. Many new DNA variants in UGT1A1 gene were discovered, including a trinucleotide (CAT) insertion in the promoter found in a subset (10%) of GS patients, but not among normal controls. *In-silico* analysis showed marked changes in the DNA-folding of the promoter and functional analysis showed a 20-fold reduction in transcription efficiency of UGT1A1 gene resulting from this insertion, thereby significantly elevating the UCB level.

CONCLUSION: The genetic epidemiology of GS is variable across ethnic groups and the epistatic interactions among UGT1A1 promoter variants modulate bilirubin glucuronidation.

Key words: Unconjugated hyperbilirubinemia; UGT1A1 gene; DNA resequencing; Luciferase reporter assay

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INTRODUCTION

Gilbert's syndrome (GS) is generally considered to be an autosomal recessive disorder (OMIM #143500) characterized by mild, chronic, non-hemolytic unconjugated hyperbilirubinemia in the absence of liver disease. Although it has long been perceived to be an innocuous clinical entity with a benign course, recent data suggest that affected individuals may be predisposed to development of liver injury following treatment with various drugs and xenobiotics and that the genetic defect in GS may influence the outcome of liver transplantation and other clinical conditions. The disorder due to a deficiency in bilirubin glucuronidation, is commonly caused by dinucleotide (TA) insertions and [TA $(TA)_7$TAA] alleles in the promoter region of the bilirubin UDP-glucuronosyltransferase 1 (UGT1A1) gene. This dinucleotide insertion reduces the efficiency of transcription of the gene and decreases hepatic UDP-glucuronosyltransferase activity to about 30% of normal levels in homozygous subjects[6-9]. The prevalence of GS, the frequency of the $(TA)_7$TAA allele in the UGT1A1 promoter and the proportion of GS patients who are homozygous for the $(TA)_7$TAA allele vary widely

across populations[10-19]. Recent data have also shown that variants in UGT1A1 gene other than the classical dinucleotide insertion in its promoter, can contribute to hyperbilirubinemia, including the milder form as in GS, although their effects appear to be variable across populations[5,20-24]. Thus, as recently emphasized[20], detailed investigations into genetic polymorphisms of UGT1A1 gene in Asian populations may provide a better understanding of unconjugated hyperbilirubinemia.

The objectives of our case-control study were to estimate the association between homozygosity for the $(TA)_7TAA$ allele and GS in India, to identify and study the impact of this and other polymorphisms in the promoter and exons of the UGT1A1 gene on serum bilirubin levels, and to estimate the frequency of the $(TA)_7TAA$ homozygous genotype in the general population in India.

MATERIALS AND METHODS

Participant recruitment and clinical biochemistry analysis

This study was conducted in 95 consecutive patients presenting to the Liver Clinic at the Department of Gastroenterology of the Institute of Postgraduate Medical Education & Research, Kolkata, India, for evaluation of persistent unconjugated hyperbilirubinemia (serum bilirubin greater than 1.2 mg/dL) documented at least twice over a period of one month. Serum total bilirubin and its unconjugated fraction were estimated in fasting condition. Each of these individuals had a normal finding on physical examination, normal hepatobiliary ultrasound, normal liver function test excluding hyperbilirubinemia and a normal reticulocyte count. A total of 95 adult healthy volunteers from the same ethnic population as the GS patients were included as controls. Each control also underwent the same set of investigations as the patients. Their fasting serum bilirubin level estimated twice at intervals of fifteen days was consistently less than 1.0 mg/dL. Patients were excluded if they were under any medication during the past one month or consumed alcohol regularly or had present or past history of hepatic/hematological disease. Additionally, quantitative estimation of hemoglobin fractions (A_0, A_2 and F) was carried out on each patient and control by cation-exchange HPLC using the Variant™ Hemoglobin Testing System Beta Thalassemia Short Program (Bio-Rad Diagnostics, Hercules, USA). A 5 mL blood sample was collected from each patient by venipuncture and DNA was isolated using a standard protocol[25]. Data and samples were collected with written informed consent of the patients and controls, after the approval was obtained from the Human Research Ethics Committee of the Institute of Postgraduate Medical Education & Research, Kolkata.

DNA re-sequencing and identification of variant alleles

DNA re-sequencing of the promoter and exon 1*1 encoding the substrate-specific region of bilirubin-UGT1 gene as well as the 4 common exons (exons 2-5) was carried out using an automated DNA sequencer (ABI-3100; Applied Bio-systems, Foster City, USA.). DNA samples were first amplified by the polymerase chain reaction technique using an ABI-9700 thermal cycler. PCR products were then cleaned using exonuclease-I (USB. Corporation, Cleveland, USA) and shrimp alkaline phosphatase (Amersham, Freiburg, Germany), and sequencing reactions were carried out. DNA resequencing was carried out in both forward and reverse directions. Raw DNA sequences were analyzed as previously described[26], and variant genotypes were identified.

Functional analysis of a novel trinucleotide insertion by luciferase reporter assay

UGT1A1 promoter fragments, 331-336 bp in length depending on the number of TA repeats and CAT insertion, were amplified using primers (F: 5'-tgtagatcttcct ctctggtaacact-3') and (R: 5'-atgaagctttgctcctgccagaggttc-3') from genomic DNA of individuals homozygous for six and seven TA repeats, and CAT insertion on $(TA)_7TAA$ background. DNA amplification was carried out with polymerase chain reaction (PCR) using FastStart Taq DNA polymerase (Roche, Mannheim, Germany) with an initial denaturation at 95℃ for 10 min, followed by 35 cycles at 94℃ for 1 min, at 56℃ for 45 s and at 72℃ for 30 s on an ABI-9700 (Applied Bio-systems, Foster City, USA.) thermal cycler. To facilitate subcloning of the PCR products in the reporter gene construct, oligonucleotides F and R were designed with a *Bgl* II and a *Hind* III restriction enzyme site at the 5' end, respectively. PCR products were doubly digested with *Bgl* II and *Hind* III, purified by Qiagen gel purification kit (Qiagen, Hilden, Germany) and subcloned into pGL3-basic vector (Promega, Madison, USA). The integrity of the resulting plasmids was confirmed by restriction mapping and sequencing analyses. Promoter activity of each construct was measured in HepG2 cell line. Cells were grown in MEM medium supplemented with 2 mmol/L L-glutamine, 0.1 mmol/L non-essential amino acids, 1 mmol/L sodium pyruvate and 10% fetal calf serum (GIBCO -BRL, Grand Island, USA) at 37℃ with 50 mL/L CO_2. Exponentially growing cells were trypsinized, seeded at 2.5×10^5 cells, and incubated overnight prior to transfection. Transfection was carried out by lipofectamin (Invitrogen, Carlsbad, USA) using 2 μg of each of the constructs (namely $(TA)_6$ TAA, $(TA)_7$ TAA and CAT insertion on $(TA)_7$ TAA background), as well as the pGL3-basic and pGL3-control as positive controls $(TA)_7TAA$, the luciferase gene was under the control of SV40 promoter and enhancer. After 48 h of transfection, the cells were lysed and centrifuged in cell culture lysis buffer (Promega, Madison, USA). Luciferase activity was assayed in the cell lysate by measuring the photoluminescence in a Monolight 2010 single channel luminometer and the total cellular protein content was measured by the standard Bradford's method. Luciferase activity was normalized to total cell protein concentration. Normalized luciferase activity of each construct was expressed as a ratio to that of the pGL3-basic vector. Three independent experiments were performed for each construct and all measurements were determined in duplicate.

Statistical analysis

Equality of proportions was statistically tested by the standard normal test procedure[27]. Equalities of mean

values of various hematological parameters for individuals belonging to different genotypes at various polymorphic loci were statistically tested using the Student *t*-test or the analysis of variance (ANOVA) procedure, as appropriate. Regression analysis was performed to test the significance of dependence of un-conjugated serum bilirubin level on some relevant variables. Allele frequencies were estimated using the gene-counting method.

RESULTS

Characteristics of patients and controls

No statistically significant ($P > 0.05$) differences in the proportions of males and females were observed between the patients (87 males, 8 females) and controls (77 males, 18 females). The mean ages of male and female patients (30.1 ± 1.1 years of males, 26.8 ± 5.1 years of females) and controls (30.2 ± 1.1 years of males, 31.1 ± 2.9 years of females) were not significantly different ($P > 0.05$). Since elevated HbA_2 levels could potentially increase the unconjugated bilirubin level, we tested the significance of the regression coefficient of HbA_2 level on unconjugated bilirubin, separately for patients and controls. In both sets, there was no statistically significant impact of HbA_2 (*P*-values for patients and controls were 0.638 and 0.106, respectively). The difference in the mean values of HbA_2 between patients and controls was not significantly different ($t = 1.76$, d.f. $= 128$, $P > 0.05$). The 8 GS patients, but none of the normal controls, who were habitual smokers were asked to refrain from smoking for 24 h prior to blood collection. We tested whether the inclusion of these 8 GS patients had a significant impact on our findings. We therefore, examined whether the mean value of un-conjugated bilirubin among patients who were smokers ($n = 8$) was significantly higher than that among patients who were not smokers ($n = 87$). No significant difference was found (mean for smoker patients $= 2.67 \pm 0.33$, mean for non-smoker patients $= 3.38 \pm 0.30$, $t = 0.696$, d.f. $= 93$, $P = 0.488$). We have performed all analyses that were reported below with and without the inclusion of the 8 GS patients who were habitual smokers. No differences in inferences were found (results not shown). Therefore, we presented all results including these 8 GS subjects.

UGT1A1 sequence variants and their relation with bilirubin level

Eleven sequence variants in UGT1A1 gene was observed, of which 3 each were in the promoter, 3 in exon 1, 2 in exon 2, 1 in exon 3 and 2 in exon 4. No variant was found in exon 5. The genotype and variant allele frequencies at these positions in patients and controls are given in Table 1. Of these 11 variant sites, 2 were non-polymorphic (frequency of the rarer allele $< 1\%$), while the remaining 9 sites were polymorphic either among patients or among controls or in both. One of the polymorphic sites was the TA insertion [$(TA)_7TAA$ allele] in the TATA box of the UGT1A1 promoter[2,3]. The frequencies of the $(TA)_7TAA$ allele (0.879) and the 7/7 genotype (80%) among the patients were significantly higher ($P < 0.005$) than those among the controls (0.384 and 10%, respectively). The un-conjugated serum bilirubin values for GS patients belonging to the 6/6, 7/6 and 7/7 genotypes are presented in Figure 1. The mean values of un-conjugated bilirubin for GS patients and normal controls were 2.5 ± 0.25 mg/dL and 0.61 ± 0.09 mg/dL respectively. The mean±SE value of un-conjugated bilirubin among patients with the 7/6 genotype was 3.46 ± 0.54 mg/dL, which was not significantly different ($P = 0.05$) from patients with the 7/7 genotype (3.31 ± 0.33 mg/dL). The mean un-conjugated serum bilirubin values for these genotypes among controls were only 0.59 ± 0.10 mg/dL and 0.64 ± 0.15 mg/dL, respectively.

No significant differences ($P = 0.05$) were found in the mean bilirubin levels among individuals belonging to the various genotypes at the remaining single nucleotide polymorphic loci. With respect to the C6844G polymorphism resulting in a non-synonymous amino acid change (A321G), all patients were CC homozygotes, while about 30% of the controls were CG heterozygotes (Table 1). We did not find any significant effect of the G71R polymorphism on un-conjugated bilirubin level. This polymorphism also showed no significant interaction with the TATA box insertion polymorphism (results not shown). These findings are discordant with those reported among the Japanese[19].

A novel human-specific trinucleotide insertion in Gilbert's syndrome patients and its impact on bilirubin level

Among the polymorphic variants described in Table 1, aside from the familiar TA insertion, the most striking was the CAT insertion (nucleotide positions -85 to -83) in the CAAT box of UGT1*1 promoter. Normally, there is one copy of the CAT trinucleotide present in human Genbank (http://www.ncbi.nlm.nih.gov/Genbank/). We found two copies of this trinucleotide in some GS patients. To confirm that this was an insertion we searched the chimpanzee (gi|6456543|gb|AF135463.1|AF135463) and gorilla (gi|6456545|gb|AF135464.1|AF135464) databases. In both species there is only copy, confirming that the single copy is the ancestral state. This CAT insertion was found only among nine (10%) GS patients, who were all homozygous for the TA insertion. We found that GS patients with the CAT-insertion had a significantly ($P < 0.001$) elevated mean level (6.13 ± 1.61 mg/dL) compared to those without the insertion (2.93 ± 0.28 mg/dL). Individuals who possessed the CAT insertion did not consistently possess a variant allele at any of the other sites. One individual who was heterozygous for the CAT insertion was also heterozygous for the I322V variant, and other three CAT-insertion heterozygotes were also heterozygotes for the H376R variant.

Functional analysis of CAT insertion

Since about 10% of the $(TA)_7TAA$ homozygotes carried the CAT insertion and the insertion significantly elevated the bilirubin level, we postulated that the insertion had a functional impact. To examine this, we studied the change in DNA-folding of the promoter region caused by this insertion. The most stable structures, namely those with lowest free energy (dG) values, are given in Figure 2 for the UGT1A1 promoter region without (dG = -14.4) and

Table 1 DNA sequence variations observed among Gilbert's syndrome patients and normal controls

Location of variant and nucleotide position (np)	Description of variant[1]	Genotype/Allele frequency (p)	Patients (%)	Control (%)
UGT1*1 promoter	CAT insertion	Insertion/Insertion	3	0
nps -85 to -83		Insertion/non-insertion	6	0
		Non-insertion/non-insertion	86	95
		p(Insertion)	0.063	0.000
UGT1*1 promoter	G→C	GG	93	95
np -63		GC	2	0
		p(C)	0.011	0.000
UGT1*1 promoter	$(TA)_6$ TAA→	$(TA)_6$ TAA /$(TA)_6$ TAA	4	32
nps -53 to -38	$(TA)_7$ TAA	$(TA)_7$ TAA /$(TA)_6$ TAA	15	53
		$(TA)_7$ TAA/$(TA)_7$ TAA	76	10
		p[$(TA)_7$ TAA]	0.879	0.384
Exon 1	G→A	GG	85	90
np +211	(G71R)	GA	9	5
		AA	1	0
		p(A)	0.058	0.026
Exon 1	T→C	TT	93	94
np +476	(I159T)	TC	2	1
		p(C)	0.011	0.005
Exon 1	T→C	TT	94	95
np +625	(*R209W*)	TC	1	0
		p(C)	0.005	0.000
Exon 2	C→G	CC	95	66
np +6844	(*A321G*)	CG	0	29
		p(G)	0.000	0.152
Exon 2	A→G	AA	87	89
np +6846	(*I322V*)	AG	7	6
		GG	1	0
		p(G)	0.042	0.032
Exon 3	G→A	GG	94	95
np +7640	(*D359N*)	GA	1	0
		p(A)	0.005	0.000
Exon 4	C→T	CC	93	95
np +7939	(*P364L*)	CT	2	0
		p(T)	0.011	0.000
Exon 4	A→G	AA	92	95
np +7975	(H376R)	AG	3	0
		p(G)	0.016	0.000

[1]Amino acid changes resulting from nucleotide changes in the exons are indicated in parentheses.

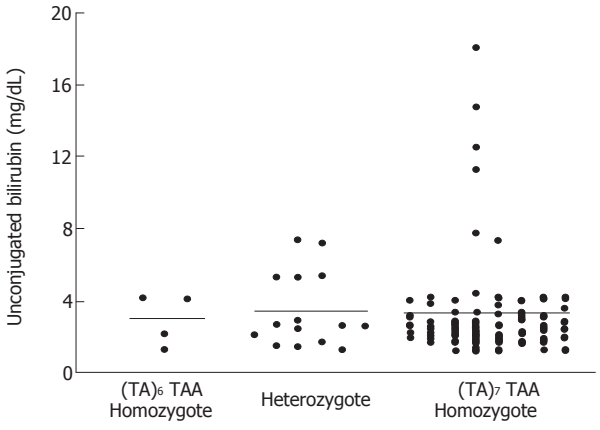

Figure 1 Distributions of un-conjugated serum bilirubin values among Gilbert's syndrome patients classified by the genotype of the common TA insertion in the TATA box of the UGT1A1 promoter.

with (dG = -14.8) the additional CAT insertion. A striking structural change was found around the region of the insertion, the loops were converted to stems. It is well known that formation of stems in the promoter could reduce transcription of the gene. A detailed functional analysis of the UGT1A1 promoter by luciferase reporter assay confirmed that there was a two-fold decrease in transcription efficiency for the variant $(TA)_7$TAA promoter allele, and a 20-fold decrease when there was a CAT insertion in the promoter on the background of the $(TA)_7$TAA allele compared to the normal $(TA)_6$TAA promoter allele (Figure 3).

DISCUSSION

Genetically determined un-conjugated hyperbilirubinemia constitutes a spectrum of clinical entities characterized by incremental serum bilirubin values related to graded reduction of UGT1A1 enzyme activity. The most novel finding of our study is that a trinucleotide (CAT) insertion in the nucleotide positions -85 to -83 of the UGT1A1 promoter was present in GS patients who were homozygous for the causal TA insertion, but not in other GS patients or in controls. This insertion significantly ($P < 0.001$) elevated the un-conjugated bilirubin level (mean = 6.13 mg/dL) in the patients to the range usually seen in Crigler-Najjar (CN) II syndrome. We have

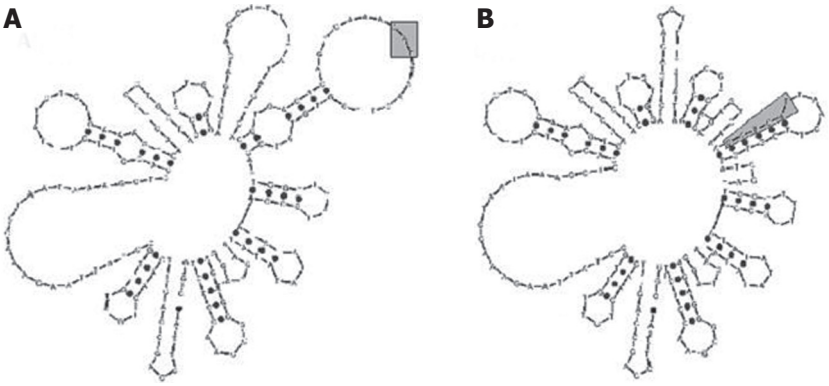

Figure 2 Folded DNA structures of the UGT1A1 promoter region with one copy of the CAT trinucleotide (shaded region) (**A**) and two copies of the CAT trinucleotide (CAT insertion allele, shaded region) (**B**).

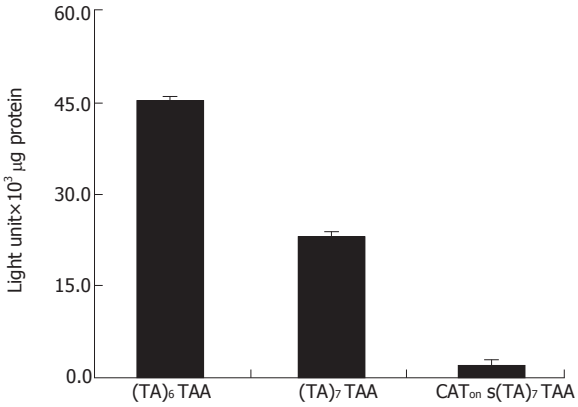

Figure 3 Transcriptional activities of the (TA)$_6$TAA, (TA)$_7$ TAA and CAT alleles as assessed by a luciferase reporter assay.

demonstrated that this insertion in the background of the (TA)$_7$ allele classically described in GS, diminishes UGT1A1 transcriptional activity to about 5% (2.4 light units/10^3 μg protein) of the normal (44.9 light units/10^3 μg protein), much lower than the 50% reduction (22.9 light units/10^3 μg protein) observed for (TA)$_7$ alone. Thus the combined biological effect of the CAT and TA insertions is a further elevation in the un-conjugated bilirubin level compared to that of the TA insertion alone, which is exactly what we have observed in the GS patients carrying the CAT insertion. This initial description of the interactive influence of a novel trinucleotide insertion and the GS-type abnormality in the promoter region alone, in the absence of a consistent exonic mutation, to quantitatively elevate un-conjugated bilirubin to levels observed in Crigler-Najjar II (CN II) provides further evidence of genetic heterogeneity and overlap of the clinical syndromes of un-conjugated hyperbilirubinemia. Thus, Gilbert's, Crigler-Najjar I (CN I) and CN II syndromes may not be mutually exclusive clinical-genetic entitities but are different windows of the quantititative spectrum of elevated serum unconjugated bilirubin levels.

The association (80%) between homozygosity for the (TA)$_7$TAA allele and GS is much stronger in India than reported earlier from most other ethnic groups[14,15,16,20]. Graded reduction of UGT1 activity has been demonstrated with increasing length of the TA repeats in a recent study[28]. It was demonstrated that 7/7 homozygotes and 7/6 heterozygotes have, respectively, a 52% and a 37% reduction of UDP glucuronyl transferase activity in liver tissue homogenates. In our study, serum un-conjugated bilirubin values among 7/6 heterozygote GS patients were high but not significantly different from the 7/7 homozygotes. The finding of increased serum bilirubin values among the 7/6 heterozygote GS patients to the same extent as those homozygous for the classically described 7/7 genotype, even in the absence of any other consistent change in the UGT1A1 gene, is intriguing. We could not explain this finding, but speculate the role of other interacting non-UGT1A1 genetic variants.

Although the insertion of additional TA repeats in the (TA)$_6$ TAA promoter, sequence of the UGT1A1 gene is the most common variation associated with Gilbert's syndrome, (TA)$_5$ and (TA)$_8$ sequences in the TATAA box have also been found[4,14]. We did not find (TA)$_5$ and (TA)$_8$ alleles in our study participants. Moreover, several mutations in the exons have been found in other populations, particularly from Asia, in association with GS and hyperbilirubinemia, either as the only abnormality or in addition to the more common promoter defect[20-22,29-31]. We have identified several promoter and coding region (all non-synonymous) variants of the gene (Table 1). The G71R variant has been reported earlier[19, 22-24], and the P364L variant has recently been reported in one Japanese GS patient[19]. Contrary to the finding among the Japanese[32], the G71R change had no impact on un-conjugated bilirubin level in our samples, nor did it interact with the dinucleotide (TA) insertion in the TATA box of the UGT1A1 gene. Many variants discovered in this study have not been reported earlier and many variants reported earlier were not found in the present sample. Although all the observed variations in exons could result in amino acid substitutions, none of these significantly altered the mean bilirubin level. Thus, the UGT1A1 gene appears to tolerate a large number of mutations without any significant deleterious effect. None of these was strongly associated with the (TA)$_7$TAA allele in our study. However, the amino acid change from isoleucine to valine at the amino acid position 322 was only found among the controls and not among the GS patients. Further studies are necessary to test whether this change helps bilirubin glucuronidation by the UGT1A1 gene.

The UGT1A1 polymorphisms have recently acquired significance because they predispose individuals to altered metabolism and enhanced toxicity of several drugs like paracetamol, propofol, irinotecan, indinavir, *etc*, which are

substrates for glucoronidation by UGT1A1[33-36]. Exons 2-5 are shared by other UGT1A transcripts and isozymes that mediate metabolism of xenobiotics apart from A1 involved in bilirubin glucuronidation[37]. The new variants in these exons may be relevant to drug metabolism, but we have not tested this. Our findings reveal that it is crucial to carry out detailed surveys on genetic variations in and around the UGT1A1 gene and functional studies on these variants for a deeper understanding of quantitative anomalies of bilirubin.

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RAPID COMMUNICATION

Ultrasonic characterization of porcine liver tissue at frequency between 25 to 55 MHz

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Abstract

AIM: To study the relation between acoustic parameters and histological structure of biological tissue and to provide the basis for high-resolution image of biological tissues and quantitative ultrasonic diagnosis of liver disease.

METHODS: Ultrasonic imaging and tissue characterization of four normal porcine liver and five cirrhotic liver tissue samples were performed using a high frequency imaging system.

RESULTS: The acoustic parameters of cirrhotic liver tissue were larger than those of normal liver tissue. The sound velocity was 1577 m/s in normal liver tissue and 1631 m/s in cirrhotic liver tissue. At 35 MHz, the attenuation coefficient was 3.0 dB/mm in normal liver tissue and 4.1 dB/mm in cirrhotic liver tissue. The backscatter coefficient was 0.00431 dB/Srmm in cirrhotic liver tissue and 0.00303 dB/Srmm in normal liver tissue. The backscatter coefficient increased with the frequency. The high frequency images coincided with their histological features.

CONCLUSION: The acoustic parameters, especially the sound backscatter coefficient, are sensitive to the changes of liver tissues and can be used to differentiate between the normal and pathological liver tissues. High frequency image system is a useful device for high-resolution image and tissue characterization.

Key words: Porcine liver tissue; Hepatocirrhosis; High frequency imaging; Tissue characterization; Acoustic parameter

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INTRODUCTION

Ultrasonic diagnosis has been widely used in the clinical medicine. However, contemporary ultrasonic diagnosis technique is limited to the qualitative description at frequency ranging between 0.5-10 MHz based on the gray scale presentation in ultrasonic image. Due to the longer wavelength at a low frequency, the image resolution is not very high. Besides, the physical quantity using the ultrasonic image is the amplitude of the echo while the spectrum of the echo is overlooked.

The research on the ultrasound scatter spectrum has begun since 1970s. Shung and Reid[1] conducted an experimental study of ultrasound backscatter in calf liver and muscle using the pulse insert-substitution method. The backscatter coefficients for calf heart, kidney, pancreas and spleen were studied by Ten and Shung[2] at frequency between 2-7 MHz using bandwidth pulse insert-substitution measurement system. The ultrasound backscatter in myocardial muscle of dog was studied by Donnell *et al*[3]. The anisotropy for ultrasound backscatter in myocardial muscle was reported by O'Mottley and Miller[4], which coincides with the theoretical prediction. The backscatter coefficient for human brain was studied at frequency between 0.5-1.5 MHz by Barger[5], who successfully differentiated the white from the gray matters in the brain. Nicholas[6] studied the backscatter coefficient of human tissues using spectrum technique. However, the frequency used in these researches is below 10 MHz. To increase the resolution of imaging, the frequency of ultrasound must be increased. D'Astous *et al*[7] and Foster *et al*[8] have achieved the high frequency imaging of breast. Turnbull[9-12] studied mouse embryos using ultrasound backscatter microscopy. Ye *et al*[13] and Pavlin *et al*[14,15] studied the ocular tissue using high frequency imaging. The ultrasonic properties of vascular tissues and blood were measued from 35 to 65 MHz by Lockwood *et al*[16] and Meyer *et al*[17]. Yano *et al*[18] and Liu *et al*[19] studied the high frequency imaging of skin and thyroid. Ultrasound backscatter microscopy images of the internal structure of living tumor spheroids have been

obtained[20,21].

Liver is the important organ for metabolism. Hepatic lobule is the basic element in liver, and is surrounded by connective tissue. Most blood capillaries pass through the boundary of its connective tissues. Hepatic tissue regeneration and connective tissue hyperplasia induce change of the normal structure of hepatic lobule. The liver becomes deformed and stiff, known as hepatocirrhosis. Common liver diseases can be diagnosed by B- ultrasound. However, it is difficult to diagnose diffuse diseases of liver.

The present study was to investigate the relation between acoustic parameters and histological structure of biological tissue and to provide the basis for high-resolution image of biological tissues and quantitative ultrasonic diagnosis of liver disease.

MATERIALS AND METHODS

Experimental system

High frequency imaging system was set up in our laboratory. The sample was fixed in the sample-holder between quartz and a thin plastic membrane and then mounted in water bath. The focused ultrasound transducer (center frequency 35 MHz, -3dB bandwidth from 25 MHz to 55 MHz, f-number 1.6, focus depth 1 mm, beam width 70 μm) was excited with a pulse (300 Vpp in amplitude, 15 ns in width). The ultrasonic pulse passing through the membrane, sample reflected from the quartz flat was received by the same transducer. The transducer and the step motor were carefully moved, the region of interest in the sample was located in the focus area and in the focal depth of the transducer. The scan parameters for raster motion were downloaded by MTM2500 pp (Newport Co, USA). The attenuation signal and backscatter signal were recorded by a 400 MHz digital scope (HP54502A, USA). The information about the C-scan imaging was acquired by sampling the backscatter signal after a specified delay corresponding to the focus and stored in a hard disk for software scan conversion after data collection was completed. An IEEE-488 bus transferred the digitized signals to the control computer for further processing

The transducer in a raster fashion over a 3 mm × 5 mm area was removed and a backscatter image of the sample was generated using C-scan mode. The region of homogenous tissue was selected for quantitative measurement. The incident direction of the sound beam was perpendicular to the surface of the sample during the measurement. The interest region containing 16 points in 4 by 4 grids was selected to measure the velocity of sound, 64 points in 8 by 8 grids were selected to measure the attenuation and backscatter coefficients of the sample and each grid was separated by 80 μm.

Sample preparation

Liver tissue samples were obtained from the Meat Processing Plant. In the experiment, the sample was clamped between plastic membrane and reflector quartz flat in a specially designed sample holder and both sides of the sample were cut at -10℃ using Cryostat 2700 (Frigocut, Reichert-june, Germany) to make the surface with a homogenous thickness between 0.5-1.5 mm. Then the sample was thawed in saline solution and sealed with a thin plastic membrane. In the study, four normal liver tissue samples and five cirrhotic liver tissue samples (hepatocirrhosis) were used. A slice from each sample was stained. The corresponding histological diagram and ultrasound image were obtained.

Table 1 Sound speed and attenuation and acoustic backscatter coefficient (mean ± SD)

		Normal liver	Cirrhotic liver
Sound speed (m/s)		1577 ± 7	1631 ± 5
Attenuation	α_1	0.121 ± 0.045	0.082 ± 0.023
(dB/mm)	α_{35}	3.0 ± 0.6	4.1 ± 0.8
	m	0.9	1.1
Backscatter coefficient	μ_1	$2.66 \times 10^{-6} \pm 1.43 \times 10^{-6}$	$4.85 \times 10^{-6} \pm 1.52 \times 10^{-6}$
(1/Srmm)	μ_{35}	0.00303 ± 0.00015	0.0043 ± 0.00025
	n	1.98	1.91

RESULTS

Images

The ultrasound imaging of normal and cirrhotic liver tissues and their corresponding pathological slices are shown in Figure 1A-1D. From the images, some microstructures of the tissue could be observed. However, the image of normal liver tissue (Figures 1A, 2B) was different from that of cirrhotic liver tissue (Figures 1C, 1D). The image of normal liver tissue was ultrasonically homogenous while the image of cirrhotic liver tissue was contrary to that of normal liver tissue. From the imaging, we could see the hyperecho of the cirrhotic liver. Some short and strong echoes caused by hyperplasia of connective tissue occurred.

Sound velocity, attenuation and backscatter coefficient

A summary of the acoustic parameters measured in the liver are listed in Table 1.The experimental results showed that the acoustic parameters from cirrhotic liver were larger than those from normal liver. The sound velocity in normal liver was 1 577 m/s and 1 631 m/s in cirrhotic liver, which coincides with the sound velocity (1 600 m/s) in bovine liver[22]. At 35 MHz, the attenuation coefficient was 3.0 dB/mm in normal liver and 4.1 dB/mm in cirrhotic liver. However, it has been reported to be 9.73 dB/mm at 70 MHz[22] and 0.89 dB/mm at 10.0 MHz respectively in liver[23]. The backscatter coefficient was 0.00431/Srmm at 35 MHz in cirrhotic liver and 0.00303/Srmm in normal liver. The power-law fits with the backscatter coefficient in normal liver as a function of frequency. However, it has been reported to be $(2.66\times10^{-6}\pm1.43\times10^{-6})^{f1.98}$/Srmm) and $(4.85\times10^{-6}\pm1.52\times10^{-6})^{f1.91}$/Srmm in cirrhotic liver[24]. The increase of backscatter coefficient is caused by hyperplasia of the connective tissue in cirrhotic liver (Figure 2). The backscatter occurs when the size of backscatter structure is similar to the wavelength of the sound beam, suggesting that the size of backscatters is within the wavelength of the ultrasound wave and the size of porcine liver lobules may be similar to it. The frequency-dependent decrease in cirrhotic liver reflects the increase of scattering elements

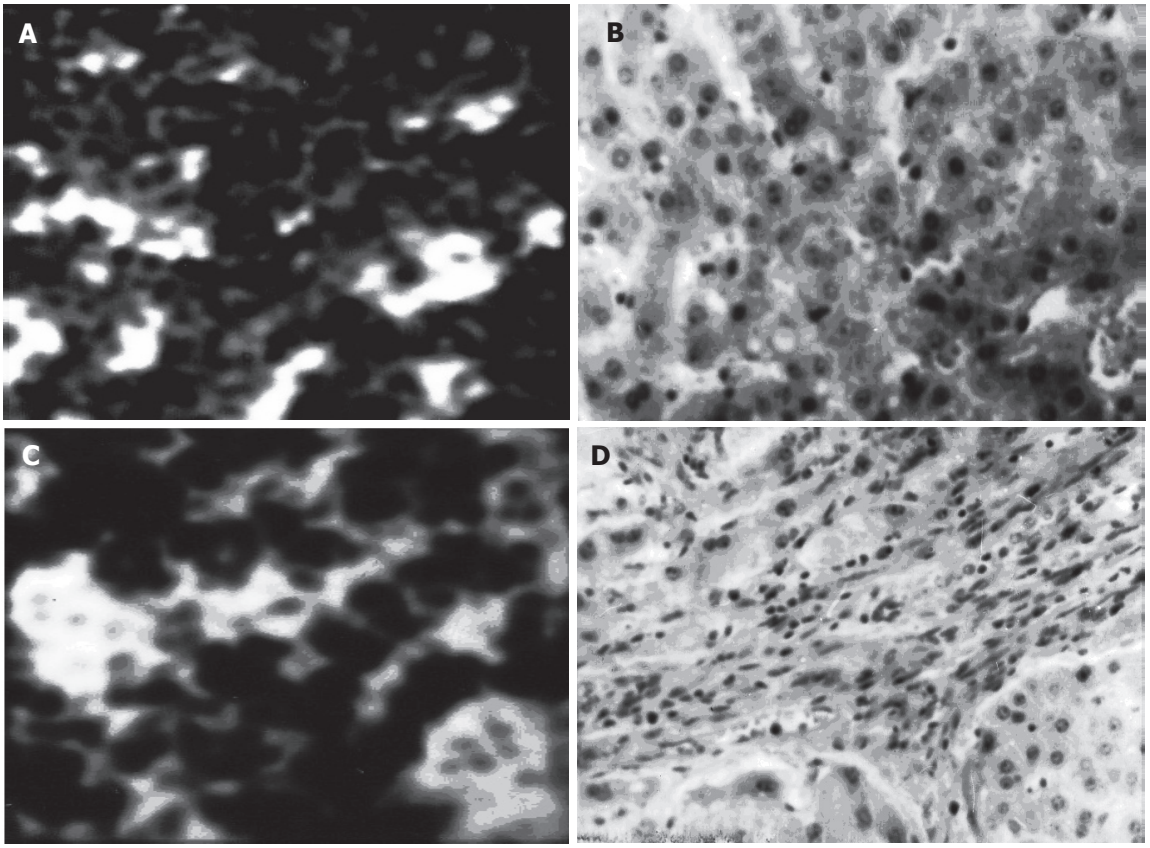

Figure 1 Ultrasound images of normal and cirrhotic liver and their corresponding pathological slices **A:** ultrasound imaging of normal liver; **B:** pathological slice of normal liver; **C:** ultrasound imaging of cirrhotic liver; **D:** pathological slice of cirrhotic liver.

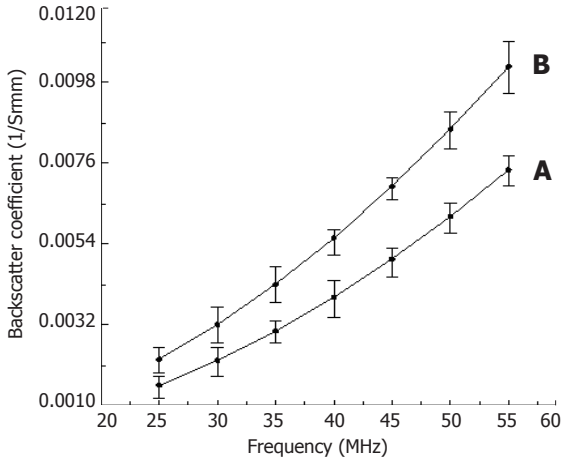

Figure 2 Backscatter coefficients changing with frequency in normal liver (**A**) and cirrhotic liver (**B**).

in the liver.

DISCUSSION

Ultrasonic tissue characterization based on spectrum analysis of backscattered radio frequency (RF) echo signals provides information regarding the acoustic properties of tissue, such as the size, concentration, and acoustic impedance of scatterers that are not available by conventional imaging methods[25,26]. The ultrasonic backscatter coefficient in human liver was measured in the range of 2.0-4.0 MHz[24]. The ultrasonic propagation properties at 100 MHz in excessively fatty rat liver were studied[27]. Strong correlation of ultrasonic speed with both water concentration and fat concentration in the liver was observed. The ultrasonic attenuation and velocity in rat liver as a function of fat concentration at 100 MHz were investigated[28] and the acoustic properties of freshly excised bovine liver were characterized at the frequency range between 20-200 MHz by the ultrasonic spectroscope system[22].

On the other hand, ultrasonic imaging systems in medicine depend fundamentally upon analysis of the ultrasonic scattering properties of the soft tissues investigated. However, the mechanism governing ultrasonic scattering within most soft tissues have not been delineated. In many cases the absolute magnitude of the scattering process has not been accurately quantified. The relationship between collagen and ultrasonic backscatter in myocardial tissue was evaluated[3]. The dependence of the ultrasonic scatter coefficient on collagen concentration in mammalian tissues was addressed[29]. And the mean-scatter spacing estimated with spectral correlation was used for tissue characterization[30]. However, there is little report on tissue characterization of hepatic tissues in the range between 25-55 MHz.

In this study, ultrasound images and sound parameters for hepatic tissues were studied by using C-scan ultrasound system at the frequency between 25-55 MHz. The above experimental results indicate that the resolution of high frequency image is much better than that obtained at low ultrasound frequency because some microstructure of the tissue can be seen. The information obtained from the ultrasound image is coincided with the histological feature of the sample. Furthermore, the samples need not to be stained. The acoustic parameters especially the sound backscatter coefficient are sensitive to the changes of the

tissues and can be used to differentiate between the normal and pathological tissues, the frequency dependence of backscatter coefficient could be reflected by the change of scattering elements and may be employed as a parameter for characterizing tissues and their physiological states. Therefore the high frequency image system is a useful device for high-resolution image and tissue characterization within the high frequency range.

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RAPID COMMUNICATION

Antiviral effect of Chinese medicine jiaweisinisan in hepatitis B virus transgenic mice

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Abstract

AIM: To study the antiviral effect of Chinese medicine jiaweisinisan (JWSNS) on hepatitis B virus (HBV) infection in transgenic mice (TGM).

METHODS: Twenty two 6-8 wk old HBV TGM in the third generation were divided into TGM control group and TGM treated group randomly. The normal control group included ten normal BC 57L/6 mice at the same age. The mice in treated group were administrated with JWSNS at the concentration of 4 g/mL and the dosage of 50 g/kg per d for 30 d, while the mice in TGM control group and normal control group were administrated with normal saline at the same dosage and the same time. Polymerase chain reaction (PCR) was used to assess the contents of HBV DNA in serum of HBV TGM before and after treatments, whereas blot hybridization was utilized to measure the contents of HBV DNA in the liver of both HBV TGM and normal BC 57L/6 mice.

RESULTS: The levels of serum HBV DNA in TGM treated group were remarkably decreased after the treatment of JWSNS (7.662 ± 0.78 *vs* 5.22 ± 3.14, $P < 0.05$), while there was no obvious change after administration of normal saline in TGM control group (7.125 ± 4.26 *vs* 8.932 ± 5.12, $P > 0.05$). The OD values of HBV DNA in the livers of the mice in TGM treated group were significantly lower than those of TGM control group (0.274 ± 0.096 *vs* 0.432 ± 0.119, $P < 0.01$).

CONCLUSION: JWSNS exerts suppressive effects on HBV DNA in the serum and liver of TGM.

Key words: Traditional Chinese herbs; Hepatitis B virus; Transgenic mice; PCR; Blot hybridization; Chronic hepatitis B

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INTRODUCTION

In this *in vivo* study, HBV transgenic mice (HBV TGM) models were established to detect the antiviral effects of traditional Chinese medicine, jiaweisinisan (JWSNS) on HBV TGM, so to further confirm the inhibitory effects of this traditional Chinese herb on HBV infection.

MATERIALS AND METHODS

Experimental animals

Normal C57BL/6 mice and the HBV transgenic mice, living in the same cote, were provided by Department of Transgenic Engineering in Hepatopathy Research Center of Guangzhou Military Hospital. All the non-transgenic mice were under close surveillance to ensure the HBV DNA in serum and tissue to be negative.

Traditional Chinese herbs

JWSNS, including buplerum chinense DC, flea body, prunus persica (L.) batsch, of 10 grams each, and radix paeoniae alba, fructus aurantii immaturus, dipsacus asper wall, rhizoma dryopteris crassirhizomae, eupatorium adenophorum sprengel, of 12 grams each, as well as 5 grams of glycyrrhizaglabral, and 30 grams of loranthus parasiticus, was prepared according to the traditional procedures. Five compounds of drugs were mixed together, 141 grams per compound, and dissolved into the water to distil twice, yielding 1500 mL distillation solution, followed by inspissation of the distillation to 180 mL. The final concentration was 4 g/mL, stored in refrigerator for use.

PCR primer and reagent

PCR primers and relevant reagents were provided by

Shanghai Bioengineering Research Center of Chinese Academy of Sciences. The sequence of PCR primer one is 5'-TGGCACTAGTAAACTGAGCC-3' and that of PCR primer two is 5'-ACATCAGGATTCCTAGGACC-3'. Other reagents such as $MgCl_2$, dNTP, buffer, Tag enzyme, and paraffin oil were purchased from Promega Company (Madison, USA). Quantitative diagnostic kit (batch number 1000-902-1) for HBV DNA was provided by Biotromcs Technological Company (San Francisco, USA).

DNA extraction kit

DNA extraction kit was obtained from Maikang Biotechnological Company of Zhongshan Medical University.

Recombinant plasmid PBR322-2.0 HBV rapid extraction reagents

The reagents included host strain, antibiotics, peptone, yeast extract, gelose, buffer Ⅰ (50 mmol/L glucose, 25 mmol/L Tris HCl, 10 mmol/L EDTA), buffer Ⅱ (0.2 mol/L NaOH, 1% SDS), and buffer Ⅲ (5 mol/L potassium acetate 60 mL, iced acetic acid 11.5 mL, water 28.5 mL).

α-^{32}P-DNA probe labeled reagents

It included NEN kit (Promega Co., Madison, USA), Sephaclex G-50 columniation, purified recombinant plasmid PBR322-2.0 HBV (100 ng/μL), 0.5 mol/L EDTA.

Blot hybridization reagents

It included nitrate fibrous membrane (aperture 0.45 μm, Amersham Co., Buckinghamshire, UK), Formamide 20×SSC buffer, 37% formaldehyde, dyestuff (25% Bromophenolblue dissolved into Ficoll), TE buffer.

Apparatus

Gene amp PCR system (Techne, England), AG-9600 equipment for analytical fluorescence microscope (USA), low-temperature freeze centrifuge (Biofuge 22R, Germany), water bath, pH meter, magnetic shaker, vacuum pump, refrigerator, balance (China), constant temperature rocker, constant temperature incubator, superclean working table (China), ZXZ-4 gyral vacuum pump (Zhejiang Linhai Vacuum Apparatus Factory), 721 spectrophotometer (The Third Spectrophotometry Factory, Shanghai) were used.

Establishment of HBV TGM model

P2.0 HBV plasmid passed through a series of *Eco*RI/*Sal*I restriction enzyme digestion, and electrophoresis, followed by the collection of 7.0 kb DNA fraction, which contained two end-to-end 3.2 kb HBV entire genes, and 0.6 kb PBR322 DNA vector. After electrophoresis quantitative analysis, the collected DNA fractions were dissolved in TE buffer under bioclean condition to ensure the concentration to be 1 mg/L, then respectively packed and stored at -20 ℃ to wait for micro- injection. C57BL/6 mice injected entire genome plasmid which contained HBV were identified to be G0 generation. Positively integrating male mice of G0 generation were selected to hybridise with infraspecific normal female mice, producing G1 generation. In similar manner, positively integrating male mice of G1 generation were selected to copulate with infraspecific normal female mice, producing G2 generation. Mice of G3 generation were produced in the same way[1].

Selecting procedures of HBV TGM

A total of one hundred and fifteen 6-8 wk old mice of G3 generation, weighing 20 g, were provided by Department of Transgenic Engineering in Guangzhou Military Hospital. HBV DNA detection kit was purchased from Baosheng Bioengineering Company (Dalian). Tissues were firstly detected to select HBV DNA positive mice, followed by serum detections. Twenty-two serum HBV DNA positive mice were eventually selected from 115 mice of G3 generation to be labeled as HBV DNA transgenic mice.

Grouping

Twenty-two HBV TGM, whose serum HBV DNA was positive, and 10 normal C57BL/6 mice were grouped into TGM treated group, 12 TGM mice; TGM control group, 10 TGM mice; normal control group, 10 normal C57BL/6 mice; and then registered. Additional six 6–8 wk old C57BL/6 mice, weighing (20 ± 2) g, whatever male or female, were also prepared. All the mice were fed in Experimental Animal Center of Guangzhou Military Hospital. Auto-supply of water and standard food were offered as well as air condition to maintain the constant temperature during the whole experimental procedures.

Administration

Mice in TGM treated group were administrated with JWSNS at 50 g/kg per d, in 0.3-0.4 mL JWSNS solution (about 4 g/mL, ig) once a day for four weeks, while the mice in the other two control groups were administrated with the same dosage of normal saline at the same time.

Sample collection

Two hours after the last administration, eyeballs were extirpated to collect peripheral blood before the execution of the mice followed and sera were then separated for detection of HBV markers. Promptly, livers were removed and broken, frozen by liquid nitrogen and stored at -70 ℃ for detection.

Detection method

PCR quantitative analysis[2, 3] was used to detect the serum contents of HBV DNA of TGM at the moment when the total contents had reached up to 1.0×10^5 kb/mL, while blot hybridization[4, 5] was utilized to analyze the contents of HBV DNA in liver of mice. The extraction of DNA in liver tissue was processed according to the instruction provided by the manufacturer of the kit. The rapid extraction of recombinant plasmid PBR322-2.0 HBV was performed by alkali fission method[6]. α-^{32}P-DNA probe was labeled according to instructions of Promega Co. reagent kit. Blot hybridization was performed as follows: 40 μL α-^{32}P-DNA probe labeled solutions was used

Table 1 Changes of serum levels of HBV DNA in HBV TGM before and after treatment of JWSNS (mean±SD)

Group		*n*	Serum HBV DNA	
			Case transformed from DNA positive to negative	Content of HBV[1]
TGM controlled group	Prior to administration of normal saline	10	0	7.125±4.26
	After administration of normal saline	10	0	8.932±5.12
TGM treated group	Prior to JWSNS treatment	10	0	7.662±0.78
	After JWSNS treatment	10	3	5.122±3.14

[1]Analyzed by *t*-test. TGM treated group, [1]$P<0.05$ *vs* TGM controlled group, [1]$P>0.05$.

Table 2 Effect of JWSNS on levels of HBV DNA in the liver of HBVTGM (mean±SD)

Group	*n*	HBV DNA blot hybridization
Normal control group	9	0
TGM control group	9	0.432±0.119
TGM treated group	9	0.274±0.096[b]

[b]$P<0.01$ *vs* TGM control group.

to dot on the membrane, which was then dipped in the metamorphic solution, and baked in the oven at 80 ℃ for two hours. After pre-interaction, membrane washing, slice nipping, the positive degree was justified according to the OD value of each blot.

Statistical analysis

Statistical data was analyzed by SPSS software. The comparability prior to and after disposal of the same sample was verified by paired-samples *t*-test, whereas the difference of the mean value among various groups was analyzed by χ^2 test. *P* less than 0.05 was taken as significant.

RESULTS

Effects of JWSNS on serum contents of HBV DNA in HBVTGM

The levels of serum HBV DNA in TGM treated group displayed considerable distinction before and after treatment of JWSNS ($P<0.05$), compared with those in TGM controlled group in which no significant difference was shown before and after administration with normal saline ($P>0.05$) (Table 1).

Effect of JWSNS on levels of HBV DNA in the liver of HBV TGM

HBV DNA blot hybridization was positive in HBV TGM both before and after treatment. The OD value showed extremely significant difference between TGM controlled group and TGM treated group ($P<0.01$), whereas blot hybridization in normal control group appeared to be negative (Table 2).

DISCUSSION

Value of HBV TGM model in the study of inhibitory effect of the traditional Chinese medicine on HBV

The host infected by HBV showed violent phyletic and tissue specificity, resulting in the remarkable restriction in the establishment of the animal model infected by HBV and anti-HBV study. In the past, data related to anti-HBV entirely came from HBV-infected patients, orangutan or cell *in vitro*. In addition, significant distinction existed between experimental data of other hepatophilic DNA virus such as duck or groundhog HBV and those of human HBV[7,8].

In 1980, microinjection HBVTGM model was successfully established which obviously overcame the limitation described above. Chissari [9] established TGM model and claimed that HBV DNA and HBsAg granules were detected in TGM blood and congregated in a fraction of hepatocyte, inducing tumefaction and dysfunction of the endoplasm as well as the ground glass hepatocytes, besides enhancing the sensitivity of hepatocytes to the lipopolysaccharide and IFN-γ, resulting in the damnification, necrosis and regeneration of the hepatocyte, or even the occurrence of hepatocarcinoma. Thus, HBVTGM model was, to a great extent, similar to the immune interaction between virus and host during natural infection of HBV[10].

HBVTGM is viewed as an immune tolerance condition. Despite limited damnification of hepatocytes in some mice, inflammation was not obvious, which is consistent with the pathologic change in human beings infected with HBV. The objective of our study emphasized on suppressive effect of JWSNS on HBV including the change of HBV contents in blood and tissue rather than the inflammatory level in liver. Thus, it is appropriate that HBVTGM served as the anti-HBV model.

Anti-HBV effect of traditional Chinese medicine and significance of HBV DNA detection

The symptom in different stage of HBV persistent infection varies from chronic asymptomatic HBV carrier and chronic hepatitis to hepatocirrhosis and hepatocarcinoma[11,12]. According to modern medicine chronic asymptomatic HBV carrier is viewed as the earlier stage of chronic persistent infection of HBV, in which the immune system is inhibited, leading to the inefficacy of interferon and lamivudine, the traditional antivirus drugs. Thus it is a widespread viewpoint that there is no effective treatment for earlier stage of HBV infection. However, it does not mean that there is no need for treatment[13,14]. Of note, at this stage, viruses copy themselves constantly, accompanied with the obvious viraemia, and approximately

normal liver function. However, as the copying process continues, a series of immune reaction of the host would be triggered to damage the liver and other organs. This stage may be a chance for traditional Chinese medicine to act as antivirus agent[15]. Pioneering clinical experiences indicated that traditional Chinese medicine such as phyllanthus urinaria L., matrine could endow the host in immune tolerance condition advantage in antivirus effect and protective effect on liver, and the detection of HBV DNA contents could help evaluate the antivirus effect of traditional Chinese medicine.

Theoretic basis for treatment of HBV infection by JWSNS

The treatment regimen of chronic hepatitis B by JWSNS is rooted in pathologic hypothesis of traditional Chinese medicine proposed by Bao Yi Liu, a famous doctor in ancient China who stated that whenever the *nephric qi* is inadequate, the protective function of human body against disease would decline, whereas *sick qi* would take the chance to invade the human body, weakening the *hepatic qi*, inducing the damp and the heat, which then attack the spleen and stomach, leading to the disfunction of both liver and spleen. This main pathologic process persists during the whole course of disease. According to this pathologic hypothesis, nothing but nourishing *nephric qi* is the key step to cure chronic hepatitis B. JWSNS, a famous compound, is used to enrich *nephric qi,* thus to reinforce the protective effect of the human body, and to overcome the state of immune tolerance.

Inhibitory effect of JWSNS on HBV of HBVTGM

In this study, HBVTGM model was used to observe the change of HBV DNA content both in serum and in hepatic tissue before and after the JWSNS treatment. The contents of HBV DNA in liver reflect the contents of HBV in hepatocyte. HBV, a hepatophilic virus, invades into the hepatocyte, in which they copy themselves, and then migrate into the peripheral circulation, inducing the diffuse chronic infection of HBV. The contents of HBV DNA reflect the level of virus copy. In the study, hepatocellular DNAs were extracted, and with the probe of P^{32} labeled plasmid P2.0 HBV, blot hybridization proceeded in nitrate fibrous membrane. The results of blot hybridization showed that no blot could be seen in normal control group, in striking contrast with the obvious blot appearing in both TGM control group and TGM treated group. The measurement of OD value of the blot demonstrated that the contents of HBV DNA in hepatocyte dramatically decreased four weeks after JWSNS treatment, compared with HBVTGM control group ($P < 0.01$).

PCR, a comparatively sensitive method was utilized in detecting the change of serum HBV DNA before and after JWSNS treatment in treated group or before and after administration of normal saline in control group. Four weeks later, no significant change could be seen in TGM control group administrated with normal saline, whereas HBV DNA of three mice was converted from positive to negative after treatment with JWSNS, and the serum contents of HBV DNA showed significant decline before and after treatment ($P < 0.05$).

The detection of HBV DNA contents in both liver tissue and serum shows that JWSNS could, to certain extent, inhibit HBV DNA, which provides the experimental proof for treatment of chronic hepatitis B with JWSNS.

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RAPID COMMUNICATION

Effect of explosive noise on gastrointestinal transit and plasma levels of polypeptide hormones

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Abstract

AIM: To investigate the effect of firing noise on gastrointestinal transit and probe its mechanism by measuring the levels of plasma polypeptide hormones.

METHODS: A total of 64 SD rats were randomly divided into a control group and three stimulating groups. Firing noise of different intensity by sub-machine guns was used as inflicting factor. The effect of firing noise on liquid substance gastrointestinal transit and solid substance gastrointestinal transit was observed by measuring the ratio of carbon powder suspension transmitting and barium sticks transmitting respectively. Plasma levels of polypeptide hormones were measured by radio-immunoassay.

RESULTS: The noise accelerated gastrointestinal transit of solid food by more than 80 db;and accelerated gastrointestinal transit of liquid food significantly by more than 120 db. Meantime, plasma levels of plasma motilin (MTL)(157.47±16.08; 151.90±17.08), somatostatin (SS)(513.97±88.77; 458.25±104.30), substance P (SP)(115.52±20.70; 110.28±19.96) and vasoactive intestinal peptide (VIP) (214.21±63.17; 251.76±97.24) remarkably changed also.

CONCLUSION: Within a certain intensity range, the firing noise changes the levels of rat plasma gastrointestinal hormones, but the gastrointestinal transit is still normal. Beyond the range, the noise induces plasma hormone levels disturbance and gastrointestinal transit disorder.

Key words: Explosive noise; Gastrointestinal transit; Gastrointestinal hormone
Mu ZB, Huang YX, Zhao BM, Liu ZX, Zhang BH, Wang QL. Effect of explosive noise on gastrointestinal transit and plasma levels of polypeptide hormones. *World J Gastroenterol* 2006; 12(14): 2284-2287

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INTRODUCTION

Explosive noise may produce an adverse effect on gastrointestinal tract, and there is higher incidence of digestive system disease in war time. Current studies mainly focus on morphological changes of gastrointestinal tract[1], and there is little information available in literature about the effect of firing noise on the gastrointestinal transit. The aim of this study was to show the effects of explosive noise on gastrointestinal transit, and probe its mechanism by measuring the levels of plasma polypeptide hormones.

MATERIALS AND METHODS

Materials

Healthy SD rats were purchased from Animal Center of the Fourth Military Medical University (FMMU). The ND2 volume level meter was provided by the School of Aerospace Medicine of FMMU. Radioimmunoassay kits were supplied by Naval Radioimmunoassay Technology Center. An FJ-2003/8PS radioimmunity counter and a high-speed and low-temperature centrifuge were used in this experiment.

Experimental procedures

After an adaptive phase of 7 d, 64 SD male rats were randomly divided into 4 groups: Group A, consisting of 16 rats, which were not stimulated; Group B, which were stimulated with 40 dB noise; Group C, which were stimulated with 80 dB noise; and Group D, which were stimulated with 120 dB noise. Different groups received different intensity noise stimulations respectively. Then 8 rats of each group (including Group A) were intragastricly administrated with carbon powder suspension[2,3], anesthetized 20 min later, and then decapitated. Blood was sampled and the ratio of carbon powder suspension transmitting measured. The other 8 rats were intragastricly administrated with barium small sticks[4], anesthetized 10 h later, and decapitated. Blood was sampled in the same way and the ratio of barium sticks transmitting observed under X-ray.

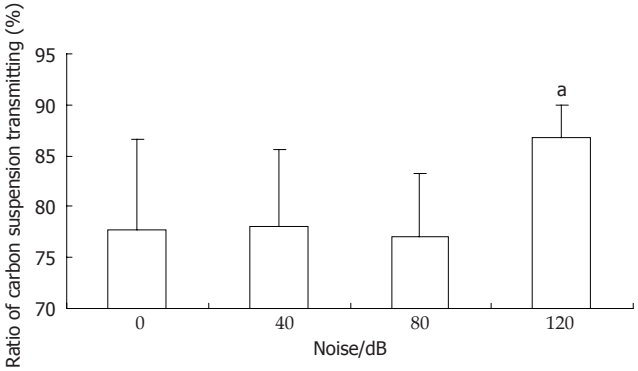

Figure 1 Effect of explosive noise on liquid substance gastrointestinal transit ($n=8$, *vs* 0 dB), $^aP<0.05$.

Noise stimulation

After 12-h abrosia, rats were put into sound-proof room. Firing noise of submachine guns acted as an inducing factor which had been recorded and was played to rats through a loudspeaker at a distance of 20-30 cm for 12 h. Examined with ND2 volume level meter and frequency spectrum analyzer, the intensity of the firing noise was measured as 0 dB (Group A), 40 dB (Group B), 80 dB(Group C) and 120 dB(Group D) respectively and their frequency as 0.25-4.00 kHz.

Liquid substance gastrointestinal transit

Rats were intragastricly administrated with 1 mL carbon powder suspension, consisting of carbon powder (50 g/L), gum arabic (100 g/L) and water(850 g/L), then anesthetized and dissected 20 min later. Small intestine was taken out of abdomen and tiled on the bench. The distance of carbon powder transmission from pyloric sphincter to the end of small intestine was measured, and the ratio of carbon powder suspension transmitting calculated [5,6] (distance of carbon powder transmission/ total length of small intestine ×100%).

Solid substance gastrointestinal transit

Rats were intragastricly administrated with 10 barium small sticks (length: 5 mm, diameter: 1 mm), then anesthetized and dissected 10 h later. The sticks were taken out of gastrointestinal tract. Barium small sticks which remained in each segment of the gastrointestinal tract were counted.

Measurement of plasma MTL, SS, VIP and SP

Rats were decapitated to sample the blood. Blood plasma was separated by centrifugation at 3500 r/min at 4 ℃, then concentrations of MTL, SS, SP and VIP were tested by radio-immunoassay[7], according to instruction of RIA kits strictly.

Statistical analysis

Analysis of variance was performed to investigate the effects of firing noise on gastrointestinal transit and plasma polypeptide hormone level in rats of four groups. All data were presented as mean ± SD. $P<0.05$ was taken as significant.

Table 1 Plasma polypeptide hormones after noise stimulation (mean ± SD, $n=8$, μg/L)

Noise/dB	MTL	SP	SS	VIP
0	128.0±5.1	52.5±20.1	184.6±49.6	254.3±129.1
40	130.6±15.6	74.4±17.4	382.8±79.1^b	471.1±145.4^a
80	133.2±30.5	86.6±15.2^b	386.6±59. 6^b	460.6±173.8^a
120	157.5±16.1^b	115.5±20.7^b	514.0±88.8^b	214.2±63.2

$^aP<0.05$. $^bP<0.01$ *vs* 0 dB(control).

RESULTS

Liquid substance gastrointestinal transit and plasma peptides levels

Ratios of carbon powder suspension transmitting in groups B and C were 78.1%±7.6% and 77.1%±6.1% respectively, and were not significantly different from that of group A (control group, 77.8%±8.8%). Ratio of carbon powder suspension transmitting in group D was 86.7%±3.3%, significantly higher than that of group A ($P<0.05$). The liquid substance gastrointestinal transit of group D (120 dB noise stimulated group) was accelerated significantly (Figure 1).

At the same time, all of the plasma MTL, SS, SP and VIP concentrations in groups B and C were increased compared with that of group A. In group D, plasma MTL, SS and SP concentrations were increased, but plasma VIP concentration decreased to some extent (Table 1). It could be seen that plasma peptides in groups B and C were increased gradually, but plasma peptides in group D were changed irregularly.

Solid substance gastrointestinal transit and plasma peptides levels

Percentage of barium sticks remained in the gastrointestinal tract in group A was 40.6%±25.4%, and that of group B was 36.9%±22.1%. There was no significant difference between groups A and B. Percentage of barium sticks remained in groups C and D was 16.2%±10.8% and 22.5%±16.1% respectively, significantly lower than that of group A(control group) ($P<0.05$) (Figure 2). There was smaller percentage of barium sticks remained in the gastrointestinal tract, indicating that barium sticks gastrointestinal transmit was faster. So the solid substance gastrointestinal transit of groups C and D (80,120 dB noise stimulated group) was accelerated.

All of the plasma peptide concentrations in group B was increased compared with that of group A. In groups C and D, plasma MTL, SS and SP concentrations were increased, but plasma VIP concentration decreased to some extent(Table 2). It could be seen that plasma peptides in group B were increased stepwise, but plasma peptides in groups C and D were irregularly disturbed.

DISCUSSION

There is a higher incidence of digestive system disease in war time than in peace time. Explosive noise is one of important factors that induce human body stress

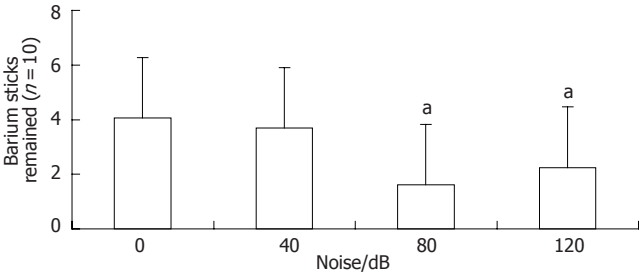

Figure 2 Effect of explosive noise on gastrointestinal transit $^{a}P<0.05$ *vs* 0 dB.

Table 2 Plasma polypeptide hormones after noise stimulation (mean ± SD, $n = 8$, μg/L)

Noise/dB	MTL	SP	SS	VIP
0	128.0±5.1	52.3±23.0	308.9±222.8	275.5±125.5
40	130.2±8.9[a]	82.6±15.7[b]	329.3±95.6	359.2±227.8
80	134.9±12.6[b]	110.3±20.0[b]	458.3±104.3[a]	251.8±97.2
120	151.9±17.1[b]	115.2±20.4[b]	503.7±65.3[b]	257.8±142.2

$^{a}P<0.05$. $^{b}P<0.01$ *vs* 0 dB (control).

in war time, and it may produce an adverse effect on gastrointestinal tract[8-10]. Liu found that explosive noise could injure gastric mucosa. Our results indicated that explosive noise could accelerate gastrointestinal transit, and that the effect was related to the intensity of the noise. There was no apparent change in gastrointestinal transit ($P>0.05$), after stimulated with lower intensity noise; however, not only solid substance but also liquid substance gastrointestinal transits were accelerated significantly ($P<0.05$), after stimulated with high level noise.

Gut hormone is a main factor in regulatory mechanism of gastrointestinal motility, and plays an important regulatory role in gastrointestinal function in stress state[11-13]. Gut hormone is generally divided into two categories, erethitic hormone and inhibitive hormone. They are contradictory regulating factors in the blood. When balance between them is lost, the disturbance of gastrointestinal motility occurs[14]. We observed plasma concentrations of two kinds of erethitic braingut peptides and two kinds of inhibitive braingut peptides, after stimulation of rats with different intensity noise, so as to probe the mechanism of explosive noise impacting gastrointestinal transit.

We found that concentrations of all four plasma peptides were increased, when rats were stimulated by low level noise, and the balance between stimulatory and inhibitory gut hormones was normal. When rats were stimulated with high level noise, three of peptides (including MTL, SS and SP) were increased in comparison with normal controls, but plasma VIP was decreased.

It is well known that MTL and SP are erethitic gut hormones. They could promote GI transit[15,16]. Our results showed that plasma MTL and SP concentration increased after stimulation with firing noise. After lower intensity sound stimulations, their plasma levels increased to some extent and after high intensity sound stimulations, their plasma levels increased obviously. In all, we found that plasma erethitic gut hormones concentration was increased and was positively correlated with intensity of noise.

SS exerts depressive effect on GI motility[17]. Previous findings showed that plasma SS level was higher in stress state[18,19]. Similarly, we found that plasma SS concentration increased along with the augmentation of the sound intensity. VIP is another kind of inhibitive gut hormone[20]. In our study, after lower intensity sound stimulation, plasma VIP concentration increased also; but it declined after stimulation by high intensity noise.

Rats, after stimulation by low intensity noise, could keep the GI transit normal, and the balance between the erethitic and inhibitive gut hormones was kept under moderate stress still, which helped to keep GI transmission function normal. At same time, we found that disturbance of GI transmission function occurred after stimulation with high intensity noise, and that the balance between the erethitic and inhibitive gut hormones was lost because of severe stress, which induced GI transmission function disorder. Therefore we presumed that changes of plasma gut hormone level were related to GI transmission function disorder induced by explosive noise and were one of the important underlying reasons.

In summary, our results indicate that explosive noise could induce stress in rats and exert some negative effect upon gastrointestinal transit. After explosive noise stimulation, secretion of many kinds of gut hormone is chaotic, which plays an important role in occurrence of gastrointestinal transmission disorder.

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RAPID COMMUNICATION

Assay of gastrin and somatostatin in gastric antrum tissues of children with chronic gastritis and duodenal ulcer

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Abstract

AIM: To study the expressions of gastrin (GAS) and somatostatin (SS) in gastric antrum tissues of children with chronic gastritis and duodenal ulcer and their role in pathogenic mechanism.

METHODS: Specimens of gastric antrum mucosa from 83 children were retrospectively analyzed. Expressions of GAS and SS in gastric antrum tissues were assayed by the immunohistochemical En Vision method.

RESULTS: The expressions of GAS in chronic gastritis Hp+ group (group A), chronic gastritis Hp- group (group B), the duodenal ulcer Hp+group (group C), duodenal ulcer Hp- group (group D), and normal control group (group E) were 28.50+4.55, 19.60+2.49, 22.69+2.71, 25.33+4.76, and 18.80+2.36, respectively. The value in groups A-D was higher than that in group E. The difference was not statistically significant. The expressions of SS in groups A-E were 15.47+1.44, 17.29+ 2.04, 15.30+1.38, 13.11+0.93 and 12.14+1.68, respectively. The value in groups A-D was higher than that in group E. The difference was also not statistically significant.

CONCLUSION: The expressions of GAS and SS are increased in children with chronic gastritis and duodenal ulcer.

Key words: Chronic gastritis; Duodenal ulcer; Gastrin; Somatostatin

Xie XZ, Zhao ZG, Qi DS, Wang ZM. Assay of gastrin and somatostatin in gastric antrum tissues of children with chronic gastritis and duodenal ulcer. *World J Gastroenterol* 2006; 12(14): 2288-2290

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INTRODUCTION

Infantile chronic gastritis and peptic ulcer are frequently encountered in children. The gastroscopic diagnostic rate accounts for 85%-94.5% for chronic gastritis and 8%-22% of peptic ulcer. The causes of these diseases and pathogenic mechanisms are not completely clear. The digestive tract is the largest and most complicated secretory organ in human bodies. Gastrointestinal hormones not only play an important regulatory role in secretory and motor functions of the digestive tract, but also have an important effect on its growth, development and damage repair. It has been shown that abnormity of GAS and SS plays an important role in causing chronic gastritis and duodenal ulcer in children[1]. We examined the expression of GAS and SS in gastric antrum tissues from 83 children with chronic gastritis and duodenal ulcer using immunohistochemical method (Table 1). The changes in GAS and SS and Hp infection and their effect on chronic gastritis and duodenal ulcer in children were studied, in order to provide a theoretical basis for the gastrointestinal hormone drugs used in the treatment of chronic gastritis and duodenal ulcer in children.

MATERIALS AND METHODS

Materials

Paraffin specimens of gastric antrum mucosa were obtained from the children during the period of 2002-2003 in our hospital. All the subjects did not receive gastrointestinal kinetic drugs and H2 receptor agonists. The biopsy tissues were fixed in 10% neutral formalin, routinely dehydrolyzed, paraffin-embedded and cut into sections which were stained with HE. Hp infection was diagnosed by rapid urease test and microscopy. The cases were regarded as strongly positive when the test paper of urease test became cherry-red from yellow within 1 min, weakly positive when the test paper turned cherry-red and negative when the test paper did not change color. The weakly positive specimens were excluded from the study. Microscopy at 400 × magnification revealed positive rod-shaped bacteria in the gastric pit and body (Figure 1). A case was considered Hp+ when it was positive in the two methods and Hp- when it was negative in the two methods. The experimental samples were divided into group A: chronic superficial gastritis, Hp+; group B: chronic superficial gastritis, Hp-; group C: duodenal ulcer, Hp+; group D: duodenal ulcer, Hp-; and group E: normal gastric antrum tissues with no obvious pathologic

Table 1 Expression of GAS and SS in gastric antrum mucosa from children with chronic gastritis and duodenal ulcer (mean ± SD)

Groups	*n*	GAS	SS
Chronic gastritis Hp+ group	20	28.50±4.55	15.47±1.44
Chronic gastritis Hp- group	19	19.60±2.49	17.29±2.04
Duodenal ulcer Hp+ group	21	22.69±2.71	15.30±1.38
Duodenal ulcer Hp- group	12	25.33±4.76	13.11±0.93
Normal control group	11	18.80±2.36	12.14±1.68

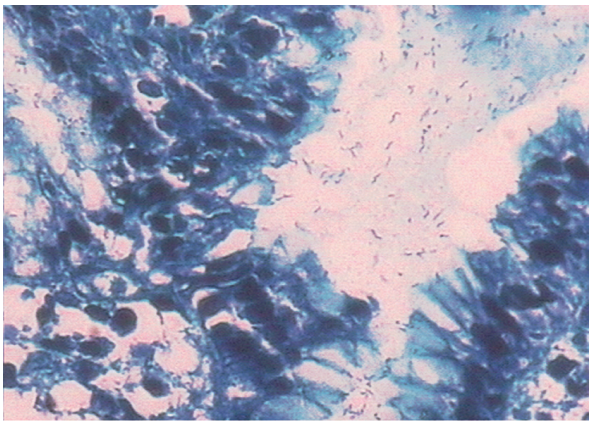

Figure 1 *Helicobacter pylori* in gastric pit (Giemasa ×400).

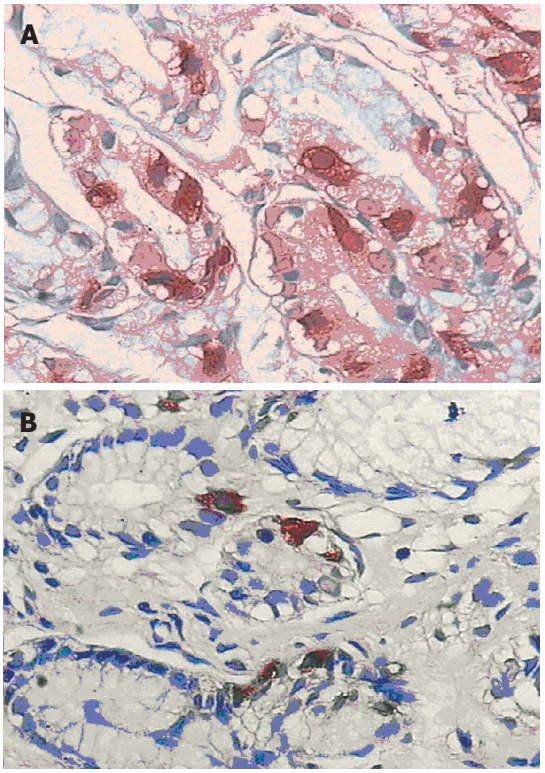

Figure 2 Expression of GAS (**A**) and SS (**B**) in glands. Brown particles were identified in cytoplasm (En Vision ×400).

changes, Hp-. Patients with other diagnoses such as chronic atrophic gastritis were excluded from the study. Thirty experimental samples were then randomly taken from each group. After specimens from patients with intestinal metaplasia or superficial specimens and very tiny specimens were excluded, the exact number of samples used in groups A-E was 20, 19, 21, 12 and 11, respectively.

Methods

Immunohistochemical method (En Vision method) was used and each paraffin sample was labeled with GAS and SS, respectively. When GAS and SS were located in cytoplasm, samples displaying brownish yellow particles or balls were regarded as positive. Positive cell bands of each sample were observed and lymph follicle areas were avoided. The number of positive cells within a sample was counted.

Statistical analysis

Statistical analysis was carried out with SPSS10.0 software package. The count of GAS and SS was in conformity with a normal distribution and expressed as mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

GAS and SS expression was higher in groups A-D than in group E with no significant difference (Figures 2A, 2B).

DISCUSSION

GAS is a kind of peptides secreted by G cells located in the gastric antrum and duodenal mucosa, whose main function is to stimulate gastric acid secretion. It is of physiopathologic importance in occurrence of duodenal diseases. Since Levi *et al*[2] found that Hp could lead to increase in serum GAS of Hp-infected persons, a flood of studies have found that the level of serum GAS is higher in Hp positive children with chronic gastritis than in Hp negative children. After anti-Hp treatment, the level of GAS in children decreases[3]. It is held that Hp infection may lead to increase in GAS released by G cells, making damages to gastric and duodenal mucosa. Hp stimulates inflammatory cell factors such as IL-21, IL-28, TNF, IFN and G cells to release GAS[4]. SS secretion is reduced by the inhibition of D cell functions by Hp infection, which results in decrease in inhibition of G cells by SS[5]. The increased Hp level on surface of the gastric antrum by ammonia interferes with the normal feedback mechanism of inhibition of GAS secretion by acids in the stomach, which results in increase in GAS secretion. Hp or its products (ammonia or peptides) have direct effects on G cells[6]. The present study showed that the expression of GAS in chronic gastritis Hp+ group (group A) and chronic gastritis Hp- group (group B) was higher than that in the normal control group (group E) and the expression of GAS in chronic gastritis Hp+ group (group A) was higher than that in chronic gastritis Hp- group (group B), indicating that chronic inflammation, especially Hp infection, can result in increase in GAS, which may be one of the pathogenic mechanisms underlying chronic gastritis.

The causes of duodenal ulcer are associated with various factors. Digestion of local mucosal tissues by gastric acid and peptase is an important cause of ulcer occurrence. Therefore, ulcer is closely associated with

GAS. Smith *et al* [7] reported that the basal and postprandial concentrations of serum GAS are increased in adults with duodenal ulcer. Wu *et al* [8] reported that the level of serum GAS in children with duodenal ulcer was much higher than that in the control group. Increased GAS release, which results in increased secretion of gastric acid and occurrence of ulcer, is one of the mechanisms underlying duodenal ulcer in children.

SS widely exists in the gastrointestinal tract, with its highest concentration in the gastric pylorus area. D cells in the gastric pylorus secrete SS. SS has an inhibitory effect on secretion of gastrointestinal hormones such as GAS, motilin and gastric acid. Therefore, SS is regarded as a defensive factor for local mucosa.

Geller *et al*[9] showed the level of fasting serum SS was higher in DU patients than in normal persons, suggesting that the increased level of SS in DU patients is a compensatory mechanism caused by the feedback stimulation with high concentrations of acids. Lucey *et al* [10] showed hydrochloric acid can increase the level of SS in DU patients. Erton *et al*[11] found that high concentrations of acids could stimulate SS release. Mihaljevic *et al*[3] showed that the level of serum SS in Hp+ patients was lower than that in Hp- patients. Zavros *et al*[12] found that the content of SS in specimens of gastric mucosa from chronic gastritis Hp+ patients was 6 times lower than that in chronic gastritis Hp- patients. Chavialle *et al*[13] showed that the content of SS in gastric antrum mucosa of DU patients was lower than that in the control group. However, Jensen *et al* [14] reported that the content of SS in bulb mucosa of DU patients was higher than that in the control group. Therefore, the true meaning of changes in the content of SS in gastroduodenal mucosal tissues of DU patients and their role in the pathogenic mechanism of DU are unclear. The extent of various damages and regulating function may be different in human tissues during different phases. Our study showed that the number of SS positive cells in groups A-D was higher than that in group E, indicating that inflammation, especially Hp infection, can stimulate release of GAS and SS as well as secretion of gastric acid under high concentrations of acids. The reason why there was no significant difference may be the insufficient cases of experiment. Due to relatively small amount of biopsy tissues from superficial or relatively superficial mucosa and the abandoned sections whose immunohistochemical staining was not clear, the valid cases for the experiment were greatly reduced. Besides, because the size of biopsy tissues from children was smaller than that from adults, the observed number of cells was also smaller. Therefore, our studies necessitate further efforts to perfect them.

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CASE REPORT

Cholelithiasis associated with haemolytic-uraemic syndrome

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Abstract

Cholelithiasis occurs infrequently in the paediatric age group. Hereditary spherocytosis, sickle cell anaemia and thalassemia are the haemolytic disorders most commonly associated with development of gall stones in paediatric age group. The question is whether an isolated episode of haemolysis can cause gallstones.

Key words: Cholelithiasis; Haemolytic anaemia; Haemolytic uraemic syndrome

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INTRODUCTION

Haemolytic uraemic syndrome (HUS) is characterised by acute haemolytic anaemia, thrombocytopenia and acute oliguric renal failure. Often the patient has a prodrome of gastroenteritic bloody diarrhoea caused by *Escherichia coli* (*E. coli*). We report a patient who presented with biliary colic four months after recovery from HUS and her abdominal ultrasound revealed gallstones.

CASE REPORT

A 15-year-old girl presented with a 10-day history of watery diarrhoea, oliguria, haematuria and vomiting. A diagnosis of HUS was made based on renal failure, anaemia, thrombocytopenia and the presence of schistocytes on peripheral smear. Stool culture grew *E. coli* O157, phage type 21. She received packed cell transfusion, but did not need any dialysis. Following satisfactory clinical and biochemical improvement, she was discharged a week after admission. On follow-up, she was doing well.

Four months after admission, she presented with an episode of intermittent colicky right upper quadrant abdominal pain and nausea. Clinical and biochemical examination was normal. An ultrasound examination revealed marginally dilated common bile duct (diameter 9 mm) and common hepatic ducts. Gall bladder contained a small amount of sludge, but was otherwise normal. She was treated conservatively with analgesics and intravenous fluids and was doing well at discharge, a couple of days later.

She was readmitted 2 mo later for another episode of colicky right upper quadrant abdominal pain and nausea. A repeat abdominal ultrasound revealed multiple small gall bladder calculi. Rest of the ultrasound examination was normal. She had a magnetic resonance cholangiopancreatography (MRCP), which revealed a normal biliary duct and a common bile duct, besides gallstones. She had an elective laparoscopic cholecystectomy 6 wk later. Multiple pigment gallstones were found. On follow-up, she remained asymptomatic and was doing well.

DISCUSSION

Gallstones are classified into three types according to their chemical composition, namely cholesterol, pigment and mixed stones. Mixed and cholesterol gallstones usually contain more than 70% cholesterol monohydrate plus an admixture of calcium salts, bile acids and bile pigments, proteins, fatty acids and phospholipids. Pigment stones are composed primarily of calcium bilirubinate containing less than 10% cholesterol.

Although cholelithiasis occurs infrequently in the paediatric age group, its incidence has increased during the last few decades. The reason why gall stones are not considered as a possible cause of jaundice or right upper quadrant discomfort in the past is the emphasis placed on the belief that haemolytic disease is a necessary prerequisite for gall stone formation in children[1].

Currently only about 20% of cholelithiasis cases in children are attributed to haemolytic conditions. Hereditary spherocytosis, sickle cell anaemia and thalassemia are the haemolytic disorders most commonly associated with development of gall stones. The question is whether an isolated episode of haemolysis can cause gallstones. The answer is yes because there are many reports in the literature.[2, 3] while the answer is no because none of the textbooks mentions it as a cause of gallstones. Hence, there is relatively low awareness that self-limiting haemolytic episodes can predispose to gall stones.

There are a few case reports of gall stones associated with HUS[2, 3]. Brandt *et al*[4] reported a high incidence of

gastrointestinal sequelae following typical HUS. Cholelithiasis might be related to haemolysis during the acute phase of HUS leading to pigment gall stones or to the use of parenteral nutrition. Parenteral nutrition was not used in the case presented.

In conclusion, neither is cholelithiasis mentioned as a complication of the HUS, nor is an acute haemolytic process listed as aetiology of gallstones. But gallstones should be suspected in both settings. An abdominal ultrasound examination can easily and readily confirm the diagnosis.

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CASE REPORT

Extended surgical resection for xanthogranulomatous cholecystitis mimicking advanced gallbladder carcinoma: A case report and review of literature

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Abstract

Xanthogranulomatous cholecystitis (XGC) is a destructive inflammatory disease of the gallbladder, rarely involving adjacent organs and mimicking an advanced gallbladder carcinoma. The diagnosis is usually possible only after pathological examination. A 46 year-old woman was referred to our center for suspected gallbladder cancer involving the liver hilum, right liver lobe, right colonic flexure, and duodenum. Brushing cytology obtained by endoscopic retrograde cholangiography (ERC) showed high-grade dysplasia. The patient underwent an en-bloc resection of the mass, consisting of right lobectomy, right hemicolectomy, and a partial duodenal resection. Pathological examination unexpectedly revealed an XGC. Only six cases of extended surgical resections for XGC with direct involvement of adjacent organs have been reported so far. In these cases, given the possible coexistence of XGC with carcinoma, malignancy cannot be excluded, even after cytology and intraoperative frozen section investigation. In conclusion, due to the poor prognosis of gallbladder carcinoma on one side and possible complications deriving from highly aggressive inflammatory invasion of surrounding organs on the other side, it seems these cases should be treated as malignant tumors until proven otherwise. Clinicians should include XGC among the possible differential diagnoses of masses in liver hilum.

Key words: Xanthogranulomatous cholecystitis; Gallbladder cancer; Gallbladder carcinoma

Spinelli A, Schumacher G, Pascher A, Lopez-Hanninen E, Al-Abadi H, Benckert C, Sauer IM, Pratschke J, Neumann UP, Jonas S, Langrehr JM, Neuhaus P. Extended surgical resection for xanthogranulomatous cholecystitis mimicking advanced gallbladder carcinoma: A case report and review of literature. *World J Gastroenterol* 2006; 12(14): 2293-2296

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INTRODUCTION

The term xanthogranulomatous cholecystitis (XGC) describes a rare inflammatory lesion of the gallbladder, characterized by marked proliferative fibrosis and infiltration of macrophages and foam cells involving the wall of the gallbladder[1]. Although a fair number of cases of this pathological condition have already been described, so far very few reports have described XGC with aggressive tumor-like inflammation pattern directly involving adjacent structures and organs[2-7].

We herein report a case of XGC presenting as advanced gallbladder carcinoma with involvement of liver, common bile duct, duodenum and transverse colon, review the characteristics of patients with XGC involving adjacent organs who underwent extended surgery, and discuss the different diagnostic and surgical options in cases of massive extra-gallbladder involvement.

CASE REPORT

A 46-year-old woman with no prior medical history was admitted to a local hospital due to progressively increasing upper abdominal discomfort for some weeks, lately associated with jaundice. No weight loss was reported. No mass was palpable at the physical examination. Ultrasound showed a gallbladder hydrops with gallstone in the infundibulum and bilateral dilation of intrahepatic bile ducts. An endoscopic retrograde cholangiography (ERC) demonstrated a filiform stenosis of the proximal common bile duct and the bifurcation with upstream intrahepatic bile duct dilation, and no visualisation of cystic duct and

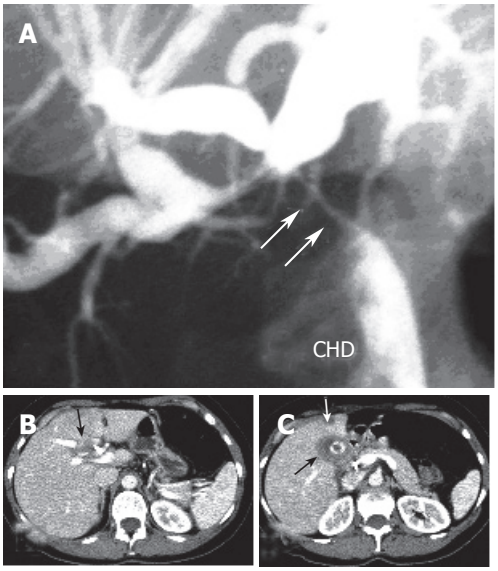

Figure 1 Proximal obstruction (arrows) of the common hepatic bile duct (CHD) with upstream dilatation of both left and right intrahepatic bile ducts (**A**), a soft tissue mass at the hepatic hilum (**B**), and thickened gallbladder wall (black arrow) with a concretion and hepatic lesion in liver segment 4 (**C**).

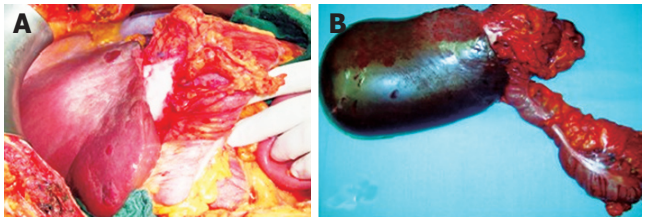

Figure 2 Intraoperative finding in situ (**A**) and surgical specimen ex situ (**B**).

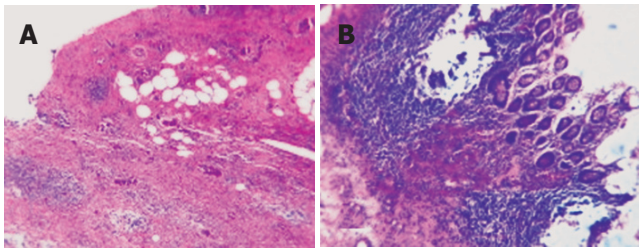

Figure 3 Xanthogranulomatous cholecystitis (**A**) and xanthogranulomatous cholecystitis involving the wall of the transverse colon (**B**). There is destruction of the submucosa and muscular coat of the transverse colon by extensive macrophage infiltrates, the mucosa is intact and exhibits no cellular atypia (HE, 200x).

gallbladder. A complete sphincterotomy was performed with insertion of an internal stent draining the left main bile duct. Magnetic resonance imaging (MRI) confirmed the presence of a hilar mass with two liver lesions in the right lobe of the liver, suspicious of metastases. MRI-vascular reconstruction excluded an infiltration of the portal vein and the hepatic artery. The patient was then transferred to our clinic with the diagnosis of advanced gallbladder cancer with differential diagnosis from Mirizzi's syndrome type I (i.e., secondary stenosis of the common hepatic duct caused by inflammatory fusion of gallbladder infundibulum and common bile duct due to gallstone impaction, without presence of a fistula). On admission to our centre, the patient was icteric after internal drainage of the left and common bile duct. Serum concentrations of total bilirubin (4.2 mg/dL, ref <1.1 mg/dL), alkaline phosphatase (325 U/L, ref <170 U/L), gamma glutamyl transpeptidase (55 U/L, ref <18 U/L), were all elevated. The tumor marker carbohydrate antigen 19-9 (CA19-9) was 42 U/mL (ref <33 U/mL) while carcinoembryonic antigen (CEA) was normal. We repeated ERC (Figure 1A) to attain cytological material and removed the internal stent. Brushing cytology revealed atypical epithelial cells with high-grade dysplasia. Computed tomography (CT-scan) with liver volumetry (Figures 1B, 1C) confirmed the presence of two hypodense lesions in the right liver lobe, suspicious of metastases, already detected by the MRI performed in the first hospital. Clinical, serological, cytological and imaging data were highly indicative of malignancy. Surgical exploration confirmed the diagnostic findings (Figure 2A): the mass completely involved the hepatic hilum, the transverse colon and the second duodenal portion, seeming to originate from the gallbladder bed, since the gallbladder itself was not distinguishable from the surrounding inflammatory tissue. The preoperatively suspected liver metastases were intraoperatively identified as hemangiomas. Assuming an advanced gallbladder carcinoma, we performed an anatomic right lobectomy en-bloc with gallbladder and extrahepatic bile duct resection, segmental duodenal resection, right hemicolectomy and partial omentectomy. Reconstruction was achieved by a hepaticojejunostomy protected by transhepatic drainage through the left biliary system, and an ileotransversostomy. Gross examination of the specimen (Figure 2B) demonstrated a bright white, hard mass, inseparable from the adjacent structures. A 2.5 cm × 3 cm × 2.5 cm biliary stone was packed in the infundibulum of the gallbladder. Histological examination unexpectedly revealed a xanthogranulomatous cholecystitis (Figure 3A), with tumor-like inflammatory tissue invading the liver as well as the proximal common bile duct, the right flexure of the colon (Figure 3B) and the second duodenal portion, with no evidence of malignancy.

The postoperative course was completely uneventful and the patient was discharged on the 15th postoperative day. One year after surgery, the patient was asymptomatic and in good health.

DISCUSSION

XGC is found after approximately 1.46% of cholecystectomies and affects men and women equally[8], almost always in presence of gallstones (91%-100%)[1]. Even if aetiology is unknown, some authors suggest that in the presence of gallstones, obstruction, and cholestasis, XGC results from the extravasation of bile into the gallbladder wall. With involvement of Rokitansky-Aschoff sinuses[8], the process is supposed to start as an initial inflammatory process, followed by a granulomatous reaction, similarly to the aetiopathogenesis of xanthogranulomatous pyelonephritis, in which chronic infection and calculi are common findings. Macroscopically, XGC is characterized by formation of multiple yellowish nodules within the gallbladder wall. Histologically, abundant so-called foam-cells (histiocytes)

Table 1 Characteristics of the patients with XGC who underwent extended surgical resections

Author Year	Age (Sex)	Symptoms	Tumor markers	Involvement of	Performed resection	Follow-up
Okamoto 1990	70 (F)	Fever, general malaise	CA19.9 ↑, CEA normal	Liver, bile duct, transverse colon	Cholecystectomy, local atypical liver resection, bile duct resection and transverse colectomy	Uncomplicated postoperative course, alive 6 mo after surgery
Maeda 1994	75 (F)	No complaints	CA19.9 ↑, CEA normal	Liver bed and transverse colon	Cholecystectomy with local atypical liver resection and transverse colectomy	Uncomplicated postoperative course, follow-up not reported
Furuta 1996	46 (M)	Epigastric pain	CA19.9 and CEA normal	Liver, bile duct, duodenum, right hepatic artery, right portal vein	Right hepatectomy and pancreatoduodenectomy	-
Natori 1997	55 (M)	No complaints	CA19.9 normal, CEA ↑	Liver (gallbladder bed) and bile duct	Cholecystectomy with local atypical liver resection and bile duct resection	-
Enomoto 2003	64 (M)	High fever, hypochondralgia	CA19.9 and CEA normal	Liver, bile duct, duodenum, transverse colon, right hepatic artery and right portal vein	Right hepatectomy, pancreatoduodenectomy and transverse colectomy	Uncomplicated postoperative course, alive 4 yr after surgery
Pinocy 2003	64 (M)	Hypochondralgia, fever	-	Right colonic flexure	Cholecystectomy and resection of the right colonic flexure	-
Present case 2005	46 (F)	Epigastric pain, sense of fullness, jaundice	CA19.9 ↑, CEA normal	Right liver, right colonic flexure, second duodenal portion	Right hepatectomy, partial duodenectomy and right hemicolectomy	Uncomplicated postoperative course, alive 1 yr after surgery

are mixed with chronic and acute inflammatory cells[9]. With regard to imaging of a liver hilar mass, ultrasound, CT, MRI and ERC can be considered. In the case of XGC, ultrasound can detect focal or diffuse thickening of the hyperechoic gallbladder wall[10] with the presence of characteristic intraluminal hypoechoic nodules in 35-73% of the cases[11]. Other commonly reported findings are cholecystitis-like fluid collections near the gallbladder and dilation of intra- and extra hepatic bile ducts in case of choledocolithiasis. Chun *et al*[12] analyzed CT-patterns of XGC in a series of patients, with emphasis on differential diagnosis of gallbladder carcinoma and stated that intramural hypodense nodules occur more often in XGC than in cancer patients. However, these characteristic findings are inconstant in patients with XGC, as highlighted by more recent studies[11, 13-14]. Visualisation of the biliary anatomy through direct (percutaneous or endoscopic) cholangiography or magnetic resonance cholangiopancreatography (MRCP) is particularly important if a direct involvement of the common bile duct is suspected. Furthermore, ERC allows brushing cytology. The potential role of positron emission tomography in the differential diagnosis of cholecystitis from gallbladder carcinoma is unclear, because only a few studies in small series are available up to now[15,16] with a patient with XGC being the only false positive case in one study[15].

Concerning tumor markers, our patient had only a slightly elevated (42 U/mL) serum level of CA 19.9 (reference = 33 U/mL), which persisted even after resolution of jaundice through placement of an endoscopic stent preoperatively. Adachi *et al*[17] argued that serum-CA 19.9 may be elevated even greatly in both carcinoma and XGC patients, thus being not helpful in the differential diagnosis between the two conditions. Even in cases of XGC with extended involvement of other organs[3,5] elevations of tumor markers concentrations (CA 19.9, CEA) are inconsistent.

Although XGC is not an exceptional finding, a direct involvement of extra-gallbladder organs and structures is very rare. To the best of our knowledge, only six cases of extended surgical resections for XGC have been reported so far. In these cases (Table 1), additional procedures such as bile duct resections, segmental resections of colon or duodenum, partial pancreatoduodenectomies have been performed. It can be discussed if such extended surgical procedures could be avoided in presence of a benign disease. Our patient exhibited severe, destructive, tumor-like xanthogranulomatous inflammation, with extensive invasion of adjacent organs (right lobe of the liver, extrahepatic bile ducts, colon and duodenum). The clinical and radiological findings were suggestive of a gallbladder carcinoma. ERC was indicative of hilar cholangiocarcinoma (Klatskin tumor). However, after confrontation with CT findings (gallbladder hydrops, gallbladder wall thickened over 1.8 cm, presence of suspect lesions in the right liver lobe, suspected infiltration of adjacent organs- all uncharacteristic findings in cases of Klatskin tumors), the most probable diagnosis was a locally advanced gallbladder cancer, with infiltration of the proximal common bile duct and the bile duct bifurcation. In addition, cytology obtained during ERC showed high-grade dysplasia and CA 19.9 was slightly but persistently elevated after stenting. Under these conditions, a radical resection of the mass was the only reasonable option. According to the published data on gallbladder cancer and hilar cholangiocarcinoma, radical surgery offers the only chance to achieve long-term survival[18-23]. The necessity of radical surgery in those cases of XGC with extensive extra-gallbladder involvement is not cleared. We agree with Houston *et al*[24] emphasizing the necessity of a complete resection of the involved structures in order to treat symptoms like jaundice and cholangitis and to prevent possible complications like bowel obstruction and perforation, potentially life threatening.

Intraoperative frozen section investigation or fine needle aspiration cytology has been suggested to confirm the

diagnosis. In cases with no invasion of adjacent organs these tools are indicated because they can change the surgical strategy (e.g, simple cholecystectomy versus associated liver resection). Nevertheless, in cases of extensive invasion of other organs, like in our patient, it can not alter our approach. Even in the case of a negative or inconclusive result, radical surgery can be performed since gallbladder carcinoma and XGC may coexist [24-26] and the patient had jaundice and cholangitis, which needed a treatment. Furthermore, even assuming an advanced gallbladder carcinoma, we believe it is essential to minimize the intraoperative tumor cell spread by avoiding any unneeded manipulation or dissection in the liver hilum.

Two different risks are linked to misjudgement of this pesudotumoral condition. On the one hand, excessive surgical resection is inevitably linked with a certain perioperative mortality and morbidity. On the other hand, this condition may be misinterpreted as not being radically operable. Especially in this latter case, before deciding for inoperability, it is essential to gain specimens for frozen section biopsy.

In conclusion, preoperative or intraoperative differential diagnosis of XGC from gallbladder carcinoma remains a challenge, especially in patients with extensive pseudotumoral involvement of surrounding structures and organs. From our point of view, radical resection is mandatory as long as malignancy can not be ruled out after a complete preoperative diagnostic examination. Preoperative fine needle aspiration biopsy and intraoperative frozen section are valuable tools for differential diagnosis when there is no invasion of adjacent organs, otherwise they would not influence the surgical strategy. The presence of symptoms such as jaundice and cholangitis, as well as the potential risk of life-threatening complications like bowel obstruction and subsequent perforation, supports a radical surgical approach.

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CASE REPORT

Development of multiple myeloma in a patient with chronic hepatitis C: A case report and review of the literature

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Abstract

An association between chronic hepatitis C virus (HCV) infection and essential mixed cryoglobulinaemia and non-Hodgkin lymphoma (NHL) has been suggested. However, a causative role of HCV in these conditions has not been established. The authors report a case of a 50 year-old woman with chronic hepatitis C (CHC) who has been followed up since 1998 due to a high viral load, genotype 1b and moderately elevated liver function tests (LFTs). Laboratory data and liver biopsy revealed moderate activity (grade: 5/18, stage: 1/6). In April 1999, one-year interferon therapy was started. HCV-RNA became negative with normalization of LFTs. However, the patient relapsed during treatment. In September 2002, the patient was admitted for chronic back pain. A CT examination demonstrated degenerative changes. In March 2003, multiple myeloma was diagnosed (IgG-kappa, bone marrow biopsy: 50% plasma cell infiltration). MRI revealed a compression fracture of the 5th lumbar vertebral body and an abdominal mass in the right lower quadrant, infiltrating the canalis spinalis. Treatment with vincristine, adriamycin and dexamethasone (VAD) was started and bisphosphonate was administered regularly. In January 2004, after six cycles of VAD therapy, the multiple myeloma regressed. Thalidomide, as a second line treatment of refractory multiple myeloma (MM) was initiated, and followed by peginterferon-α2b and ribavirin against the HCV infection in June. In June 2005, LFTs returned to normal, while HCV-RNA was negative, demonstrating an end of treatment response. Although a pathogenic role of HCV infection in malignant lymphoproliferative disorders has not been established, NHL and possibly MM may develop in CHC patients, supporting a role of a complex follow-up in these patients.

INTRODUCTION

Numerous clinical syndromes have been reported in association with hepatitis C virus (HCV) infection. Some are well established while others remain a speculation (Table 1)[1], Fourteen to seventy percent of patients with HCV have detectable cryoglobulins in their serum even in the absence of rash, weakness or arthralgias[1], whereas 50-90% of patients with mixed cryoglobulinaemia are reported to have HCV infection[2]. It is suggested that approximately 10% of type II mixed cryoglobulinaemiae can evolve into malignant lymphoma several years after diagnosis.

Epidemiological studies from Europe, Japan and North America also implicate that HCV plays a role in the pathogenesis of non-Hodgkin lymphomas (NHL)[3-7]. HCV infection is detectable in a significant proportion of NHL (14%-52%), nonetheless, it has not been confirmed[8]. A 50-fold elevation in the risk for NHL of the liver or salivary glands has been reported in an Italian case-control study, which is greater than the relative risk of hepatocellular carcinoma. The relative risk for NHL of other sites is increased about 4-fold[9]. An association between multiple myeloma (MM) and chronic HCV infection has been suggested by some epidemiological studies[2,9].

CASE REPORT

The authors report a case of a 50 year-old woman with chronic HCV infection who has been followed up since 1998 due to high viral load (13.47 MEq/mL), genotype 1b and moderately elevated liver function tests (LFTs). The patient received treatment of chronic backache and tonsillectomy prior to admission to our hospital. Laboratory data and liver biopsy revealed moderate activity (grade 5/18, stage I). In April 1999, one-year interferon therapy (3x3ME/wk) was initiated. HCV-RNA became negative with normalization of the LFTs. However, the

Table 1 Extrahepatic diseases associated with hepatitis C virus infection

Association: strong	Intermediate	Weak
-Cryoglobulinemic syndrome (Cutaneous leukocytoclastic vasculitis, arthritis, weakness) -Renal disease (membranoproliferative glomerulo- nephritis) -Peripheral neuropathy -Non-Hodgkin lymphoma -Sjörgen's syndrome	-Porphyria cutanea tarda -Diabetes	-Thyroid disease -Corneal ulcers -Lichen planus -Pulmonary fibrosis

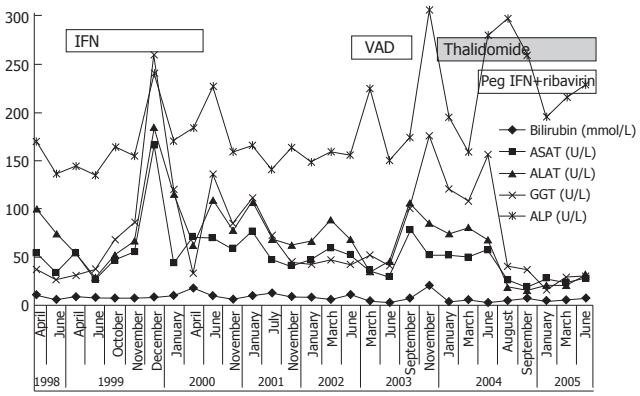

Figure 1 Liver function tests from April 1998 to July 2005.

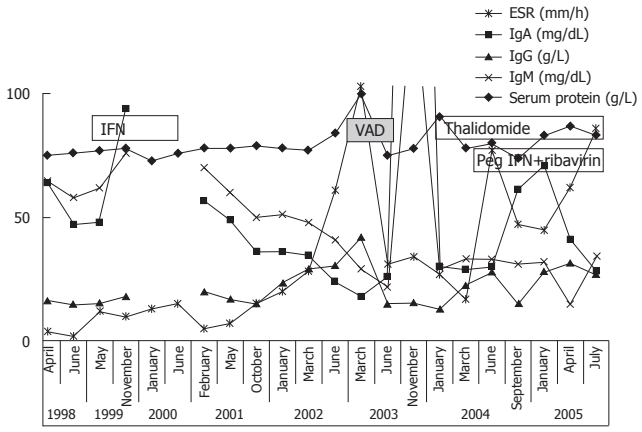

Figure 2 ESR and serum immunoglobulin levels from April 1998 to July 2005.

patient relapsed in the 7th mo of treatment (HCV-RNA became positive, LFTs increased). The liver function tests are summarized in Figure 1. In February 2000, hypothyroidism was diagnosed, substitution was initiated and follow-up was scheduled.

In September 2002, the patient was admitted for chronic backpain. CT examination revealed degenerative changes of the 5th lumbar vertebral body. Subsequently, the patient was not monitored until March 2003, when she was again hospitalized due to chronic backache and weakness of the right lower limb. Based on laboratory results, IgG-κ multiple myeloma was diagnosed (erythrocyte sedimentation rate; ESR): 104 mm/h, IgA: 0.18 g/L, IgG: 41.86 g/L, IgM: 0.29 g/L and 12.8% M-component on serum immunoelectrophoresis; bone marrow biopsy: 50% plasma cell infil). Mixed cryoglobulins were detected in the serum. Anemia, low platelet count or hypercalcaemia did not occur during follow-up. Serum IgG was elevated from 1998. However, monoclonality was not detected prior to March 2003. The serum immunoglobulin data and ESR are presented in Figure 2. MRI revealed a compression fracture of the 5th lumbar vertebral body and an abdominal mass in the right lower quadrant, infiltrating the canalis spinalis, ileum and sacroiliac joint (osteolytic lesions).

An aggressive treatment regimen was implemented with vincristine, adriamycin and dexamethasone (VAD). From April to October 2003, she received six cycles of VAD therapy followed by multiple myeloma's regression. The presence of compression fracture also prompted the regular adminis of bisphosphonate (Aredia). In January 2004, 22.2% M-components were detected by immunoelectrophoresis, yet the ESR was normal (17mm/h). Methyl prednisolone (100 mg b.i.d) and cytoxane (100 mg o.d.) p.o. therapy was started.

In April 2004, she was admitted to the Haematological Department for paraparesis. Laminectomy (Th VII-IX) with myelin decompression and tumor resection was performed. Therapy was amended with thalidomide (100mg o.d.) orally. In August, repeated CT scans demonstrated compression fractures at L5 and S2 accompanying narrowing of the spinal canal at L5. In September, due to worsening of the paraparesis, a second laminectomy was performed (L5-S1) followed by insertion of a stabilizing prosthesis (L4-S1).

In June 2004, weekly peginterferon-α2b (1.5 μg/kg) and 800 mg ribavirin (daily) were prescribed (HCV PCR 145000 IU/mL, Roche TaqMan). No further dose adjustment was necessary. The LFTs became normal and PCR returned negative after three months of therapy, indicating an early viral response (EVR). In June 2005, the LFTs were normal with a negative HCV-PCR, demonstrating an end of treatment response (ETR).

DISCUSSION

In recent years, major advances have been made in the treatment of HCV infection with the sustained response rate of 52%-63% achieved[11-13]. A major disadvantage in Hungary is that the hard-to-treat genotype 1 is almost universal (90%-95%) and occurs much more frequently compared to that in other European countries[13, 14].

Apart from hepatocellular carcinoma, HCV infection is also associated with various extrahepatic diseases, including mixed cryoglobulinaemia and NHL. Fourteen to seventy percent of patients with HCV have detectable cryoglobulins in their serum[1, 2], while 50%-90% of patients with mixed cryoglobulinaemia are reported to have HCV infection.

Epidemiological case-control studies from the 1990s suggest that chronic HCV infection is associated with the development of NHL. It was reported that 9% and 11.5% of NHL patients are HCV-antibody positive[15,16]. Germanidis *et al*[4] investigating 201 NHL patients found that the prevalence of HCV infection was 2-fold higher

than that in controls[4]. More recent studies have further confirmed this finding. A Japanese study showed that 17% of patients with B-cell NHL were HCV-antibody positive compared to 6.6% of controls[6]. Moreover, an East European study[7] reported that 6 out of 42 (24.3%) NHL patients were HCV-antibody positive. In contrast, Rabkin *et al*[8] investigated the stored sera of 57 NHL patients, 24 MM patients and fourteen Hodgkin's disease patients, and found that only four patients were HCV-antibody positive.

An association between chronic HCV infection and MM was also found in epidemiological studies. Gharagozloo *et al*[2] showed that HCV antigens were detectable in 11% of patients with MM, 69% of patients with essential mixed cryoglobulinaemia and 4.3% of patients with NHL, using recombinant immunoblot assay (RIBA) and enzyme-linked immunosorbent assay (ELISA). Another study revealed that HCV infection was found in 32% of MM patients[10]. The former was associated with 4.3-fold risk for MM.

The possible mechanism by which HCV infection leads to stimulation of B cells is starting to unravel. It is well documented that HCV infection, as observed in our case, usually precedes NHL by many years[9]. Hepatitis C is lymphotropic and may replicate in lymphocytes and hepatocytes[17]. The second portion of the HCV envelope (E2 protein) binds to CD81[18], suggesting that this phenomenon is associated with CD19 and CR2 as well as MHC class II molecules on lymphocytes. The binding of CD81 to B cells can activate this complex, which lowers the antigen threshold necessary for antibody stimulation, thus rendering the B cell hyper-responsive. Sequencing of the antigen-binding region of immunoglobulin produced by malignant lymphocytes demonstrates that it has a high degree of homology to both antibodies specific for E2, as well as the antibodies produced by B cells that secrete RF. Furthermore, 88% of patients with HCV infection and cryoglobulinaemia demonstrate over-expression [t(14,18)translocation] of the anti-apoptotic *bcl-2* gene, compared with 8% of patients with HCV infection, 2% of patients with other liver diseases, and 3% of individuals with other rheumatoid disorders, which cause enhanced B cell survival[19]. In addition, over-expression of NF-kB has been reported in lymphocytes and liver samples of patients with chronic HCV infection and those with NHL[20, 21]. This is an important finding, as NF-kB plays a key role in virus-induced lymphomagenesis. Mutations of the NF-kB gene are common in lymphoid malignancies[22] and alterations of NF-kB could initiate changes in downstream regulatory pathways. A second mutation (e.g. myc, NF-kB) could possibly initiate the progression to lymphoma[23].

In conclusion, although a pathogenic role of HCV infection in malignant lymphoproliferative disorders has not been established, NHL and possibly MM may develop in cases of CHC, supporting the need for a complex follow-up in these patients.

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CASE REPORT

A rare case of enteropathy-associated T-cell lymphoma presenting as acute renal failure

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Abstract

Enteropathy-associated T-cell lymphoma (EATCL) is a high grade, pleomorphic peripheral T-cell lymphoma usually with cytotoxic phenotypes. We describe a first case of patient with EATCL that is remarkable for its fulminant course and invasion of both kidneys manifested as acute renal failure. The patient was a 23 year old woman with a long history of celiac disease. She was presented with acute renal failure and enlarged mononuclear infiltrated kidneys. Diagnosis of tubuloi-nterstitial nephritis and polyserositis was confirmed with consecutive pulse doses of steroid therapy. After reco-very, she had disseminated disease two months later. Magnetic resonance imaging showed thickened intestine wall, extremely augmented kidneys, enlarged intra-abdominal lymph nodes with extra-luminal compression of common bile duct. Laparotomy with mesenterial adipous tissue and lymph glands biopsy was done. Consecutive pathophysiological and immunohistochemical analyses confirmed the diagnosis of EATCL: CD45RO+, CD43+, CD3+. The revision of renal pathophysiology sub-stantiated the diagnosis. The patient received chemotherapy, but unfortunately she died manifesting signs of pulmonary embolism caused by tumor cells.

Key words: EATCL; Acute renal failure; Celiac disease; Anti-endomisial antibodies

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INTRODUCTION

Intestinal enteropathy associated T-cell lymphoma (EATCL) is highly aggressive, pleomorphic peripheral T-cell lymphoma (PTL), usually with cytotoxic immunological phenotypes (TdT-, CD3+, CD5-, CD7+, CD4-, CD8±, CD45RO+, CD103+, HLA-DR-)[1-3]. This type of PTL originates from intraepithelial T-lymphocytes of small intestine mucosa and is associated with celiac disease (CD) in about 50% of the time[4,5]. Intestinal lymphoma develops in 7%-12% of CD cases[6]. It may even occur without any previous CD[6]. Infiltration of the kidneys is commonly found in disseminated lymphoma but rarely in primary renal lymphoma[7]. Acute renal failure (ARF) arising from bilateral renal infiltration is also uncommon[8,9]. Primary renal failure may occur and is usually of B-cell lineage[7]. It is rare for patients with lymphoma to develop ARF as their initial clinical presentation. The first report in which the phenotype of non-Hodgkin's lymphoma was established using renal biopsy was published by Miyake *et al*[10]. Renal lymphoma is commonly secondary due to lymphomatous infiltration of the kidneys in disseminated lymphoma and advanced stage IV of the disease. Different pathophysiological aggressive types of B-cell lymphoma such as Burkitt[11], precursor B-lymphoblastic lymphoma/leukemia[12], mantle cell lymphoma, diffuse large B-cell lymphoma[13] as well as PTL[14,15], T-cell rich B-cell lymphoma[8], make parenchymal neoplastic invasion of the kidneys. We present, as far as we know for the first time, ARF as the initial manifestation of EATCL.

Nevertheless, the intestinal lymphomas account for 20%-35% of non-Hodgkin's lymphomas (NHL) of the gastrointestinal tract (GIT) and serological screening for CD is not recommended generally in people with lymphoma[16]. In patients with primary small intestinal disease, 9% are found to have small intestinal lymphoma[17]. Lymphomas involving the small intestine represent a heterogenous group with diverse pathogenic mechanisms[18] and high-risk MALT lymphoma is most frequently considered for differential diagnosis. EATCL, a type of PTL is most commonly localized in jejunum (70% of cases) but rarely in the colon and stomach. Gross examination can reveal multiple ulcers of jejunal

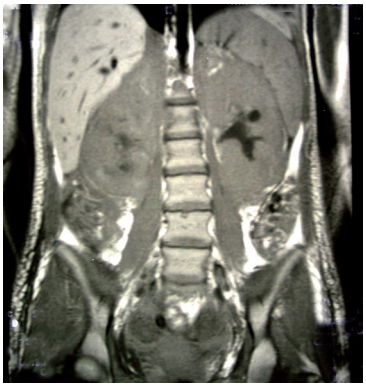

Figure 1 Magnetic resonance imaging shows extremely augmented kidneys.

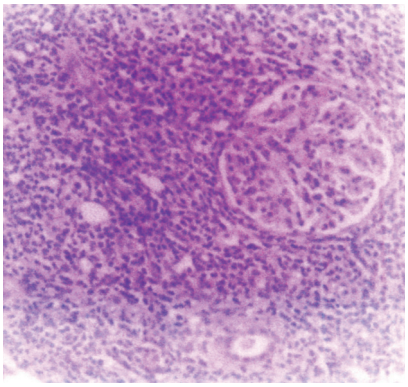

Figure 3 Renal infiltration with monomorphous lymphoid infiltration (H&E, x 40).

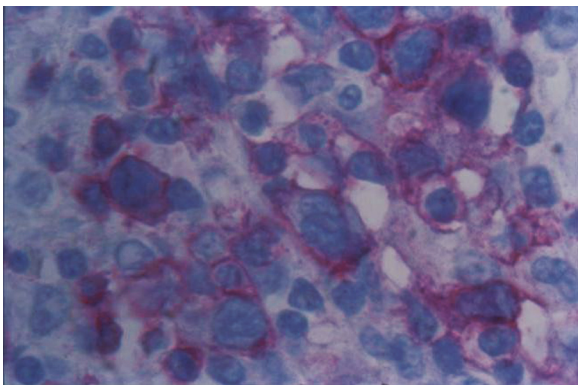

Figure 2 Neoplastic mesenterial lymph node infiltration with staining of lymphoma cells (APAAP with anti-CD3 Ab x 400).

mucosa frequently associated with perforation of intestinal wall or solitary lymph nodes[19]. Microscopy can show solitary epithelial lesions in the form of microabscess or lymphoepithelial lesions. In the adjacent mucosa, alterations are seen in CD such as crypt elongation, flattened mucosa and villi which are shortened, blunted or missing[2]. Tumor cells of the respective immunological phenotype have genetic characteristics as clonally rearranged TCR β and γ[5,6] can be arrested in varying stages of activation[1].

CASE REPORT

We report a case of female patient who was 23 years old with CD diagnosed at her age of 3 and had regular gluten-free diet regime. In March 1999, she was admitted to the Institute of Nephrology due to anemia and ARF. Clinical examination revealed excessive pallor of the skin and apparent mucous membranes with fever, splenomegaly +1 cm and ascites. A history was negative for exposure to nephrotoxins and hereditary renal diseases. Laboratory tests showed Hb = 65 g/L (120-170), WBC = 5.3×10^9/L (4-10), Platelet = 401×10^9/L (150-450), MCV = 90 fl (80-100), Hct = 0.34 (0.36-0.42), and normal differential count. In biohumoral status, the following pathological values were found: ESR = 90 mm/h, urea = 17.9 mmol/L (2.5-7.5), Cr = 476 μmol/L (53-106), ClCr = 13.3mL/min, tubular proteinuria=0.82 g/24h without erythrocyturia, total protein = 60 g/L (62-81), albumin = 6 g/L (40-55), Fe = 4.6 μmol/L (7-26), TIBC = 36.3 μmol/L (44.8-75.1), LDH = 590 IU/L(160-320). Immunological analyses showed CRP 28.9 mg/L (<9), ANA negativity, RF 37.3 IU/mL (<25) WR 1:40+. Virusological and bacteriological analyses were normal. Hemostasis screening did not display any detectable abnormalities. X-ray of the lungs and heart was normal, too. Abdominal echosonography showed craniocaudal splenomegaly of 14 cm, ascites and enlarged kidneys, the right of 14.5 cm and the left of 13.7 cm, without corticomedullary border. Echosonography of the heart showed pericardial effusion. Thereupon, ultrasound-guided biopsy of kidneys was performed and pH finding indicated tubulointerstitial nephritis (TIN). There were no criteria for connective tissue disease, drug-or infection-induced acute TIN**.** The diagnosis of idiopathic acute TIN with polyserositis was made and urbason at pulse doses of 3×1 g/d was administered, followed by prednisolone 45 mg/d for another month. After a short recovery, in May 1999, the patient was rehospitalized for jaundice, pains under the right costal arch, nausea and vomiting. Endoscopic retrograde cholangiopancreatography showed extra-luminal compression of ductus pancreaticus and ductus hepaticus which was moved to the right, while choledochus was filamentary narrowed distally from the site of compression. Proximal of stricture was a huge biliary duct dilatation with changes of intrahepatical biliary ducts [as in cholangitis]. Magnetic resonance imaging of the abdomen and small pelvis revealed the enlarged retroperitoneal and mesenterial lymph glands, thickened small intestine wall, extremely augmented kidneys with destroyed corticomedullary structure, as well as infiltration of the right sacral bone (Figure 1). Choledochotomy and cholecystectomy with choledochojejunostomy and biopsy of mesenterial lymph glands and adipous tissue were performed. Pathohistological (PH) and immunohistochemical (IHC) analyses of mesenterial lymph nodes and adipous tissue from transfersal mesocolon showed the PTL infiltration (anti-CD45RO+, anti-CD43+, CD3+ as well as anti-EMA- /epithelial membrane antigen/, anti-vimentin-) (Figure 2). The revision of renal PH findings substantiated the diagnosis (Figure 3). Neoplastic lymphoid cells were also found in the ascites (Figure 4). Antiendomysial serum IgA antibodies (EmA) in high titer +++ were (Figure 5) detected by indirect immunofluorescence on monkey oesophagus. Anti-nuclear, anti-microsomal, anti- tireoglobulin antibodies were negative and serum levels of IgM, IgG and IgA were normal. Upon complete staging of the disease, it was

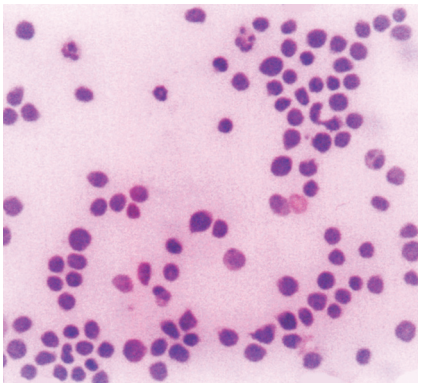

Figure 4 Neoplastic lymphoid cells in ascites (MGG, x100).

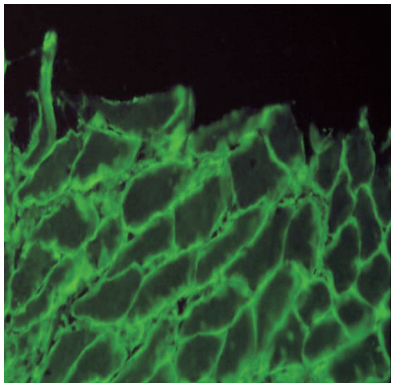

Figure 5 IgA antiendomysial antibodies surrounding sarcolemma of smoth muscle fibers in lamina muscularis mucosae of monkey esophagus (IIF, x 400).

verified that it was EATCL with infiltration of intestines, intraabdominal lymph glands, kidneys and sacral bone (CS IVB). The patient was transferred to the Institute of Hematology, where she received chemotherapy with cyclophosphamide (1200 mg, D1), doxorubicin (90 mg, D1), vincristine (2 mg, D1), prednisolone (100 mg, D1-5. On the second day of therapy, the patient died manifesting the signs of cardiorespiratory insufficiency and picture of pulmonary embolism, most probably caused by tumor cells.

DISCUSSION

EATCL is a relatively rare disease with the incidence rate less than 1% of all NHL[20]. By nature, it is aggressive whether it occurs as de novo disease or results from long-term untreated or refractory CD. Usually, the jejunum is involved with presence of multiple circumferential ulcerations but without formation of homogenous tumor mass. In addition, the involvement of mesenterial lymph glands is frequently seen. In fact, refractory CD is considered as lymphoma of low grade malignancy resulting from clonal expansion of intraepithelial lymphocytes and represents an intermediate stage between CD and EATCL[21,22].

The moment when CD converts to intestinal lymphoma is sometimes difficult to recognize. Diffuse bilateral infiltration of the kidneys by lymphoma cells is a rare but well documented cause of ARF[23]. The diagnosis should be suspected in a patient with ARF, bilateral enlargement of the kidneys, minimal proteinuria, non-specific findings on urine analysis, and absence of other features (fever, skin rush, eosinophilia) typical of drug-induced TIN. Renal imaging techniques may suggest the possibility of lymphomatous infiltration, but only renal biopsy or autopsy can provide a definitive diagnosis[24]. Diffuse bilateral renal cell lymphoma sometimes presents as ARF of unknown cause. Signs of extrarenal lymphomatous involvement were detected in 44% patients at the time of kidney biopsy or shortly thereafter[25] and often clinically mimic primary renal disease or systemic connective disease.

In our patient who was diagnosed with CD 20 years prior to the development of lymphoma, CD was in subclinical form for years. This is a key point for misdiagnosis of TIN instead of lymphoma renal infiltration. At the time of aggravation of general condition, renal failure with signs of TIN and polyserositis predominated in the clinical picture of disease. Short-term improvement after the corticosteroid treatment was followed by stasis icterus but without any symptoms typical of intestinal lymphoma. Diagnostic dilemma was resolved by complete PH and IHC examinations of bioptic specimens of mesenterial lymph glands and mesocolic adipous tissue tumor. Time loss due to unrecognized intestinal lymphoma, and dissemination into abdominal organs and small pelvis, brought about the extreme progression of the disease. Because of huge tumor mass, it led to lethal outcome with pulmonary embolism by tumor cells after the chemotherapy. Actually, this was a long-term CD, which like the premalignant condition gave rise to highly aggressive T-cell lymphoma with striking propagation and short survival median.

Our case demonstrated that in this point of view, serological follow-up was very useful and could reduce the time of possible subclinical or refractory CD. It is necessary to determine serum IgA EmA not only to monitor the activity but also to predict the gluten-free diet compliance. IgA EmA should disappear after a gluten free diet is started and about 90% of patients who have characteristic findings of CD respond to complete dietary gluten restriction[26]. In the early stages of EATCL, serological, immunohistochemical and molecular biological analyses may lead to the correct diagnosis and better prognosis[27].

The target cells in EATCL as a member of uniform subclass T-cytotoxic lymphocytes are also target cells in the intestinal lesion in CD patients, indicating that the evolution of genetically controlled autoimmune disease as pre-malignant condition to highly risk and aggressive lymphoma of small intestine is the evidence confirming that autoimmunity is a risk factor for malignancy.

Our case revealed very aggressive atypical clinical onset of EATCL patient, which was presented as ARF without intestinal symptoms at presentation. This is a first case in published literature, which describes ARF as an initial manifestation of EATCL. Although it was a case of misdiagnosis of idiopathic TIN with polyserositis instead of lymphoma, our study has confirmed that subjects with persistent high IgA EmA should be regularly re-examined concerning possible evolution of CD towards EATCL. Although good serological predictors are not yet available, IgA EmA assessment may help to detect individuals at higher risk to develop EATCL.

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CASE REPORT

An interesting cause of esophageal ulcer etiology: Multiple myeloma of IgG kappa subtype

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Abstract

Multiple myeloma is a neoplasm of mature and immature plasma cells. A 50-year-old woman with lumbago, dysphagia, and left arm pain was presented. Upper endoscopical examination was performed. There was an exudate-covered ulcer in the distal esophagus, located at 30-32 cm from the incisors, covering the whole mucosa. Histopathological examination of the specimens obtained from the lesion showed the involvement of plasma cells consistent with multiple myeloma of IgG kappa subtype. Esophageal involvement of multiple myeloma should be kept in mind in patients presenting with dysphagia.

Key words: Multiple myeloma; Esophageal ulcer; Dysphagia; Endoscopy

Pehlivan Y, Sevinc A, Sari I, Gulsen MT, Buyukberber M, Kalender ME, Camci C. An interesting cause of esophageal ulcer etiology: Multiple myeloma of IgG kappa subtype. *World J Gastroenterol* 2006; 12(14): 2305-2307

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INTRODUCTION

Multiple myeloma (MM) is a plasma-cell neoplasm characterized by skeletal destruction, renal failure, anemia and hypercalcemia. The most common symptoms at presentation are fatigue, bone pain and recurrent infections. New diagnostic criteria require the presence of at least 10% plasma cells on examination of the bone marrow (or biopsy of tissue with monoclonal plasma cells), monoclonal protein in the serum or urine, and evidence of end-organ damage[1].

Apart from the bone marrow, MM may involve any part of the body including spleen, liver, lymph nodes, thyroid, adrenal glands, ovary, testis, lung, pleura, pericardium, skin, pancreas and intestinal tract. Esophageal involvement by multiple myelomas is uncommon in the English literature. Here we report an extremely rare involvement in MM.

CASE REPORT

A 50-year-old woman was admitted to Gaziantep University Hospital with lumbago, dysphagia and left arm pain. She complained of lumbago in the last 3 mo, which deteriorated but was relieved by non-steroid anti-inflammatory drugs (NSAIDs). Dysphagia was particularly associated with solid foods over a period of month. Her severe arm pain began after lifting a heavy weight. She also complained of fatigue.

Her medical history revealed a 10-year history of hypertension and one year of type 2 diabetes mellitus. No abnormal signs were detected apart from the pale conjunctivas and the left arm pain during physical examination. She was taking gliclazide 3 mg/d and amlodipine 10 mg/d.

Biochemical examination and complete blood count showed (normal reference ranges are given in parenthesis) hemoglobin:102 g/L (110-180 g/L); hematocrit: 0.32 (0.35-0.60); ESR: 110 mm/h (1-18 mm/h); glucose: 160 mg/dL (70-110 mg/dL); total serum protein: 144 g/L (64-82 g/L); albumin: 22 g/L (34-50 g/L); globulin: 122 g/L (14-30 g/L); calcium: 12.6 mg/dL (8.5-10.1); (BUN, Cr, Na, Cl, and P were in the normal limits). IgG: 46.7 g/L (7-16); IgA:0.29 g/L (0.7-4); IgM:0.171 IU/mL (0.4-2.3); and IgE:184 g/L (0-100). Bone marrow aspiration and biopsy confirmed multiple myeloma (30% plasma cells) besides hyperglobulinemia, hypercalcemia, anaemia, elevated ESR, and bone pain. Multiple lytic lesions were encountered on radiographic examination of the arms, the vertebras, and the cranium. Endoscopic examination showed exudate-covered ulcer in the distal esophagus located at 30-32 cm from the incisors, covering the whole mucosa (Figure 1) and no pathological finding in the stomach. Histopathologic examination of the specimens obtained from the lesion showed the involvement of plasma cells in the ulcerated esophageal mucosa (Figure 2). Immunohistochemically, plasma cells revealed positive reaction with CD38, CD56, and kappa light chain and (Figure 3)

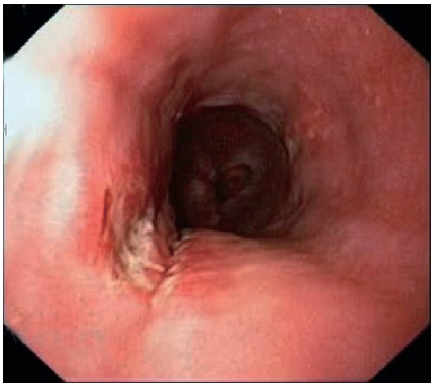

Figure 1 Exudate-covered ulcer in distal esophagus.

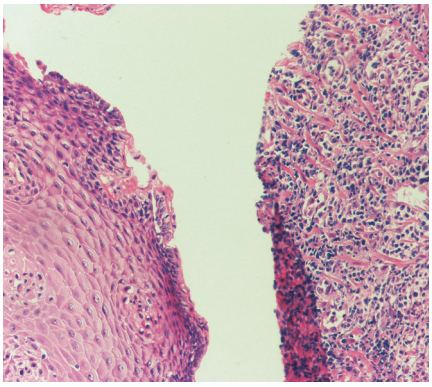

Figure 2 Plasma cells infiltrating esophageal mucosa (hematoxylin & eosin staining, x 200).

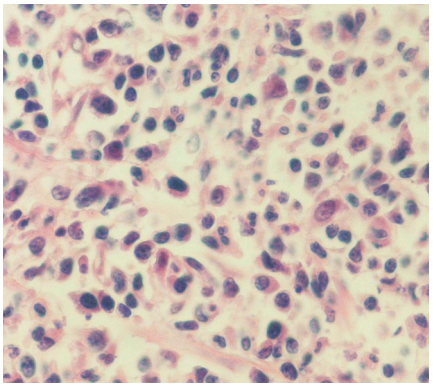

Figure 3 Myeloma cells in lamina propria of esophagus (hematoxylin & eosin staining, x 400).

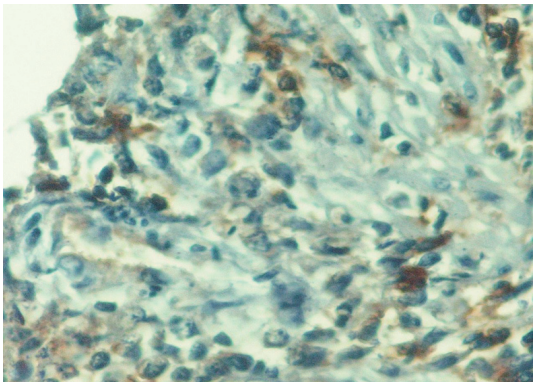

Figure 4 Immunohistochemically stained kappa light-chain showing monoclonality of plasma cells (x400).

negative reaction with CD20 and lambda light chain both in the bone marrow and in the esophagus (Figure 4). It was consistent with MM of IgG kappa subtype. Left upper extremity MRI revealed an irregularly shaped 5 cm × 4 cm × 6 cm mass on the humeral head. Total excision of the mass in the left arm was performed. It was also consistent with MM.

The patient was diagnosed with MM of IgG kappa subtype, and chemotherapy regimen of VAD was applied (vincristine 0.4 mg for 24 h infusion on days 1-4, adriablastina 9 mg/m^2 for 24 h infusion on days 1-4, dexamethasone 40 mg po on days 1-4, 9-12, 17-20). Dysphagia was relieved after administration of the chemotherapy regimen. Control endoscopical examination revealed that the formerly detected ulcers on admission were improved.

DISCUSSION

Multiple myeloma is a neoplastic proliferation of monoclonal plasma cells that can result in osteolytic bone lesions, hypercalcemia, renal impairment, bone marrow failure, and the production of monoclonal gammopathy. Although it is usually restricted to the bone marrow, extramedullary involvement can occur in the form of plasmacytomas in up to 20% of cases. The most common site of extramedullary involvement is the upper respiratory tract, including the oropharynx, nasopharynx, nasal cavity, paranasal sinuses, and larynx[1,2]. Gastrointestinal involvement by plasma cells is rare, representing less than 5% of all extramedullary plasmacytomas. Presentation may be either primary or secondary. Before a diagnosis of multiple myeloma is made it is mandatory to exclude primary extramedullary plasmacytoma (PEMP) by performing the necessary investigations. The differential diagnosis of PEMP from multiple myeloma is important because these two entities are thought to be biologically different and their prognoses are not the same.

Most of the reported cases represent involvement of the gastrointestinal tract by solitary plasmacytomas. All segments of the gastrointestinal tract may be involved by plasma cell infiltration. Small bowel is the most common site of involvement, followed by stomach, large bowel and esophagus[2-5]. Esophageal involvement by multiple myeloma is uncommon (Figure 3). A search of the English literature for plasmacytoma of the esophagus has revealed only four previous reports. The case reported by Morris and Pead[6] occurred in a 59-year old woman who presented with weight loss and intermittent dysphagia that progressively worsened over a 4- 5-mo period. Plasmacytoma is a protuberant mass occurring in the lower esophagus and involving the full thickness of the wall. Ahmed *et al*[7] encountered an esophageal PEMP in a 67-year-old man who, in addition to dysphagia, had weakness and weight loss. The lower third of the esophagus was invaded by an 8 cm tumour that extended to the gastric cardia and penetrated the entire thickness of the esophageal wall. Davis and Boxer[8] described a PEMP of esophagus in a 69-year-old man who only had a two-week history of dysphagia. Furthermore, this patient did not have weight loss, malaise or constitutional symptoms. The tumour was polypoid,

measured 4 cm in diameter, and involved the distal third of the esophagus. Recently, Chetty *et al*[9] described a PEMP of esophagus in a 58-year-old man. Dysphagia occurs particularly with consumption of solid foods over a period of 2 mo. Gross examination of esophagus can reveal large polypoid tumour. In the present case, however, dysphagia was particularly with solid foods over a period of one month. The lesion was in the form of ulcer and was probably induced by impairment of the esophageal mucosa due to replacement of the stromal tissue by the plasma cells (Figure 4).

The presenting features of esophageal plasmacytoma are similar to esophageal carcinoma, i.e., progressive dysphagia, profound weight loss, signs of malnutrition, and anemia. Diagnosis on clinical grounds alone is impossible and the radiologic appearance does not seem distinctive. Endoscopic biopsy is the earliest opportunity for diagnosis.

Diffuse plasma cell infiltration may also be seen in Barrett's esophagus that should be ruled out in the differential diagnosis. Plasma cells are positive for monoclonal light chain in MM as in the present case, but they are polyclonal in Barrett's esophagus. Additionally, there was no intestinal glandular metaplasia in the present case.

In conclusion, multiple myeloma is a systemic disease which may be rarely presented with esophageal involvement. It should be kept in mind that esophageal ulcers in patients diagnosed with MM may be related to the myeloma involvement and these patients should be examined further.

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Meetings

MAJOR MEETINGS COMING UP

Digestive Disease Week
107th Annual of AGA, The American Gastroenterology Association
20–25 May 2006
Loas Angeles Converntion Center, California

American College of Gastroenterology
Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24–25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3–8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26–30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14–16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1–5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4–5 May 2006
Berlin
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symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6–7 May 2006
Berlin
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ILTS 12th Annual International Congress
3–6 May 2006
Milan
ILTS
www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006–1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15–18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006–1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6–8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
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www.icsi2006.se/9/23312.asp

7th World Congress of the International Hepato-Pancreato-Biliary Association
3–7 September 2006
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www.edinburgh.org/conference

Society of American Gastrointestinal Endoscopic Surgeons
26–29 April 2006
Dallas – TX
www.sages.org

Digestive Disease Week 2006
20–25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25–26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3–7 June 2006
Seatlle - Washington
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EVENTS AND MEETINGS IN 2006

10th World Congress of the International Society for Diseases of the Esophagus
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Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyr*A, *arg* 1, *c myc*, *c fos*, *etc.*

Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*

Biology: *Helicobacter pylori, H pylori, E coli, 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_2SO_4	0.5 mol/L H_2SO_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_r18 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 $\cdot L^{-1}$	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	A_{260} nm	*A* should be in italic.
	^bP<0.05	^aP<0.05	In Table, no note is needed if there is no significance instatistics: ^aP<0.05, ^bP<0.01 (no note if *P*>0.05). If there is a second set of *P* value in the same table, ^cP<0.05 and ^dP<0.01 are used for a third set: ^eP<0.05, ^fP<0.01.
45	**F*=9.87, $^§F$=25.9, $^\#F$=67.4	^1F=9.87,^2F=25.9, ^3F=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	$10\times10\times10cm^3$	10 cm×10 cm×10 cm	
66	N·km	KN·m	moment
67	$x\pm s$	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 *et al.*	
72	EcoRI	*Eco*RI	*Eco* in italic and RI in positive. Restriction endonuclease has its prescript form of writing.
73	Ecoli	*E.coli*	Bacteria and other biologic terms have their specific expression.
74	Hp	*H pylori*	
75	Iga	*Iga*	writing form of genes
76	*igA*	IgA	writing form of proteins
77	-70 kDa	~70 ku	

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EDITORIAL

Third-line rescue therapy for *Helicobacter pylori* infection

Rossella Cianci, Massimo Montalto, Franco Pandolfi, Giovan Battista Gasbarrini, Giovanni Cammarota

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Abstract

H pylori gastric infection is one of the most prevalent infectious diseases worldwide. The discovery that most upper gastrointestinal diseases are related to *H pylori* infection and therefore can be treated with antibiotics is an important medical advance. Currently, a first-line triple therapy based on proton pump inhibitor (PPI) or ranitidine bismuth citrate (RBC) plus two antibiotics (clarithromycin and amoxicillin or nitroimidazole) is recommended by all consensus conferences and guidelines. Even with the correct use of this drug combination, infection can not be eradicated in up to 23% of patients. Therefore, several second line therapies have been recommended. A 7 d quadruple therapy based on PPI, bismuth, tetracycline and metronidazole is the more frequently accepted. However, with second-line therapy, bacterial eradication may fail in up to 40% of cases. When *H pylori* eradication is strictly indicated the choice of further treatment is controversial. Currently, a standard third-line therapy is lacking and various protocols have been proposed. Even after two consecutive failures, the most recent literature data have demonstrated that *H pylori* eradication can be achieved in almost all patients, even when antibiotic susceptibility is not tested. Different possibilities of empirical treatment exist and the available third-line strategies are herein reviewed.

Key words: *Helicobacter pylori*; Third-line rescue therapy; Antimicrobial resistance; Levofloxacin; Rifabutin; Furazolidone; Doxycycline

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INTRODUCTION

Helicobacter pylori (*H pylori*) is a spiral-shaped bacterium that is attached to or just above the gastric mucosa. The organism can persist in the stomach indefinitely and may not cause clinical illness for many years after infection. Indeed, a large number of infected patients never develop any symptoms. However, a large body of literature has associated *H pylori* infection with gastritis and gastric malignancies (gastric adenocarcinoma and MALT-lymphoma)[1]. Chronic *H pylori* infection has also been associated with several extra intestinal diseases, such as autoimmune thrombocytopenia, sideropenic anemia and chronic urticaria but the pathogenesis is still not known [2].

H pylori gastric infection is one of the most prevalent infectious diseases worldwide with an estimation of 40%-50% of the world population. Remarkable differences are due to geographical, socio-economical and demographic factors [3, 4]. *H pylori* transmission is still not completely understood. In addition, among infected patients, the reasons why only some develop symptoms is still a matter of speculations. The more generally accepted point of view is that bacteria are likely spread from person to person by fecal or oral transmission. Humans are the primary reservoir of *H pylori* infection [5].

Several tests are available to detect *H pylori* in patients with ulcer or dyspepsia. The more commonly used tests are the evaluation of bioptic specimens during upper GI endoscopy, the detection of serum anti *H pylori* antibodies and breath tests with ^{13}C-labeled urea [6].

The discovery that most upper gastrointestinal diseases are the consequence of *H pylori* infection and can be treated with antibacterials is an important medical advance [7]. In the last few decades, *H pylori* eradication has been standardized. The occurrence of resistance to therapeutic regimens is a growing problem.

Selection of papers was based on those papers thought to be more relevant to the authors based on two criteria: larger studies and novel studies even if based on limited series of patients, in which case this limit was stated.

FIRST-LINE THERAPY

The first-line therapy protocol is now generally accepted [8-12], which consists of proton pump inhibitor (PPI) (b.i.d.) or ranitidine bismuth citrate (RBC) plus two antibiotics: clarithromycin (500 mg, b.i.d.) and amoxicillin (1 g, b.i.d.) administered for 7 d. Metronidazole (500 mg, b.i.d.) can be used as an alternative to amoxicillin. However, even with

the correct use of these drug combinations, infection is not eradicated in 10%-23% of patients [13].

FACTORS DETERMINING PRIMARY ERADICATION FAILURE

H pylori may develop resistance to the prescribed antibacterials and may acquire resistance by acquisition and recombination of genes from other bacteria [14]. Chromosomic mutations can also induce resistance [15]. Gene acquisition is unlikely because *H pylori* lives alone in a unique ecological niche and is equipped with multiple restriction systems to avoid the introduction of hexogenous DNA [16]. Therefore, resistance is generally thought to be the consequence of point mutations. Indeed metronidazole targets DNA and a high mutation rate is observed [15].

After the development of eradication therapies, *H pylori* resistant strains have rapidly disseminated [17-21]. Several mechanisms are involved in the development of resistance. First, the lack of patient compliance is assumed to be a key factor in eradication failure, which occurs because adverse events are relatively frequent and lead to treatment discontinuation [22, 23]. Second, insufficient antibiotic concentration at the site of infection contributes to the spreading of resistant strains [22, 23].

An emerging problem is that general practitioners prescribe treatments without adequate diagnosis and do not adhere to eradication guidelines [24, 25]. Given the importance of host immune response in *H pylori* infection, the role of immunity in eradication failure can be hardly argued. However, data are anecdotal. Borody *et al* [26] suggested that IL-4 is important in *H pylori* eradication and hypothesized that IL-4 defect contributes to eradication failure.

Cytochrome P450, isoenzyme 2C19 [27] and interleukin-1-beta polymorphisms can interfere with acid secretion and have the activity of antimicrobial agents [28].

Finally, socio-economic factors (smoking habit), geographical factors, gender, histological changes also affect the eradication success [23-25]. Disease phenotypes also contribute to eradication failure. In fact, the failure rate in duodenal ulcer is 21.9%, lesser than in nonulcer dyspepsia (33.7%). In addition, the presence of histological fibrosis and lympho-epithelial lesions leads to poor eradication rates [23, 29].

Large studies on all these possible mechanisms of failure are lacking, but clarithromycin resistance appears to be the most important mechanism [30, 31].

SECOND-LINE THERAPY

Second-line therapy has been extensively reviewed by several authors[30, 32-34]. Therefore, we herein only discuss the Maastricht guidelines and some of more recently proposed protocols using new antimicrobial drugs, such as levofloxacin, rifabutin and furazolidone.

Most authors concord that culture after a first eradication failure is not thought to be necessary to start the second-line therapy. The assessment of *H pylori* sensitivity to antibiotics may be useful only after failure of the second-line therapy [8, 9, 35]. As second-line therapy, the Maastricht 2-2000 Consensus Report suggests a quadruple therapy based on bismuth (120 mg, q.i.d.), tetracycline (500 mg, q.i.d.), metronidazole (500 mg, t.i.d.) and antisecretive agent (PPI, b.i.d.) for a minimum of 7 d [12].

Further trials have shown that replacing the proton pump inhibitor and the bismuth compound of the quadruple therapy by RBC also achieves good results, with an eradication rate ranging between 57%-95% [36-39]. The failure of second line quadruple therapy is associated with its discontinuation because of the high incidence of side effects (6%-68%) [40]. Low compliance for the high number of pills to be taken each day also affects the clinical results [24]. However, in second-line regimens, new combination of drugs has been used. A triple therapy with the combination of levofloxacin, rabeprazole and tinidazole or amoxicillin has been proposed as an alternative to Maastricht [41]. This protocol shows an eradication rate higher than 90% compared to quadruple therapies given for 7 d (63%) with a lower incidence of side effects [42].

Rifabutin has been shown to have a good eradication rate (87%), if administered at a high dose (300 mg) in combination with amoxicillin and PPI, as compared to quadruple therapy [43-47]. Rifabutin shows an important side effect (mielotoxicity) [46]. Wong *et al* [43] showed that a combination of levofloxacin, rifabutin and rabeprazole has a high efficacy with an eradication rate >90% [43].

Furazolidone is also used to replace metronidazole in quadruple therapy [48-51]. Different *in vivo* studies have confirmed the efficacy of regimens containing a high-dose furazolidone [200 mg, b.i.d.] as the second-line therapy in patients with metronidazole-resistance [48-51]. Many other combinations have been used [31] with various rates of success. Bacterial eradication may fail in up to 40% of cases after the suggested second-line regimens. As a consequence, to treat patients who have already undergone the first- and second-line therapies is a common challenge.

THIRD-LINE RESCUE THERAPY

Currently, a standard third-line therapy is lacking. Different groups have tested various therapeutic protocols [33, 52, 53]. When available, endoscopy with culture and consequent antibiotic susceptibility testing remains the most appropriate option for patients with two eradication failures [54-56] to avoid a widespread use of expensive antibiotics such as rifabutin. The use of these drugs may also induce severe side-effects and development of *H pylori* resistant strains [30]. However, systematic use of culture is questionable [57]. Culture implies general endoscopic risks and is expensive as well as time-consuming due to *H pylori* difficult growth and not always available on a routine basis [58].

The sensitivity of bacterial culture is not 100% even in expert hands [6]. Moreover, amoxicillin and tetracycline rarely induce resistance [58, 59]. On the other hand, most of *H pylori* isolates after two eradication failures are resistant to metronidazole and clarithromycin, respectively [55]. Therefore, these two drugs are not recommended for third-line therapy [22, 56]. Our own previous data also show high resistance rates to metronidazole and clarithromycin even if the previously used regimens did not include either

of these two drugs [55]. In addition, *in vitro* susceptibility cannot predict eradication success [60-62]. Taken together, these data suggest that cultures are not strictly necessary to decide upon a third-line protocol.

The third-line therapy should avoid metronidazole and clarithromycin and antibiotics that are likely to have contributed to development of resistance. A consensus for third-line therapies has not been presently reached. Herein we discuss those based on levofloxacin, rifabutin, furazolidone and doxycycline.

Levofloxacin-based therapy

Levofloxacin is a broad-spectrum fluoroquinolone, active against Gram-positive and negative bacteria and atypical respiratory pathogens [63]. Levofloxacin inhibits the DNA synthesis, has a good oral absorption and is well tolerated [64]. Fluoroquinolones are active against *H pylori in vitro* [65] and have a synergistic effect with PPIs [66]. Primary resistance to levofloxacin ranges between 8%-31% in different countries or regions [55, 67, 68].

Recently, Gatta *et al* [69] have proposed a third-line treatment after two eradication failed courses without fluoroquinolones, with standard dose of PPIs (b.i.d.), levofloxacin (250 mg, b.i.d.) and amoxicillin (1 g, b.i.d.) for 10 d. The eradications rates of 76.2% and 84.6% according to ITT and PP analysis, respectively, have been achieved in 151 enrolled patients in a prospective open study. The levofloxacin-based treatment could eradicate most of the strains (92.3%) which are resistant *in vitro* to both clarithromycin and metronidazole, but susceptible to levofloxacin. The primary resistance to levofloxacin found in this study was 14%. Furthermore, this drug combination, successfully employed as rescue therapy [70], is well tolerated and has no major side-effects [71].

A more recent prospective multicentric study [72] reports data of 100 patients who have failed two eradication courses without fluoroquinolones. This study demonstrated that a regimen of levofloxacin (500 mg, b.i.d.), amoxicillin (1 g, b.i.d.) and omeprazole (20 mg, b.i.d.) for 10 d can achieve an eradication rate of 60% or 66% according to ITT and PP analysis. The treatment was given without previous sensitivity test.

Low compliance with the current regimens is one of the main causes of failures [24]. Therefore, Coelho *et al* [73] have proposed a combination of rabeprazole (20 mg), levofloxacin (500 mg) and furazolidone (200 mg) (two tablets) administered at a single dose for 10 d. Twelve patients who failed at least two eradication courses are successfully treated. Per-protocol and intention-to-treat eradication rates were 100% and 83.3%, respectively. However, because of the paucity of patients in third-line therapy, these data have to be confirmed in larger series. Furthermore, cultures obtained before treatment from some patients show no resistance to furazolidone, while 87% of the samples analyzed are sensitive to levofloxacin. No severe adverse effects are observed [73]. Therefore, the results after the levofloxacin-based triple therapy for ten days in patients with two eradication failed courses with amoxicillin, clarithromycin, metronidazole, tetracycline and bismuth, are encouraging. However, the resistance to quinolones is easily acquired, and the resistance rate is relatively high in countries with a high consumption of these drugs [55, 68]. Therefore, it seems advisable to reserve levofloxacin to third-line rescue treatment to avoid the increase of the resistance phenomenon [72].

Rifabutin-based therapy

Rifabutin is a spiropiperidyl derivative of rifamycin-S, an antitubercolar compound. Rifabutin inhibits the beta-subunit of *H pylori* DNA-dependent RNA polymerase encoded by the rpoB gene [74]. Rifabutin is expensive and unavailable in various countries and has side effects (leukopenia and thrombocytopenia, with myelotoxicity) [46]. It has been suggested to reserve the use of rifabutin for the treatment of multiresistant *Mycobacterium tuberculosis* strains [53, 75]. *H pylori* is highly susceptible *in vitro* to rifabutin and no resistant strains have been isolated from patients treated or untreated for *H pylori* infection [74, 75]. Furthermore, rifabutin is chemically stable at a wide pH range [76].

Three different trials have shown that rifabutin (300 mg o.d. or 150 mg b.i.d)-based therapies in combination with amoxicillin (1 g, b.i.d.) and standard dose of PPIs (b.i.d.) are a good third-line strategy, achieving the eradication rate of at least 70% [46, 47, 53]. On the other hand, Qasim *et al* [77] have achieved only a 38% eradication rate [77].

A more recently single centre prospective study [78] studied 67 patients who failed to respond to two or more courses. The result showed that when the rifabutin dose is reduced from 300 mg to 150 mg, it results in a signficant drop in eradication rate from 86.6% to 66.6% [45]. Borody *et al* [78] have shown that a 12 d regimen with low-dose rifabutin (150 mg a day) in combination with increased frequency of amoxicillin (1 g, t.d.s.) and pantoprazole (80 mg, t.d.s.) could achieve an overall eradication rate of 92.1% in patients harbouring double resistance strains to metronidazole and clarithromycin, with an eradication rate of 95.7%. Mild side effects are found in 40% of patients. Unlike regimens which use higher doses of rifabutin, no patients develop drug-related neutropenia or thrombocytopenia after treatment. Nevertheless, the main problem with a widespread use of rifabutin is the concern that antibiotic resistance may develop against *Mycobacterium avium* in HIV-infected patients. Therefore, the use of this drug for *H pylori* is questionable.

Furazolidone-based therapy

Furazolidone is a broad-spectrum nitrofuran, active against Gram-negative and positive bacteria and protozoa by inhibiting bacterial enzymes [79]. It is widely used in low income populations because it is inexpensive. It kills *H pylori* [80,81]. Strains resistant to furazolidone are rare [82, 83] and its potential to develop resistance is as low as bismuth compounds or amoxicillin [84]. Furthermore, it has no cross-resistance to metronidazole [83] and is effective in populations with a high prevalence of metronidazole resistance [85]. It has poor oral absorption and presents some side effects, especially gastrointestinal ones [79]. Concomitant intake of alcohol and MAO-inhibitors should be avoided as other interacting drugs. Furazolidone may induce a disulfiram-like reaction to alcohol and is an MAO-inhibitor. One week quadruple regimen with lansoprazole (30 mg, b.i.d), bismuth (240 mg, b.i.d.), tetracycline (1g, b.i.d.) and fura-

zolidone 200 mg (b.i.d.) has shown an eradication rate of 90% as third-line therapy in 10 patients with metronidazole resistance by culture [48]. Furthermore, 7 d triple-regimen comprising of furazolidone (200 mg, b.i.d), amoxicilin 1 g (b.i.d.) and standard dose of PPI (b.i.d.), achieves an eradication rate of 60% in 10 patients who failed first-line, second-line and rifabutin-based triple therapy [77].

In conclusion, in developing countries where resistance to metronidazole is usually very high [12], furazolidone in combination with tetracycline, bismuth and PPI for one week is very effective, safe and cost effective against *H pylori* as the third-line therapy.

Doxycycline-based therapy

Doxycycline is a widely used tetracycline antibiotic for several infections. With respect to tetracycline, doxycycline requires the administration of only two tablets per day, leading to a better compliance in patients undergoing eradication therapies. Furthermore, Heep *et al* [19] have found no secondary resistance to doxycycline in *H pylori* isolates from patients who failed one or more eradication therapies.

Quadruple regimens represent the most widely used rescue therapy. Yet, it is limited by lack of patient compliance due to the large number of tablets and by several side-effects. The classic quadruple therapy includes bismuth salts which have a synergistic effect on antibiotics possibly by decreasing the bacterial load, PPI which facilitates antibiotic activity by increasing the gastric pH, tetracycline with a low rate of resistance in *H pylori* isolates, and metronidazole [58, 59]. Induction of metronidazole resistance has suggested a new protocol, namely replacing tetracycline with doxycycline (because it requires the administration of only two tablets per day) and metronidazole with amoxicillin (because its resistance is less 1%), 1-week-quadruple therapy with doxycycline (100 mg, b.i.d.), amoxicillin (1 g, b.i.d.), omeprazole (20 mg, b.i.d.) and bismuth salts (120 mg, two tablets b.i.d). This treatment has proved to be a highly effective third-line 'rescue' therapy, achieving 91% eradication rate in patients harbouring metronidazole and clarithromycin resistant *H pylori* strains (by ITT analysis) [55]. This regimen, showing excellent compliance (99%) and mild side-effects, may well constitute the test available option for the third-line rescue treatment.

Rifampicin-based therapy

Rifampicin is a semisynthetic derivative of rifamycin B. The target is the DNA-dependent DNA polymerase, mainly the beta subunit [86]. Rifampicin inhibits the growth of most Gram-positive and negative microorganisms. The clinical efficacy of rifampicin against *H pylori* has been discovered by the observation of the decrease of anti-*H pylori* antibodies in patients on rifampicin-containing antitubercular therapy [87]. Rifampicin has an excellent *in vitro* efficacy against *H pylori* [88, 89] and a favorable pharmacokinetics. Less-expensive rifabutin is available in many countries. A single-center study has shown that 10 d rifampicin (450 mg o.d.)-triple therapy in combination with esomeprazole (40 mg b.i.d.) and tetracycline (1 000 mg b.i.d.) can achieve an eradication rate of 32.1% and 31.6% (by ITT analysis), if given as second-line or third-line therapy, respectively. Side effects are common but minor.

In conclusion, rifampicin-based rescue therapy is not as effective as a salvage-based therapy for *H pylori* eradication [86].

CONCLUSION

An undisputed third line strategy to cure *Helicobacter pylori* is still lacking. Eradication rates >90% can be achieved following the Maastricht guidelines for first- and second-line therapies. New first-line alternative strategies are needed, considering the development of primary and secondary resistances. Second-line therapy depends on which regimen is used initially, the re-administration of any antibiotics against which *H pylori* has probably become resistant, as metronidazole and clarithromycin or drugs with cross-resistance to these or previously used antimicrobial are not recommended. To face treatment failures, several third-line 'rescue' therapies have been tested, achieving good eradication rates. In our opinion, levofloxacin-triple (eradication rate of 92%) [69] and doxycycline-quadruple (eradication rate of 91%) [55] are more active on resistant strains. They are safe, better tolerated and less expensive than rifabutin-based regimen. Moreover, the widespread use of rifabutin may be a major concern due to the possible development of antibiotic resistance. We believe that the worldwide aid tubercular emergency and the risk to develop *Mycobacterium*-resistant strains strongly suggest a conservative approach reserving rifabutin to antitubercular therapy. This is especially recommended in countries where alternative drugs are available. In developing countries where resistance to metronidazole is usually very high, the 7 d furazolidone-quadruple third-line therapy is effective against *H pylori* (with eradication rates of 90%), safe and cost-effective.

In conclusion, our review shows that *Helicobacter pylori* eradication can be eventually obtained even in the few patients who experience up to 8 consecutive failures [78, 90, 91]. This can be done by different drugs as reported in the different protocols discussed above.

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EDITORIAL

Pathogenesis of primary biliary cirrhosis: A unifying model

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Abstract

Primary biliary cirrhosis (PBC) is a disease of unknown etiology leading to progressive destruction of small intrahepatic bile ducts and eventually to liver cirrhosis and failure. It is characterised by female predominance and serum auto-antibodies to mitochondrial antigens targeting the E2 components of the 2-oxoacid dehydrogenase complex. Although they are associated with disease pathogenesis, no concrete evidence has been presented so far. Epidemiological data indicate that a geographical clustering of cases and possible environmental factors are implicated in pathogenesis. A number of genetic factors play a role in determining disease susceptibility or progression, although no definitive conclusion has been reached so far. A key factor to immune pathogenesis is considered to be the breakdown of immune tolerance, either through molecular mimicry or through the so called determinant density model. In this review, the available data regarding the pathogenesis of primary biliary cirrhosis are described and discussed. A new unifying hypothesis based on early endothelin overproduction in primary biliary cirrhosis (PBC) is presented and discussed.

Key words: Primary biliary cirrhosis; Pathogenesis

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INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic cholestatic disease of unknown aetiology characterized by progressive destruction of small intrahepatic bile ducts eventually leading to cirrhosis. It is considered to be an autoimmune disease due mostly to the presence of well characterized auto-antibodies. These auto-antibodies target the components of 2-oxoacid dehydrogenase complexes. Antibodies against components of the nuclear pore complex have also been described. The disease may be considered as an example of the vanishing bile duct syndrome.

Although auto-antigens have been molecularly identified and epitope-mapped and auto-reactive T and B cells have been characterized, the exact mechanism of liver tissue damage remains unclear. Recent reviews have summarised the present theories of PBC pathogenesis [1,2]. In this review, we first examined the evidence and then the current models concerning disease pathogenesis. Finally, a unifying hypothesis based on recent observations is proposed.

PRIMARY BILIARY CIRRHOSIS AS A GENETIC AND ENVIRONMENTAL DISEASE

Following earlier case reports of familial cases of PBC, a more comprehensive cohort study has estimated the sibling relative risk for PBC at 10.5, similar to other classical autoimmune diseases [3]. Recently, a pairwise concordance rate of 0.63 for PBC in monozygotic twin pairs has been published [4], which is one of the highest reported in autoimmunity. Taken together these reports indicate a significant genetic contribution to the disease pathogenesis. However, studies on specific genes have provided only weak associations. Extensive reviews on genetic factors in PBC have been recently published [5, 6].

Geographical disease clusters have been reported [7-9] and provide evidence for an as yet unidentified environmental factor in PBC pathogenesis. Although these studies are criticized and the case control studies are not able to identify putative environmental factors [10,11], there is enough evidence that environmental susceptibility does play a role.

PRIMARY BILIARY CIRRHOSIS AS A HUMORAL IMMUNE RESPONSE DISEASE

High titers of antibodies against mitochondrial elements are characteristic of the disease. Anti-mitochondrial antibodies (AMA) target the E2 component of the

pyrurate dehydrogenase complex (PDC-E2), which belongs to the family of the 2-oxoacid dehydrogenase complexes (2-OADC) [12]. The main epitopes have been localised within the inner lipoyl-binding domain of the subunit overlapping amino acids 212-226. The AMA response is polyclonal and these antibodies also react with the dihydrolipoamide dehydrogenase binding protein [13].

Based on the in vitro fact that 2-OADC activity is inhibited by AMA and 10% of the portal B cells produce antibodies reactive with PDC [14,15], a pathogenetic role of AMA has been proposed.

Sera from over 50% of patients do contain AMA with a different specificity. They react with the E1α component of PDC [16]. Their reactivity is directed to the C-terminus of the molecule which contains the active site of the enzyme and therefore these antibodies are also inhibitory of PDC activity [17,18]. Antibodies against the branched chain of 2-oxoacid dehydrogenase complex E1α have also been identified [19]. Auto antibodies against the nuclear pore proteins gp210 and p62 are associated with more active or severe disease [20]. Perhaps the best evidence for the pathogenetic role of auto antibodies in PBC comes from the description of secretory IgA anti-PDC in saliva, bile and urine of patients which retain their enzyme inhibitory capacity [21-24].

In PBC, both biliary epithelial cells and salivary epithelial cells (the main targets of the disease process) demonstrate an apical surface up-regulation of PDC or an antigen cross reacting with it [25]. This expression appears earlier than the reported up-regulation of other surface molecules like MHC class II, or ICAM-1 [26]. It seems, however, that PDC is released from apoptotic mitochondria to the cytoplasm within six hours of the induction of apoptosis and that auto-reactive epitopes are present on the still intact cell surface at later time points during the process of apoptosis [27]. However, convincing evidence for a role of AMA in the pathogenesis of PBC has yet to be produced [28]. Moreover, the very existence of the so called autoimmune cholangitis or AMA-negative PBC, a disease similar in every aspect to PBC but without detectable AMA, strongly argues against a pathogenetic role of AMA.

T CELL RESPONSES IN PBC

CD4 and CD8 T-cells reactive with PDC have been identified in the peripheral blood and liver of PBC patients [29-31]. These cells are reactive with the native human antigen [32, 33]. PDC- E2 specific T-cells are present in the liver of PBC patients [29, 34], mostly during the earliest disease states [30, 31]. Epitope mapping studies have identified HLA DR4*0101-restricted T cell epitope, spanning residues 163-176 of PDC-E2 [35, 36]. Recently HLA-A2-restricted CD8 T cell lines reactive with PDC-E2 residues 159-167 have been characterised [37, 38]. Interestingly, CD8 T cells from livers of PBC patients demonstrate cytotoxicity against PDC-E2 159-167 pulsed autologous cells [39].

APOPTOSIS IN PBC

There is concrete evidence that apoptosis is possibly the most important mechanism of biliary epithelial cell loss. Markers of ongoing apoptosis have been reported within affected portal tracts [40,41], including down regulation of the anti-apoptotic protein bcl-2 [42]. Apoptosis is considered the result of the attack of effector cells like CD8 T cells [39]. Interestingly, *in vitro* caspase cleavage of PDC-E2 has been shown to generate immunologically active protein fragments [43].

ROLE OF REACTIVE OXYGEN SPECIES (ROS) in PBC

Data on the role of oxidative stress in the pathogenesis of PBC are scarce. In the damaged bile ducts of PBC, glutathione-S-transferase expression is markedly reduced, reflecting reduction of intracellular glutathione, while perinuclear expression of 4-hydroxynonenal is increased, reflecting active lipid peroxidation associated with biliary epithelial damages [44]. Levels of the antioxidant vitamin E have been found to be decreased in PBC, together with other fat soluble vitamins [45-47], while serum total antioxidant capacity (measured with an enhanced chemiluminescent technique) is significantly reduced in PBC patients [48].

A number of antioxidant substances including retinol, alpha-tocopherol, total carotenoids, lutein, zeaxanthin, lycopene, alpha and beta-carotene are reduced in PBC patients compared to normal controls [49]. However, we have reported highly corrected total antioxidant capacity in PBC [50], a fact that may reflect a compensatory but probably not sufficient increase to counteract an increased ROS production.

Evidence for a role of ROS in the liver damage of PBC is provided by *in vitro* reports that ursodeoxycholic acid (UDCA), a drug commonly used in PBC, has extensive ROS scavenging properties and prevents mitochondrial oxidative stress and lipid peroxidation in a dose-dependent manner [51-53]. Finally, evidence from the rat bile duct-ligated model may have relevance to PBC.

Lipid peroxidation is a relatively late event in this model and a close link seems to exist between lipid peroxidation and the activation of inflammatory cells [54,55]. Free radicals triggering hepatic injury in this model, involve overproduction of the pro-inflammatory cytokines TNFα, IL-6 and IL-1b via enhanced activation of nuclear factor kB [56]. Moreover, *in vitro* experiments have shown that several bile acids including taurochenodeoxycholic acid and taurocholic acid cause hepatocyte injury with a concomitant generation of hydroperoxide by mitochondria [57,58] and also induce hepatocyte apoptosis in a time- and concentration-dependent manner via ROS generation by mitochondria [59]. An increased bile acid concentration is a feature of at least late PBC.

CURRENT VIEWS ON THE PATHOGENESIS OF PBC

There are two fundamental facts that should be interpreted in every model trying to explain the pathogenesis of PBC. First, the PBC auto-antigen PDC is located on the inner surface of the inner mitochondrial membrane and is therefore normally separated from the extra-cellular immune system by three membranes. It is difficult to understand how such an antigen is exposed to antigen presenting cells, eliciting an autoimmune reaction. Second, PBC is a disease with very limited tissue distribution, yet the putative autoimmune response is directed at an antigen with an extremely widespread localization.

So far, the models developed to explain the pathogenesis of PBC suggest that the key step in disease pathogenesis is the breakdown of T cell self-tolerance to PDC, since the induction of anti-PDC antibodies is not enough by themselves to produce liver disease [60]. The mechanisms of the disease pathogenesis have been elegantly reviewed elsewhere [61].

Molecular mimicry model of self-tolerance breakdown

Infection, either viral or bacterial, can either directly induce apoptosis of biliary epithelial cells or more probably trigger an immune attack on epithelial cells as a result of molecular mimicry. A T-cell response is initiated and mediated by toll-like receptor interaction with a pathogen epitope cross-reactive with a self-PDC epitope. An immune attack on biliary epithelial cells is mediated by these T-cells leading to apoptosis. However, the evidence for the initiating micro-organism is conflicting. Studies implicating mycobacteria as the source of cross-reactive targets are not reproducible and recent reports on Chlamydia pneumoniae as the potential microbial factor require confirmation [62].

Non PDC-E2 microbial sequences with a high degree of similarity to PDC-E2 212-226 epitope, mostly *E coli* mimics, are described as the major targets of cross-reactivity with human PDC in the sera of PBC patients [63]. Recently, the cross-reactive target has been reported to be the myobacterial hsp65 sharing a common motif with PDC-E2 212-226 epitope [64]. IG G3 antibodies to mimics from Lactobacillus delbrueckii with the same motif cross-reactive target can react with the PDC-E2 212-226 epitope in PBC sera [65]. Therefore, this motif may be a candidate epitope in the molecular mimicry model.

An alternative explanation for the molecular mimicry model would be a retroviral infection. The retroviral etiology of PBC has been recently reviewed in detail [28], but still remains controversial [66].

Determinant density model

This model has been described in detail by Jones [1]. According to this model, potentially self-PDC reactive T cells survive negative selection in the thymus, because their T cell receptor (TCR) shows low affinity for the complex of self-peptide and MHC. Sporadic self-PDC-derived epitopes presented by antigen presenting cells (APC) to these low TCR affinity T-cells, are unable to activate T cells. However, enrichment of APC presentation of self-PDC-derived epitopes could give sufficient low affinity presentation to overcome a triggering threshold and induce a proper CD4 T-cell activation.

In this model also, the initial trigger of antibody response cross-reaction with self-PDC could be either viral or bacterial epitopes with a structural homology to PDC. An interesting feature of this model is that the state of activation of APC mediated through toll-like receptors may determine the efficacy of antigen presentation and promote tolerance breakdown [67]. Peripheral blood monocytes from PBC patients produce higher levels of pro-inflammatory cytokines (TNFα, IL1b, IL-6, IL-8) when they are challenged with specific ligands for TLR2, TLR3, TLR4, TLR5 and TLR9. These findings indicate that monocytes in PBC (and possibly APC) are hyper-responsive to signalling through TLRs, a fact that may help in tolerance breakdown.

PATHOGENESIS OF PRIMARY BILIARY CIRRHOSIS: A UNIFYING HYPOTHESIS

We recently reported a significant increase of endothelins, particularly ET2 (and to a lesser extent of ET1) both in peripheral blood and in the hepatic vein, occurring at an early stage of the disease. Moreover, UDCA treatment caused a significant reduction of all three endothelins, its effect being most pronounced in early stage PBC.

Based on our observations, a new unifying hypothesis for the pathogenesis of PBC is proposed (Figure 1). In this model, there is a primary dysfunction of endothelial cells overproducing ET-2 (and to a lesser extent ET-1). This could be a primary genetically determined event. Endothelial cells express scavenger receptor type B [68] and internalise foreign antigens. Indeed lipoteicholic acid, a strongly antigenic component of gram positive bacteria, has been found in endothelial cells [68], while Helicobacter and lipopolysaccharide have also been described in PBC livers [69, 70]. ET2 may in turn stimulate Kupffer cells to produce pro-inflammatory cytokines, such as IL-1 and IL-6 from mouse peritoneal macrophages (but not TNFα or NO in this particular model) [71]. ET2 is also a potent macrophage chemoattractant [72] via the ETB receptor. ET2 shares the similar peptide sequence with CXC chemokines.

In accordance with this hypothesis, macrophages constitute 30% of the cellular infiltrate on portal areas and around damaged bile ducts [73]. Activated macrophages have also been observed by electron microscopy near epithelial cells of the bile ductules and seem to develop into epitheliod cells [74]. Epitheliod granulomata of PBC patients contain most MCP2 and MCP3 positive cells at their edge and more than 60% of them co-express CD68, indicating that they are derived from macrophages [75].

In stages 3 and 4, PBC Kuppfer cells and myofibroblasts are increased in periportal and periseptal areas, possibly indicating that Kupffer cells interact with stellate cells and lead to fibrosis [76], thus forming the connecting element to the development of cirrhosis.

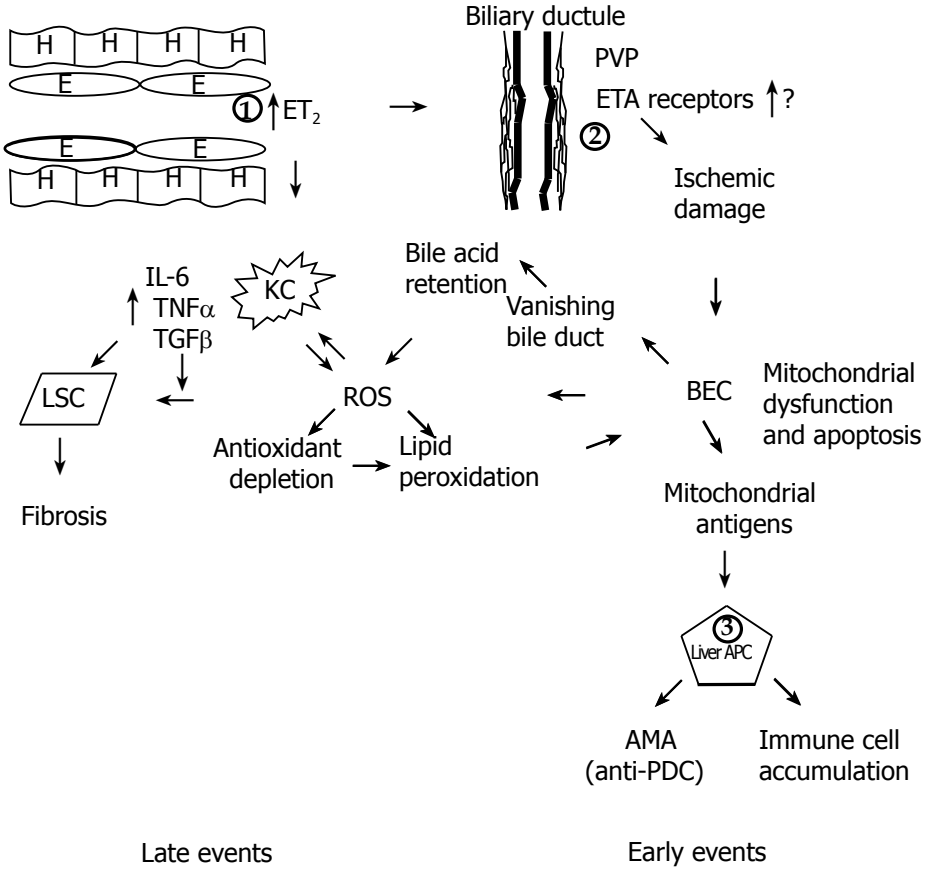

Figure 1 Figure 1 Occurrence of late and early events in the unifying model. The fundamental early defect in PBC is the overproduction of ET2 by endothelial cells (1) possibly driven by virus [95-98] or other microbial pathogens in genetically predisposed individuals. ET2 is a chemoattractant for Kupffer cells and also causes contraction of stellate cells leading to early portal hypertension. ET2 leads to ischemic damage of BEC (2) through constriction of the PVP with resultant BEC mitochondrial dysfunction and membrane disruption by ROS generation and eventually apoptosis leading to the vanishing bile duct syndrome. Mitochondrial antigens possibly generated through caspase cleavage, are presented by hyper-responsive liver dendritic cells (APC) (3) and lead to immune cell accumulation and AMA production. The second fundamental defect occurring later in the disease process is the production of ROS. Together with ingestion of BEC apoptotic bodies, ROS drives the accumulated Kupffer cells to produce more ROS and pro-inflammatory cytokines and TGFβ, which in turn leads to fibrosis. ROS is also produced after development of the vanishing bile duct syndrome as a result of bile acid retention. Finally through antioxidant depletion or through an insufficient increase of antioxidants ROS leads to lipid peroxidation and further BEC apoptosis and mitochondrial dysfunction. Genetically determined control may be exercised at levels 1,2 and 3.

Endothelins also cause contraction of stellate cells [77] and possibly help their differentiation to myofibroblasts. This contradiction may play an important role in the development of early portal hypertension in PBC as has been suggested in rats with biliary cirrhosis [61, 76-78].

Intrahepatic bile ducts receive their blood supply from a periductal network of minute vessels, known as the peribiliary vascular plexus (PVP). This plexus originates from hepatic artery branches accompanying its intrahepatic bile duct [79] and drains mostly into the sinusoids [80]. The peribiliary space also contains dendritic cells and stellate cells. Insufficient perfusion of this system can cause profound bile duct damage [81]. ETs and NO seem to be the principal molecules that regulate circulation of the PVP [82]. It is suggested in our model that patients with PBC have damage to the biliary endothelium as an initiative event caused by ischaemia due to ET-2 driven vasoconstriction. There is direct evidence that the peribiliary capillary plexus is indeed damaged in PBC. It was reported that the peribiliary plexus is significantly reduced in PBC (and interestingly also in auto-immune hepatitis), while there is proliferation of the plexus in other liver diseases [83]. There are also other reports indicating vascular impairment in PBC [61,78]. The increased circulating ET-2, observed both in early and late stage PBC seems to be specific for this disease, since it has not been found in the disease control groups of cirrhotics and patients with chronic viral liver disease.

Biliary ischemia might then lead to apoptosis of biliary epithelial cells, which is indeed a mechanism proposed for biliary epithelial destruction in PBC [84, 85]. Moreover biliary epithelial cells (BEC) undergoing apoptosis release the pyruvate dehydrogenase complex (PDC) from mitochondria into the cytoplasm as early as 6 hours after induction of apoptosis and auto-reactive epitopes are present in BEC, while other cells efficiently delete cytoplasmic PDC by glutathione [43,86]. Such a mechanism may also explain the similarities between PBC and graft *vs* host disease (GVHD) [87-90]. GVHD is associated with endothelial cell injury [91] and IL-1 has been implicated in its pathogenesis [92]. More importantly, in GVHD after small bowel transplantation, ET1 levels are increased before the induction of GVHD and have been histochemically shown to be increased in endothelial and epithelial cells some days before GVHD, implicating a pathogenetic significance [93]. Immunoreactive epitopes, self-PDC generated during apoptosis, possibly through the action of caspase 3 [43] are taken up by either the peribiliary dendritic cells or by BEC expressing MHC II (this could be either genetically determined or alternatively be caused by pro-inflammatory cytokines [94]). In the first instance this leads to the production of auto-antibodies and possible the determinant density model as elegantly described by Jones [1].

There are many questions that have yet to be answered regarding the above suggested model of liver injury in PBC. The most important issue is that the increased concentration of ET-2 has been found in

systemic circulation. This means that the vasoconstriction and the consequent ischemic injury ought to happen in many organs apart from liver and PVP. A possible explanation for this selectivity is an increased expression of ET receptors in the PVP of PBC patients but this suggestion needs to be further studied.

The suggested model is diagrammatically outlined in Figure 1. However, the proposed model has the following advantages. It implicates both innate and adaptive immunity. The former is the initiating event while the latter is the element that causes perpetuation of the disease even after disappearance of the initial event (if this is environmental infections). It explains the role of infective agents and the similarity of PBC with graft vs host disease. Interaction of endothelial and Kupffer cells with stellate cells explains the progress to fibrosis and cirrhosis. It predicts that most infiltrating cells should be CD4 helper T cells participating in B cell differentiation as it is indeed the case [31-35] but the role of CD8 is limited [37,38]. Since AMA production is a secondary phenomenon rather than pathogenetically related to liver damage, the presence of AMA negative PBC is also explained. Ursodeoxycholate (UDCA) may act mostly as an R0S scavenger preventing the mitochondrial oxidative stress. Most importantly, it offers 3 levels for a genetically determined control, namely the level of endothelial cells (and possibly Kupffer cells), the level of perivascular plexus and ET receptor expression, and the level of peribiliary dendritic cells that might be genetically hyper-responsive. All of them may well be estrogen dependent, thus explaining the extreme female prevalence of the disease, but this requires further research.

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REVIEW

Eosinophilic esophagitis: A newly established cause of dysphagia

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Abstract

Eosinophilic esophagitis has rapidly become a recognized entity causing dysphagia in young adults. This review summarizes the current knowledge of eosinophilic esophagitis including the epidemiology, clinical presentation, diagnostic criteria, pathophysiology, treatment, and prognosis. An extensive search of PubMed/Medline (1966-December 2005) for available English literature in humans for eosinophilic esophagitis was completed. Appropriate articles listed in the bibliographies were also attained. The estimated incidence is $43/10^5$ in children and $2.5/10^5$ in adults. Clinically, patients have a long history of intermittent solid food dysphagia or food impaction. Some have a history of atopy. Subtle endoscopic features may be easily overlooked, including a "feline" or corrugated esophagus with fine rings, a diffusely narrowed esophagus that may have proximal strictures, the presence of linear furrows, adherent white plaques, or a friable (crepe paper) mucosa, prone to tearing with minimal contact. Although no pathologic consensus has been established, a histologic diagnosis is critical. The accepted criteria are a dense eosinophilic infiltrate (>20/high power field) within the superficial esophageal mucosa. In contrast, the esophagitis associated with acid reflux disease can also possess eosinophils but they are fewer in number. Once the diagnosis is established, treatment options may include specific food avoidance, topical corticosteroids, systemic corticosteroids, leukotriene inhibitors, or biologic treatment. The long-term prognosis of EE is uncertain; however available data suggests a benign, albeit inconvenient, course. With increasing recognition, this entity is taking its place as an established cause of solid food dysphagia.

Key words: Eosinophilic esophagitis; Allergy; Dysphagia

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INTRODUCTION

The esophagus normally is rather devoid of eosinophils. Not merely a simple conduit for swallowing food and liquids, the esophagus is being recognized as an immunologically active organ that can respond to a variety of stimuli like gastric acid and allergens by recruiting eosinophils and eliciting an inflammatory response. Eosinophils, for example, commonly infiltrate the lower esophagus in conjunction with gastroesophageal reflux disease (GERD)[1] A relatively new entity, eosinophilic esophagitis, with more extensive eosinophilic infiltration, particularly in the proximal esophagus[2] possesses clinical features that differ from GERD[3]. This entity is becoming increasingly recognized as a cause of dysphagia, often with a history of food impaction[4]. Since its original description in 1978[5], EE has exploded onto the clinical scene, becoming a recognized cause of solid food dysphagia, first identified in the pediatric and now the adult population[6-8]. Eosinophilic esophagitis is a disorder in which eosinophils infiltrate the superficial mucosa of the esophagus. Previously thought to be a rarity, case reports of eosinophilic esophagitis are rapidly accumulating in the literature[3,7]. Clinical presentation and the endoscopic-[7] and histological[7] features have become more firmly established, although a consensus is still lacking for an absolutely clear-cut diagnosis. Eosinophilic esophagitis has been associated with food allergies and atopic conditions such as asthma and atopic dermatitis[6,8-10].

Eosinophilic esophagitis is better known in the pediatric population through several published studies and reviews[11-14]. Infiltration of eosinophils into the esophagus may result from conditions such as food allergy, infection, gastroesophageal reflux disease (GERD), or systemic eosinophilic conditions. The mechanism of dysphagia from eosinophilic esophagitis has yet to be defined.

This review summarizes the epidemiology, clinical presentation, possible pathophysiological mechanism, diagnosis, treatment, and prognosis for eosinophilic esophagitis, primarily focusing on adults with this condition.

Table 1 Clinical features of eosinophilic esophagitis

	Adult	Pediatric
Common	Dysphagia Food impaction/foreign body Esophageal stricture Nausea/vomitting/regurgitation Heartburn Food allergy	Abdominal pain Failure to thrive Nausea/vomitting Dysphagia Food allergy Heartburn
Uncommon	Hematemesis Globus Waterbrash Weight loss Chest pain Abdominal pain	Food impaction
Associated Conditions	History of atopy Asthma Allergic rhinitis	Asthma Allergic rhinitis Eczema Atopic dermatitis Strong family history of atopy

EPIDEMIOLOGY

Epidemiological studies on eosinophilic esophagitis are lacking, likely from inadequate recognition and a paucity of established diagnostic criteria. Most publications are case reports or case series. Information is more widely available for the pediatric population compared to adults. This may be due to increased aggressiveness in investigating children with GI symptoms, or practice habits of pediatric gastroenterologists performing random biopsies in all cases. Fox *et al*[15] estimated that 6.8% of children with esophagitis had eosinophlic esophagitis, while Liacouras *et al*[16] indicated 3.4% of such children experienced reflux symptoms. A previous estimate of frequency was 1 per 100 000[17]. A recent population based study by Noel *et al*[13] in Ohio based on 103 children suggested a much higher figure: an annual incidence of 1 per 10 000 and a prevalence of 4.296 per 10,000 children, which rose over the period of the study - from 2 000 to 2003. Whether this represented a true increase in the entity or bias from improved detection is unknown. A strong familial pattern was evident. In Italy the prevalence was reported to be 3.5%[18]. A worldwide pediatric registry has been established[19].

Population-based data is lacking in the adults. Croese *et al*[20] identified eosinophilic esophagitis in 19 adult patients from a population of 198 000 over a 21 months period. The study, however, used 30 eosinophils per high power field as its criteria for diagnosis, a value higher than in most studies (usually >20/high power field). Therefore the incidence may be underestimated. The study also included both pediatric and adult populations with an age range of 14-77. Nevertheless, eosinophilic esophagitis appears to be an increasingly recognized entity with an accelerating frequency[13,21,22].

DIAGNOSIS

The diagnosis of eosinophilic esophagitis is based on clinical presentation, endoscopic or radiographic features, and histopathological criteria.

Clinical features

Clinical features (Table 1) of eosinophilic esophagitis have been previously well defined[13,20,22,23]. There is a male predilection and a wide range of ages from pediatric to adult populations. Mean age in children ranges from 7-10 years, and 30-40 years in adults. Dysphagia is the most common symptom in adults and is usually longstanding. Food impaction, reflux symptoms, vomiting or regurgitation, and food allergy are also common. Abdominal pain (30%), vomiting (30%) and failure to thrive (20%) are more common in the pediatric population compared to only 3% adults with abdominal pain, however there may be a selection bias based on more aggressive evaluation of these symptoms in children compared to adults. The majority of the pediatric population will have a history of atopic conditions, such as asthma, allergic rhinitis, eczema, or atopic dermatitis[24]. Noel *et al*[13] found 57.4% of children with eosinophilic esophagitis had a history of rhinoconjunctivitis, 36.8% wheezing, 46% possible food allergy, and 73.5% a family history of atopy. Adults also may have a history of atopy, but this is not as prevalent as in children. Croese *et al*[20] found that 46% of adults with eosinophilic esophagitis had a history of atopy, and only 25% food allergy. No relation has been found to connective tissue diseases such as scleroderma, rheumatoid arthritis, or lupus. Uncommon symptoms include hematemesis, globus, and waterbrash.

Laboratory features have not been extensively reported in eosinophilic esophagitis, therefore sensitivity and specificity of laboratory tests are unknown. Peripheral blood eosinophilia range from 5%-50% in the adult population with eosinophilic esophagitis. Increased serum IgE, positive skin prick or radioallergosorbent test (RAST) may be found in 40%-73% of patients[6,20]. In a study of 26 children, 19 tested positive for skin prick testing, and 21/26 had positive patch testing[25]. Skin testing may therefore help to identify causative food agents. These cases of rather overt immediate hypersensitivity are often not apparent in the adult patient. Indeed, food allergies in childhood may not persist to adulthood. Limited studies are available on the use of these laboratory values for the diagnosis of eosinophilic esophagitis.

Radiological features

The most common diagnostic imaging test that to date has detected eosinophilic esophagitis is a barium study[26,27]. Zimmerman *et al* retrospectively assessed 14 patients with confirmed eosinophilic esophagitis and found 10 with strictures (mean length 5.1 cm), of whom 7 had multiple fixed ring-like indentations. Four patients had esophagitis, 10 hiatus hernia, and 9 with evidence of reflux[27].

Endoscopic features

The "feline esophagus", also known as the "corrugated esophagus", "ringed esophagus", or "concentric mucosal rings", is the classic endoscopic description of eosinophilic esophagitis (Table 2, Figure 1)[7,20]. A small caliber esophagus with a narrow fixed internal diameter, with or without a proximal esophageal stenosis, may also be the major feature[28,29]. Adherent white exudates, vesicles, or papules along with loss of vascular pattern may indicate focal areas

Table 2 Endoscopic features of eosinophilic esophagitis

Endoscopic feature	Description
Feline esophagus (corrugated, ringed esophagus)	Multiple concentric rings, may be fine in nature, web-like or thickened
Small calibre esophagus	Narrow, fixed internal diameter Featureless, unchanging column Poor expansion on air insufflation Proximal and/or distal stenosis
Adherent white papules	White exudates 1-2 mm in diameter which do not wash off (similar to candidiasis) Speckled patches Vesicles Loss of vascular pattern
Esophageal furrows	Vertical esophageal lines
Crêpe paper mucosa	Fragile esophageal mucosa Delicate, inelastic Mucosal abrasions or tear with minimal contact

Table 3 Histopathology of eosinophilic esophagitis

	GERD	Eosinophilic esophagitis
Eosinophilic infiltration (squamous epithelium)	<10/hpf	>20/HPF
Other features	Esophagitis (usually distal)	Esophagitis (proximal and/or distal, may be patchy or segmental)
	Intestinal metaplasia	Basal zone hyperplasia
		Increased papillary size
		Superficial eosinophilic layering or aggregates
		Microabscesses

GERD: Gastroesophageal reflux disease; HPF: high power field.

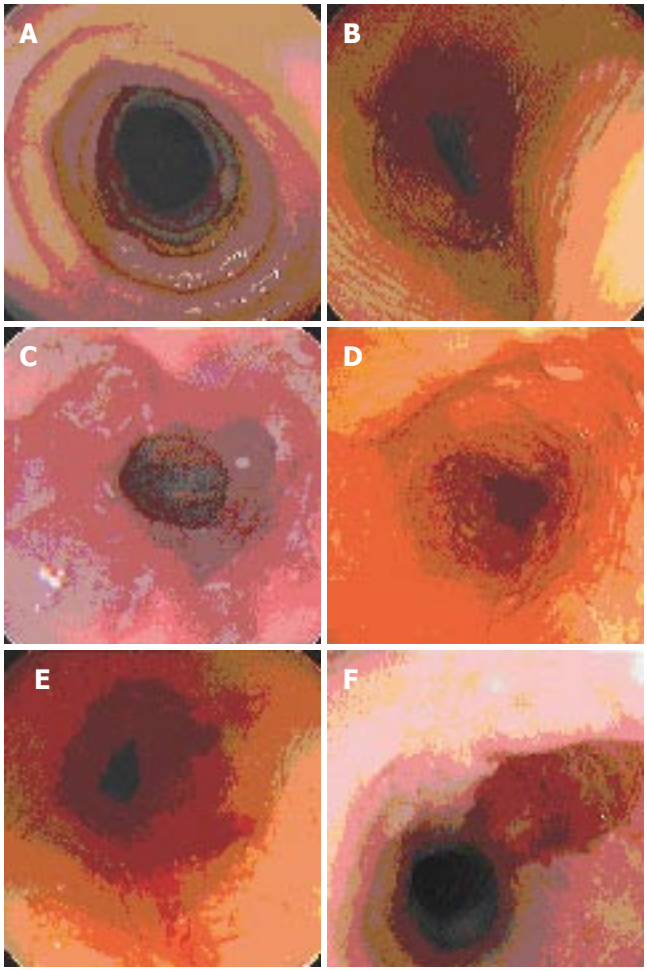

Figure 1 Classic endoscopic features of EE. **A**: Coarse, corrugated, narrow esophagus with papules; **B**: Fine feline esophagus; **C**: Distal esophageal stricture; **D**: Diffusely narrow esophagus with adherent white papule; **E**: Friable, crêpe paper mucosa with linear furrows; **F**: Large esophageal tear after biopsy.

of eosinophilic infiltration[30,31]. Vertical esophageal lines also may indicate eosinophilic esophagitis[32]. Finally, the esophageal mucosa may be fragile, or the so called "crêpe paper mucosa"[33]. This would explain the frequency of esophageal tears following dilation when treating the dysphagia associated with an apparently narrowed esophagus or its ringed structure (appearing like stricture). Thus the fragile esophagus is also characteristic. Endoscopic ultrasound, when performed, will show circumferential but asymmetric thickening of the muscularis propria[34]. The most common endoscopic findings in one relatively large series[35] were, in order of frequency: mucosal rings (81%), furrows (74%), strictures (31%), exudates (15%), small caliber (10%) and edema (8%). The endoscopic appearance is helpful but not diagnostic without a confirmatory biopsy. All patients with endoscopic features of eosinophlic esophagitis should have distal and proximal eosophageal biopsies to confirm eosinophilic esophagitis. Furthermore, this should be assessed prior to mechanical dilatation of strictures, as medical treatment for eosinophilic esophagitis should be the initial treatment. There have been no studies assessing the histopathological diagnosis of eosinophilic esophagitis in those with dysphagia and normal endoscopy. Therefore, it is unclear whether all these patients should have the proximal esophagus biopsied.

Histopathology

The diagnosis of eosinophilic esophagitis is dependent on eosinophilic infiltration of the squamous epithelium (Table 3). Although there is no consensus statement, most studies agree that >20 eosinophils per high power field (HPF) are diagnostic of eosinophilic esophagitis[6,36,37]. GERD can increase eosinophilic infiltration in the distal esophagus and therefore, mid or upper esophageal biopsies with increased eosinophils is more specific for eosinophilic esophagitis. Nevertheless, increased tissue eosinophils associated with GERD occur at a lower density <10/HPF[36,37] (Table 4).

Other features that are helpful but not essential for the diagnosis include basal zone hyperplasia, increased papillary size, and superficial layering of eosinophils with aggregates or microabscesses (aggregate of 4 or more contiguous eosinophils).

PATHOPHYSIOLOGY

Eosinophils originate in the bone marrow. When mature, only a small number circulate in the peripheral blood; rather they are predominantly tissue-dwelling cells. In health, other than hematopoietic sites, eosinophils only reside in the lamina propria of the gastrointestinal tract, the exception being the esophagus. Resident in the

Table 4 Differential diagnosis [40]

Primary	Idiopathic eosinophilic esophagitis Familial eosinophilic esophagitis Atopic esophagitis
Secondary: Eosinophilic related	Eosinophilic gastroenteritis Hypereosinophilic syndromes
Secondary: Non – eosinophilic related	GERD Recurrent vomitting Infection (helminth, parasitic, fungal) Esophageal GI stromal tumor Myeloproliferative disorders Carcinomatosis Allergic vasculitis Scleroderma Drugs/Iatrogenic

Table 5 Treatment regimens for eosinophilic esophagitis

Treatment option	Protocol
Elimination Diet	Avoidance of allergen depending on results of food allergy testing Oligoantigenic diet: Eliminate large number of suspected foods and allow limited nutritionally balanced diet Elemental diet: Various formulas such as Neocate (free amino acids, corn syrup solids, medium chain triglycerides)
Topical corticosteroids: Mayo Clinic protocol	Fluticasone 220 μg puffer 4 puffs BID x 6 wk, swallowed, no spacer Rinse mouth with water and spit out No food or drink for 3 h after dose
Systemic (oral) corticosteroids	Methylprednisolone 1.5 mg/kg per day (or equivalent dose prednisone) Divide into bid dosing for 4 wk then taper over 6 wk
Montelukast	Initial dose: 10 mg *po* daily Titration: Dose up to 100 mg/d depending on symptoms and tolerance Maintenance: Once symptoms relieved titrate down to minimal dose to maintain remission (usually 20 - 40 mg/d)
Mepolizumab	10 mg/kg *iv* infusion q 4 wk x 3 doses

BID: twice daily; PO: oral; IV: intravenous.

gastrointestinal tract, eosinophils normally do not evoke either an inflammatory reaction or tissue damage[38]. Under inflammatory conditions, eosinophils can infiltrate several organs (e.g., lung, esophagus and GI tract, and skin), playing a major role in causing tissue damage and organ dysfunction, and being mediators of allergic responses such as atopic dermatitis, allergic rhinitis, and asthma. Eosinophilic esophagitis characteristically has a dense eosinophilic infiltrate confined to esophageal tissues[21,36]. Activation of eosinophils results in degranulation, upregulated cytokine production, and IgE production. Recruitment and activation is regulated by cytokines including interleukin 5 (IL-5), eotaxin, interleukin 13 (IL-13), and interleukin 4 (IL-4)[38,39,40].

IL-5 is a critical cytokine for the differentiation and activation of eosinophils[38,41]. In eosinophilic esophagitis, a Th2 allergic response and production of IL-5 is key in recruitment of eosinophils to the esophagus. Mice devoid of IL-5 or lacking the receptor for IL-5 have a significant reduction in GI eosinophils. Overexpression of IL-5 can promote eosinophilic accumulation[38]. Eotaxin is constitutively expressed in the GI tract and has a critical role in eosinophilic recruitment[38]. Transgenic IL-5 mice deficient in eotaxin fail to recruit eosinophils to the GI tract. It seems to be more important for chemotaxis to the stomach and intestine[40,42]. Fujiwara, however, showed significant staining of eosinophils with anti-eotaxin-antibodies in patients with EE[43].

IL-13 is a profibrotic cytokine and its production has been demonstrated in eosinophils. Likely Th2 mediated, IL-13 contributes to an inflammatory response and bronchial hyperreactivity[10,44]. Intratracheal IL-13 was shown to induce eosinophilic esophagitis, linking pulmonary to esophageal eosinophilic inflammation[45]. It appears that IL-13 is a key mediator of eosinophilic inflammatory pathways and in the recruitment of eosinophils to the esophagus[10,45]. IL-13 may be a serologic indicator of systemic inflammation[46]. IL-13 induction of eosinophilic esophagitis seems to be dependent on IL-5, eotaxin, and STAT-6[45]. Interestingly, mepolizumab, an anti-IL-5 antibody, was shown to be beneficial for symptomatic and histologic improvement in eosinophilic esophagitis[47]. It is possible that the interaction of IL-13, IL-5 and eotaxin may be a component to the development of eosinophilic esophagitis. IL4 and IL-13 share a signal transduction pathway involving IL-4 receptor α chain and STAT-6[43]. IL-4 has been implicated in eosinophilic accumulation, regulating trafficking, and promoting adhesion to endothelial surfaces. Increased IL-4 secreting T cells in esophageal lesions were evident in one trial of patients with secondary eosinophilic esophagitis[48], but do not induce eosinophilic infiltration into the murine esophagus[43].

The net result of such chronic inflammation is irreversible structural change with loss of mucosal elasticity and the development of fibrosis in the subepithelial layers[21].

TREATMENT

The majority of reports on eosinophilic esophagitis are case reports or series. Therefore, randomized controlled trials (RCTs) for eosinophilic esophagitis treatment are not available. Indeed, a recent Cochrane review did not yield any RCTs, nor were the authors able to make any conclusions on benefits and harms of treatment regimens[49]. Treatment falls into two categories: (1) avoidance/removal of stimulation and (2) immune modulation. The majority of studies have been published in pediatric literature (Table 5). Avoidance of stimulation involves dietary changes with the elimination of foods or an elemental diet. Given that skin testing may help identify causative foods[25,27], this may help in avoidance of the specific culprit in some cases. Skin prick and skin patch testing may be more effective than skin prick testing alone. As shown by Spergel *et al*[25] in 26 children with documented eosinophilic esophagitis, 68 foods were identified in 19/26 by skin prick testing, and 67 foods in 21/26 by skin patch testing, for an average

of 2.7 foods per patient. With specific food avoidance, 18 had complete resolution and 6 partial improvement. Kelly *et al*[50] used an elemental diet in 10 children with eosinophilic esophagitis, and showed partial or complete resolution of symptoms in all 10. Markowitz *et al*[51] conducted a study in 346 children with chronic GERD symptoms of which 51 were eventually diagnosed with eosinophilic esophagitis. They were then given an elemental formula (Neocate 1+, SHS Northa America, Gaithersburg, MD) consisting of free amino acids, corn syrup solids, and medium chain triglyceride oil. Forty-eight patients were fed via a nasogastric tube, 49/51 patients improved symptomatically and there was a significant decrease in the number of eosinophils in the distal esophagus. Average time to improvement was 8.5 d. Unknown is if any of these measures of food avoidance or elemental diets are effective in adults.

Topical steroid therapy has been shown to be helpful in a number of uncontrolled case series reports for both the pediatric[52,53] and adult populations[54]. Arora *et al* treated 21 adult patients with eosinophilic esophagitis (diagnosed via solid food dysphagia, ringed esophagus, and eosinophils >20/hpf in mid to distal esophagus) with a 6 wk regimen of fluticasone 220 μg 4 puffs swallowed twice daily. All patients had complete symptomatic relief for at least 4 mo. The only side effect was dry mouth, with no oral candidiasis reported. Three out of 21 patients had relapse at 4 months and 50%-60% of patients had recurrence of symptoms at 1 year[6,54]. Systemic steroid therapy was first reported by Liacouras *et al* in the pediatric population[16]. Of 1809 patients with reflux, 20 had documented eosinophilic esophagitis and were treated with 1.5 mg/kg oral methylprednisolone divided twice daily for 4 wk. Steroids and anti-reflux medications, such as proton pump inhibitors, were then tapered and withdrawn after 6 wk. Thirteen out of 20 patients had a complete response and 6/20 marked clinical improvement (total 19/20 responders). Average time to improvement was 8 d. All had histologic evidence of improvement and a significant decrease in peripheral eosinophil counts and quantitative IgE levels. At 1-year follow-up, 10/20 were asymptomatic and 9/20 relapsed. Relapsers were treated with dietary changes, of which two required a second course of oral steroids. A randomized controlled trial comparing oral to inhaled corticosteroids is ongoing.

Leukotrienes promote eosinophilic trafficking, smooth muscle constriction, and mucous hypersecretion. Eosinophils generate large quantities of leukotriene C4, which is then metabolized to leukotriene D4 and E4 (LTD4 and LTE4 respectively). Montelukast is a selective inhibitor of the LTD4 receptor. Attwood *et al* reported 12 adult patients with dysphagia secondary to eosinophilic esophagitis and investigated the use of montelukast in 8/12 [55]. Patients were given an initial dose of montelukast 10 mg orally once daily and titrated up to a total of 100 mg daily. Once symptoms were relieved, dose was reduced to a "maintenance level" (20-40 mg/d). All patients were previously treated with proton pump inhibitors and 2 previously responded to corticosteroid treatment. All patients had symptomatic improvement, with only 2 having residual discomfort. Patients have been treated for a median of 14 months with no relapse. Six out of 8 experienced recurrence of symptoms within 3 wk of dose reduction or cessation. Important side effects were nausea and myalgias. Treatment did not change the density of eosinophils on repeat biopsy.

The central role of IL-5 in eosinophilic regulation and activation makes it a viable target for therapy. Mepolizumab is a humanized anti-IL-5 monoclonal antibody shown to be safe and effective in reducing sputum eosinophils in asthma but ineffective in outcome measures[56]. Garrett *et al*[47] performed an open label pilot study on 4 patients with hypereosinophilic syndromes, of which 3 had idiopathic hypereosinophilic syndrome and only 1 patient had eosinophilic esophagitis. This patient had dysphagia, esophageal narrowing on endoscopy with marked eosinophilia on biopsy, and was unresponsive to dietary elimination, topical, and oral corticosteroid treatment. Three doses of mepolizumab (10 mg/kg intravenous) infused at 4 wk intervals were given and patients followed for 18 wk after first infusion. Remarkable symptomatic improvement was achieved. Endoscopic and histologic improvement was seen at 4 wk after the last infusion. Peripheral eosinophils were reduced immediately after the first infusion and continued to the end of follow up. No serious adverse events were noted. No larger trials have been published. Other medications successfully used in eosinophilic gastroenteritis such as cromolyn and ketotifen (mast cell stabilizing medications), and suplatast tosilate (selective Th2 IL-4 and IL-5 inhibitor) have not been studied in eosinophilic esophagitis[19].

PROGNOSIS

Esposito *et al*[18] followed 7 children with eosinophilic esophagitis for 4 years, ages ranging from 6 months to 14 years old. All were treated with inhaled fluticasone. Two children experienced relapse at 1 year and 4 years post treatment, respectively, which improved with a second course of inhaled corticosteroid. Compliance was low in 2 patients and both had poor clinical and histologic response. Repeat treatment with appropriate dosing cured their symptoms. All children had normal growth after treatment. Interestingly, density of eosinophilic infiltrate was inversely proportional to age, and progressively reduced with time. This may explain the higher incidence in children compared to adults. Liacouras *et al*[16] found a 50% one year relapse rate after a course of oral steroids. Finally, Orenstein *et al*[14] found that 1/3 of patients were asymptomatic without any therapy.

Straumann *et al*[21] documented the natural history of eosinophilic esophagitis in 30 adult patients. Mean age was 40.6 and mean follow up time was 7.2 years. None were treated with dietary changes or medical therapy. Only those with severe and frequent attacks were treated with dilatation. No patients died and all were in "good health" with maintenance of body weight. Twenty-nine of 30 (96.7%) patients had dysphagia throughout follow up: 7 experienced increasing dysphagia, 11 persistent but stable dysphagia, 11 decreasing dysphagia, and 1 complete resolution. Eleven out of 30 required dilatation, of which 10 had reduction or cure of dysphagia. In terms of the impact of

dysphagia on quality of life, 1/30 reported a significant negative impact on socioprofessional activities, 15/30 minor, and 14/30 reported no significant impact. No increased risk of malignancy was found and no eosinophilic gastroenteritis was documented. It appears that in the adult population disease tends to be stable with no significant effect on morbidity or mortality, at least for up to 11 years' follow up. Whether a persistent inflammatory state will affect motility, mechanical obstruction, inflammatory bowel disease, malignancy, or mortality in the long term has yet to be seen.

SHORTFALLS

A lack of consensus for diagnosis hinders research progress in eosinophilic esophagitis. Case descriptions to date have used variable cutoffs for eosinophilic infiltration ranging from >15/HPF to >30/HPF. This not only affects the epidemiological data for incidence and prevalence, but also affects the inclusion/exclusion into trials and histological response to therapy. Furthermore, no distinction is made between proximal and distal esophageal biopsies, which might influence the pathologist into the diagnosis of esophagitis secondary to reflux rather than idiopathic eosinophilic esophagitis. Biopies are essential for the accurate diagnosis of eosinophilic esophagitis and are best taken from the proximal esophagus to better distinguish this entity from reflux esophagitis, even though the latter has a less dense eosinophilic infiltrate.

No objective criteria have been developed to assess the response to treatment. All studies to date have employed subjective improvement in symptoms, which is prone to bias. No randomized controlled trials to date have validated the efficacy of any treatment regimen. Elimination diets are inconvenient and result in low compliance. Topical steroid treatment seems safe[54] and may be the most convenient and effective therapy in adults, though technique may be an issue. Oral corticosteroid has its myriad of side effects and complications. Montelukast may be effective but would be long-term treatment given its relapse rates off treatment. Given its benign natural history in which the majority of patients have minor or no impact on quality of life, these therapies should be validated with vigorous clinical trials that include an analysis of cost effectiveness.

Finally, what should be done with the non-responsive patient? Compliance should definitely be confirmed. Repeat treatments may be beneficial. Combination therapy has yet to be explored. New biologic agents may be beneficial, but efficacy needs to be confirmed, and cost will be a limiting factor.

CONCLUSION

Since its original description, most publications have emphasized the clinical and histopathological presentation of eosinophilic esophagitis. The epidemiology is being better understood as clinicians are recognizing this unique disease entity. Diagnostic criteria are evolving which will improve the quality of future research. A consensus on the diagnosis of eosinophilic esophagitis is urgently needed. Despite a plethora of case reports, case series, case cohorts and reviews, randomized placebo-controlled trials are needed to confirm the efficacy of treatment regimens. In adult patients, topical corticosteroid appears to be the most convenient and efficacious treatment. As the pathophysiology of eosinophilic recruitment and activation in the esophagus is further elucidated, future treatment targets are possible. The long term natural history and response to treatment is awaited. Eosinophilic esophagitis may be a relatively new entity, undoubtedly overlooked in the past but this disease is here to stay. With better recognition, it has moved into the forefront of esophageal diseases.

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Helicobacter infection in hepatocellular carcinoma tissue

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Abstract

AIM: To investigate whether *Helicobacter* species (*Helicobacter* spp.) could be detected in hepatocellular carcinoma (HCC) tissue.

METHODS: Liver samples from 28 patients with hepatocellular carcinoma (HCC) diagnosed by histopathology were studied. Twenty-two patients with other liver diseases (5 with liver trauma, 7 with cavernous liver hemangioma, 6 with liver cyst and 4 with hepatolithiasis), 25 patients with gastric cancer, 15 with colonic cancer and 15 with myoma of uterus served as controls. Two pieces of biopsy were obtained from each patient. One was cultured for *Helicobacter* spp. and extraction of DNA, the other was prepared for scanning electron microscopy (SEM) and *in situ* hybridization. The samples were cultured on Columbia agar plates with microaerobic techniques. *Helicobacter* spp. in biopsy from the studied subjects was detected by polymerase chain reaction (PCR) with *Helicobacter* spp. 16S rRNA primers. Amplified products were identified by Southern hybridization and sequenced further. Besides, other genes (*vacA, cagA*) specific for *Helicobacter pylori* (*H pylori*) were also detected by PCR. *Helicobacter* spp. in biopsies was observed by SEM. Transmission electron microscopy (TEM) was performed to identify the cultured positive *Helicobacter* spp. The presence of *Helicobacter* spp. was detected by *in situ* hybridization to confirm the type of *Helicobacter*.

RESULTS: The positive rate of *Helicobacter* cultured in HCC and gastric cancer tissue was 10.7% (3/28) and 24%(6/25), respectively. *Helicobacter* microorganisms were identified further by typical appearance on Gram staining, positive urease test and characteristic colony morphology on TEM. The bacterium was observed in adjacent hepatocytes of the two HCC samples by SEM. The number of cocci was greater than that of bacilli. The bacterium was also found in four gastric cancer samples. PCR showed that the positive rate of HCC and gastric cancer samples was 60.7% and 72% respectively, while the controls were negative ($P < 0.01$). The PCR-amplified products were identified by Southern hybridization and sequenced. The homology to 16S rRNA of *H pylori* was 97.80%. The samples were verified by *in situ* hybridization for *Helicobacter* spp. 16S rRNA–mRNA and proved to be *H pylori* positive. There was no statistical significance between HCC and gastric cancer ($P > 0.05$), but the positive rate of HCC and controls had statistical significance ($P < 0.01$). Only 3 HCC samples and 2 gastric cancer samples of the cagA genes were detected. None of the samples reacted with primers for vacA in the two groups. As for the genotype of *H pylori*, type II had preference over type I.

CONCLUSION: *Helicobacter* infection exists in liver tissues of HCC patients. *Helicobacter* spp. infection is related with HCC, which needs further research.

Key words: Hepatocellular carcinoma; *Helicobacter* infection

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INTRODUCTION

The profound impact of HCC on human health is known worldwide[1]. In China, the death rate ranks the third in malignant tumors. Persistent hepatitis B virus (HBV) and hepatitis C virus (HCV) infection and aflatoxins are the main causes of HCC[2]. The real risk factors for HCC may be far more than the known causes. A new infectious agent, *Helicobacter hepaticus* (*H hepaticus*) causing chronic active hepatitis and associated liver tumors has been described by Ward *et al*[3]. Recently, other *Helicobacter* species (*Helicobacter* spp.), including *H pylori* and other bacteria associated with the pathogenesis of gastric[4-7] and extradigestive manifestations[8,9], have been detected in the liver of patients suffering from cholestatic diseases and HCC arising from non-cirrhotic liver[10,11]. We have previously reported a high frequency of *Helicobacter* spp.

in the liver of patients with HCC[12]. *Helicobacter* spp. DNAs have been detected in paraffin-embedded tissue sections of HCC by PCR[13]. Whether *Helicobacter* spp. promotes liver tumors or acts as a cofactor in the process of carcinogenesis in humans with hepatitis virus needs further research. This study was to determine whether *Helicobacter* spp. could be detected in HCC tissue and to investigate the potential significance of *Helicobacter* in HCC carcinogenesis.

MATERIALS AND METHODS

Samples

All samples were immediately frozen in liquid nitrogen and stored at -80℃ before testing. Liver samples from 28 patients with HCC (25 males, 3 females, mean age 54 years) were studied. Twenty patients (18 males, 4 females, mean age 48 years) with other liver diseases (5 with liver trauma, 7 with cavernous liver hemangioma, 6 with liver cyst and 4 with hepatolithiasis), 25 with gastric cancer (20 males, 5 females, mean age 61 years), 15 with colonic carcinoma (9 males, 6 females, mean age 55 years) and 15 with myoma of uterus (mean age 49 years) served as controls. Two pieces of biopsy were obtained from each patient, one was cultured for *Helicobacter* spp. and extraction of DNA, the other was prepared for scanning electron microscopy (SEM) and *in situ* hybridization.

Germ culture

To culture *Helicobacter* spp., the diluted homogenates of biopsy specimens were smeared on the surface of Columbia agar plates (Oxoid Company, France) supplemented with 7% sheep erythrocytes, 6 μg/mL vancomycin, 2 μg/mL amphotericin B and 0.32 μg/mL polymyxin B. The plates placed in an anaerobic jar together with a GENbox microaer paper sachet (Biomerieux, Marcy l'Etoile, France) were incubated to generate a microaerophilic environment containing 5 mL/L oxygen, 10 mL/L CO_2, and 85 mL/L N_2), then the biopsy specimens were incubated at 37℃ for 7 d under a humid condition. *Helicobacter* colonies were identified further by their typical morphology, characteristic appearance on Gram staining, positive urease test. PCR was performed to examine the 16S rRNA, cagA and vacA genes of *Helicobacter* spp. The PCR products were sequenced.

Detection of 16S rRNA, vacA and cagA genes

Approximately 5 mm × 5 mm × 5 mm of tissue was cut and lysed in 0.25% pancreatic RNase, 0.2% collagen enzyme and 0.1 mg/mL proteinase K (Sigma, St Louis). The proteins were extracted with phenol:chloroform, and the genomic DNA was recovered by precipitation with ethanol. Initially, the samples were amplified by *Helicobacter* spp. 16S rRNA primers as previously described[13]: sense primer: 5'-AAC GAT GAA GCT TCT TCT AGC TTG CTA G-3' (28 bp); antisense primer: 5'-GTG CTT ATT CGT TAG ATA CCG TCA T-3' (25 bp) (Shanghai BioAsia Biotechnology Co., Ltd, China). The forward and reverse primer amplified a product of approximately 400 bp. Thirty-five cycles of amplification were performed, each consisting of an initial denaturation at 94℃ for 4 min, followed by denaturation at 94℃ for 1 min, primer annealing at 55℃ for 1.5 min, extension at 72℃ for 2 min, and a final extension step at 72℃ for 10 min. *H pylori* (NCTC11637) was used as the positive control and double-distilled water was used as the negative control. The samples generated a positive result in *Helicobacter* spp. PCR was subsequently performed with another two different sets of primers. A primer pair amplifying a 352-bp product, based on the partial DNA sequence of a species-specific gene encoding *vacA* of *H pylori* was previously described elsewhere[14]. Primers based on *cagA*, amplifying a 297-bp product, were used as previously described [15], including sense primer: 5'-GGA GCC CCA GGA AAC ATT G-3'; antisense primer: 5'-CAT AAC TAG CGC CTT GCA C-3'; sense primer: 5'-ATA ATG CTA AAT TAG ACA ACT TGA GCG A-3'; antisense primer: 5'-TTA GAA TAA TCA ACA AAC ATC ACG CCA T-3'. The *vacA* and *cagA* genes of *H pylori* (NCTC11637) were used as the positive control and double-distilled water was used as the negative control.

Southern hybridization

In order to prove the characteristics of *Helicobacter* spp. 16S rRNA, Southern hybridization was carried out using probes for *Helicobacter* spp., *H pylori*, *H hepaticus*, and *H fennellia* (Beijing AoKe Biotechnology Co. Ltd, China). These probes were designed using Battle software: *Helicobacter* spp. 16S rRNA-cDNA probes: 5'-CGC CGC GTG GAG GAG GAT GAA GGT TTT AGG ATT GTA-3' (36bp): *H pylori* 16S rRNA-cDNA probes: 5'-GAG GGC TTA GTC TCT-3' (15bp); *H hepaticus* 16S rRNA-cDNA probes: 5'-CCT TGC TTG TCA GGG -3' (15bp); and *H fennellia* 16S rRNA-cDNA probets: 5'-CCT TGC TTG ACA GGG-3'(15bp). Hybridization was performed by the above-mentioned probes using the digoxygenin DNA labeling kit (Boehringer Mannheim Company) according to the manufacturer's instructions, then the PCR products were transferred to a nylon membrane (Amersham, Buckinghamshire, United Kingdom) with the capillary blotting technique. The membrane was prehybridized, hybridized, then anti-digoxin (1:5000) and CSPD were added. The bound probes were finally detected by autoradiography after 2 h at room temperature (Kodak Scientific Imaging Film, XK-1 REF 6535009, Rochester, New York).

Sequence analysis of PCR products

The PCR products were purified from agarose gels by the JETsorb DNA extraction kit (Genomed, GmbH, Bad Oeynhausen, Germany). The purified products were sequenced and analysed (Dalian Bao biotechnology Co., Ltd, China). The sequences were compared with the known 16S rRNA of *Helicobacter spp.* and the other bacteria using the GenBank.

Scanning microscopy and in situ hybridization

The appropriate specimens were examined by SEM (JEOL JSM-840). For each hybridization reaction, the biopsy specimens were frozen, cut into 4 μm thick sections using

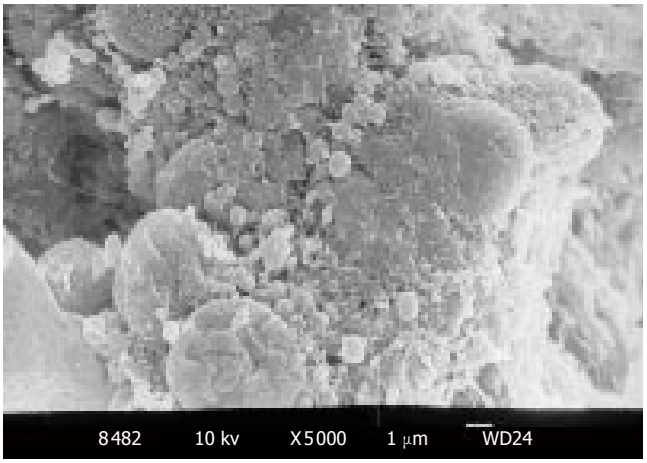

Figure 1 Cocci in adjacent hepatocytes (SEM × 5000).

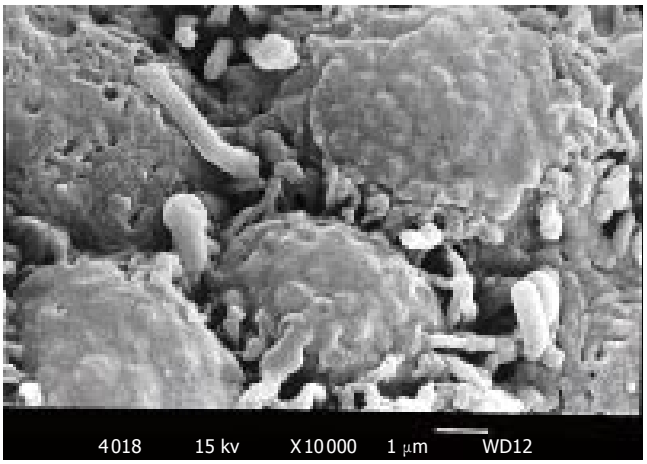

Figure 2 Spiral-shaped bacteria in vicinity of pit cell lineage (SEM × 10 000).

a cryomicrotome (Leica, Wetzlar, Germany) and placed on glass slides. The slides were placed in 4% polyformaldehyde for 20 min, washed and treated with proteinase K (100 μg/mL) at 37℃ for 30 min, washed again and marinated with 0.2% glycin for 10 min, and finally washed with 2 × SSC for 5 min. The sections were covered with 20 μL of 4 kinds of probes mentioned above. The slides were then hybridized overnight at 60 ℃ in a humid chamber and washed with SSC. The sections were incubated in protein blocking buffer. After washed, the slides were incubated with anti-digoxin-alkaline phosphatases complex (1:3000) for 1 h at 37 ℃ and marinated with buffer. After marinated, the slides were counterstained with DAPI overnight, marinated with double-distilled water for 5 min, dehydrated in a graded series of ethanol, hyalined with xylene, covered with balata and observed under microscope.

Statistical analysis

The results of PCR and hybridization were analyzed. $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of germ culture

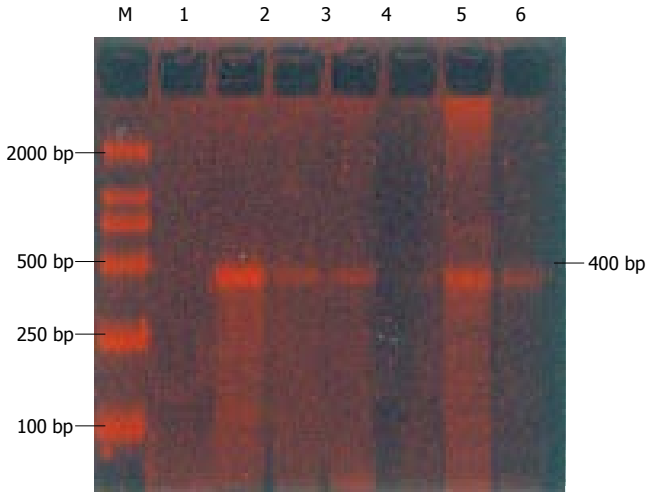

Figure 3 Analysis of *Helicobacter* spp. PCR products from HCC samples. The 400-bp fragments were analyzed by 1.5% agarose gel electrophoresis. Lane M: nucleotide marker; lane 1: negative control (double-distilled water); lane 2: positive control (*H pylori* DNA); Lanes 3, 4, 6: positive samples.

Helicobacter microorganisms were identified by typical appearance on Gram staining, positive urease test and characteristic colony morphology on TEM. After cultured for 48 h, the small and grey colonies were detected in Columbia agar plates, and the mean colony diameter was 1 mm. *Helicobacter* was cultured in 3 HCC and 6 gastric cancer tissue specimens. The positive rate was 10.7% and 24%, respectively. The bacterium was positive in urease test. TEM showed that the bacterium could be divided into two types, one was bacillus with flagellum, the other was coccus with flagellum. All the flagelli were located in one side.

Scanning microscopy

The cocci were observed by SEM in adjacent hepatocytes of 28 HCC samples, and the mean diameter cocci was 0.7 - 0.8 μm (Figure 1). In the 25 gastric cancer samples, 4 were found to have spiral-shaped bacteria (2.0-4.0 μm in length and 0.5-1.0 μm in width). The spiral-shaped bacteria partly adhered to the surface of gastric epithelial cells (Figure 2).

PCR of 16S rRNA,vacA and cagA

The positive rate of HCC and gastric cancer tissue samples was 60.7% and 72%, whereas no sample was positive in the other groups. The size of PCR products corresponded to the expected 400 bp (Figure 3). The positive rate of HCC and gastric cancer tissue samples had no significant difference ($P > 0.05$). Amplified products were identified by Southern hybridization. Besides, the other genes (vacA, cagA) specific for *H pylori* were also detected. The sizes of PCR fragments generated with the vacA (352 bp) and *cagA* primers (297 bp) corresponded to the expected sizes. The cagA gene was detected only in 3 HCC and 2 gastric cancer samples (Figure 4). None of the samples reacted with primers for *vacA* in the two groups.

Sequence analysis and in situ hybridization

The amplified products were sequenced (Figure 5) and compared with *Helicobacter* spp. The *Helicobacter* spp.

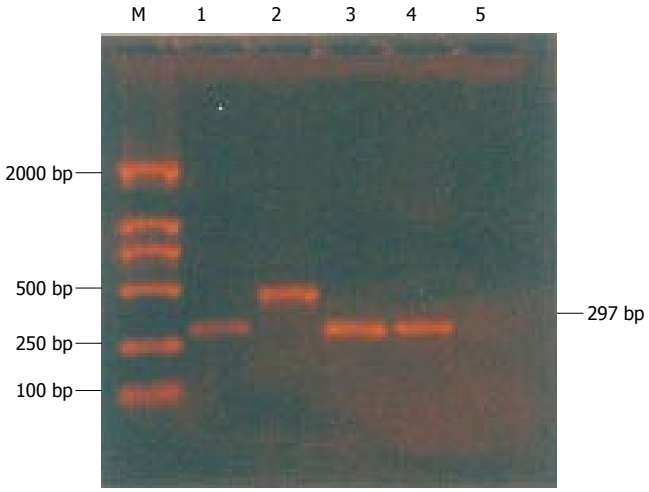

Figure 4 Analysis of *vacA* and *cagA* PCR products from HCC samples. The 352-bp and 297-bp fragments were analyzed by 1.5% agarose gel electrophoresis. Lane M: nucleotide marker; lane 1: positive control (*cagA* DNA); lane 2: positive control (*vacA* DNA); Lanes 3, 4: *cagA* positive samples; lane 5: negative control (double-distilled water).

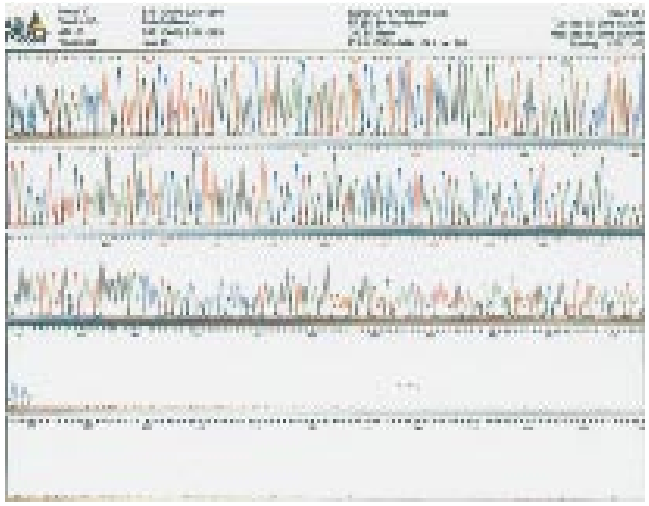

Figure 5 Sequencing results of 16S rRNA in *helicobacter* genus-positive products from HCC.

searched from the GenBank, includeed *H hepaticus, H bilis, H felis, H canis, H muridarum, H mustelae, H nemestrine, H fennelliae, H cinaedi, H acinoyx* (Figure 6). The homology of base pair and amino acid sequence was 97.8% between the amplified products and *H pylori* and 80.9% in *H fennelliae*.

In situ hybridization showed that *Helicobacter* spp. 16S rRNA-mRNA was positive in 17 patients with HCC-with a positive rate of 60.7%. The black-brown granules distributed in cholangioles of hepatocytes were positive hybridization signal. The positive signal was also found in intracellular fluid close to the hepatocyte membrance. All the granules were dispersedly distributed. The number of granules was not different in carcinoma and its adjacent tissues. Seventeen patients with gastric cancer were positive and the positive rate was 68%. The positive granules residing within the epithelium plasma membrane were symmetrically distributed and the number of the granules was not different in carcinoma and its adjacent tissues. *Helicobacter* spp. was negative in patients with colonic cancer (except for one case) and in those with other liver diseases and myoma of uterus.

Table 1 *Helicobacter* detection by different probes

Group	cDNA probes				
	Helicobacter spp.	*H pylori*	*H hepaticus*	*H fennellia*	*P*
HCC	17/28	17/28	0	0	
Gastric cancer	17/25	17/25	0	0	0.40[a]
Other liver diseases	0/22	0	0	0	0
Colonic cancer	1/15	1	0	0	0
Myoma of uterus	0/15	0	0	0	0

[a]$P > 0.05$ *vs* HCC group and gastric cancer group.

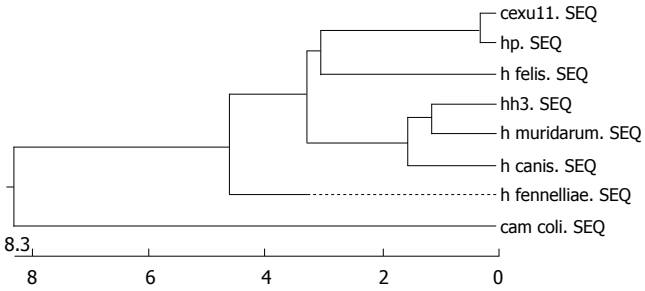

Figure 6 Genic phylogenetic tree of *Helicobacter* spp. and deduced protein.

The positive rate of *Helicobacter* spp. 16S rRNA-mRNA was comparable in HCC and gastric cancer patients with no statistical significance ($P > 0.05$). When HCC and gastric cancer patients were compared with those with other liver diseases, there was a statistical significance($P < 0.01$, Table 1).

In order to confirm the type of *Helicobacter*, the probes for *H pylori*, *H hepaticus* and *H fennellia* were used to detect the positive samples of *Helicobacter* spp. 16S rRNA-mRNA by *in situ* hybridization. The result revealed that the hybridization by *H pylori* probe was consistent with that by *Helicobacter* spp. probe. The positive rate was 60.7% in HCC patients and 68% in gastric cancer patients while being negative in patients with other liver diseases.

DISCUSSION

H pylori is one of the most common bacteria worldwide found in more than 50% of human population[16]. The presence of *H pylori* is the main cause of several gastroduodenal diseases, including peptic ulcer[4,6], gastric cancer[17], and gastric MALT lymphoma[18], and has been designated as class I carcinogen by the World Health Organization[19]. Chronic *H pylori* or *Helicobacter* spp. infection is related with a variety of extragastric diseases, including ischaemic heart disease, liver disease, skin disease, blood disorder and others[8]. The relationship between *Helicobacter* spp. infection and liver disease in humans needs to be further studied. Fan *et al*[20] demonstrated that the seroprevalence of *H pylori* is high in Chinese

patients with HBV-related chronic hepatitis. Experimental infection with *H hepaticus* in mice causes chronic hepatitis and HCC[3]. Thus *H hepaticus* infection of mice provides a uniquely valuable animal model for exploring the mechanisms underlying liver cancer[21]. Avenaud *et al*[11] have demonstrated the presence of genomic sequences of *Helicobacter* spp. in patients with HCC. Ponzetto *et al*[22] reported that the *cagA* gene can be obtained from liver tissue of cirrhotic patients with HCC. By PCR, hybridization and partial DNA sequencing, Nilsson *et al*[10] found that *Helicobacter* genus-specific primers are positive in patients with PBC or PSC. Furthermore, the gene sequence obtained from positive PCR of *Helicobacter* spp. 16S rRNA is usually analogous to *H pylori*. *Helicobacter* spp. has been successfully cultured in liver samples from patients with Wilson's disease by Queiroz and Santos[23]. The isolate is closely related to *H pylori* by biochemical and 16S rRNA analysis. There is evidence that hepatobiliary *Helicobacter* spp. may exist in liver of severe HCV-infected and HCC patients[19]. High rates of human HCC are reported in Southeast Asia, which are caused by hepatobiliary *Helicobacter* spp. and other environmental agents[24]. Pellicano *et al*[25] reported that the presence of genomic sequences of *H pylori* is infrequent in patients who have undergone surgery for metastasis of colon cancer to the liver. In contrast, the presence of genome of *Helicobacter* spp. is higher in patients with HCV-related cirrhosis and HCC. Huang *et al*[26] reported that *Helicobacter* spp. 16S rDNA can be found in patients with primary liver carcinoma. Verhoef *et al*[27] found that gastric colonization with a specific subset of *Helicobacter* strains is associated with the induction of HCC, either directly via colonization of the liver or indirectly via secretion of specific toxins by *Helicobacter* residing in the stomach. Rocha *et al*[28] reported that the presence of *Helicobacter* species DNA in liver is associated with hepatitis C. These observations prompted us to explore a possible association between *Helicobacter* spp. and HCC in Chinese patients.

In this study, the relationship between *Helicobacter* spp. and HCC was investigated by isolation and culture of strains from biopsies, electron microscope, PCR, hybridization techniques and partial DNA sequencing assay. The potential significance of *Helicobacter* spp. in HCC carcinogenesis was investigated. *Helicobacter* spp. was 10.7% (3/28) and 24% (6/25) cultured in HCC and gastric cancer tissue samples, respectively. *Helicobacter* microorganisms were identified by their typical appearance on Gram staining, positive urease test and characteristic colony morphology on transmitting electron microscopy. The bacterium was observed by SEM in the adjacent hepatocytes of HCC samples. The number of cocci was greater than that of bacilli. Our study showed that *Helicobacter* spp. DNA could be found in liver tissue from 60.7% patients with HCC, which might be related to a variety of environmental factors, host characteristic bacteria virulence determinants, and the small size of samples. Eighteen of 25 (72%) liver samples from patients with gastric cancer were positive by PCR analysis using *Helicobacter* spp. primers. Patients with HCC and gastric cancer had a higher positivity with no statistical significance ($P > 0.05$), suggesting that *Helicobacter* spp. infection is closely related with diseases of the digestive system. Amplified products were identified by Southern hybridization, suggesting that *Helicobacter* spp. infection might occur in HCC patients. Sequencing showed that PCR-amplified products and *H pylori* had a 97.8% homology. To make sure that the bacteria was *H pylori*, the vacuolating cytotoxin gene A (*vacA*) and cytotoxin-associated gene A (*cagA*) specific for *H pylori* were also detected by PCR. *H pylori* can be divided into $cagA^+$- *H pylori* and $cagA^-$-*H pylori* in clinical medicine, leading to a different result in gastrointestinal disease. $CagA^-$-*H pylori* usually colonizes the mucous gel or the apical epithelial surface, whereas $cagA^+$- *H pylori* colonizes the immediate vicinity of epithelial cells or the intercellular spaces[29]. Strains possessing the *cag* pathogenicity island are more likely to cause disease rather than those lacking this locus[30]. The *vacA* and *cagA* help gastric epithelial cells to form vacuolation, injury, necrosis, ulcer, etc. *H pylori* strains possessing *cagA* are associated with the development of peptic ulcer, gastric cancer and extragastric diseases. Most of the strains could be divided into two major types. Type Ⅰ bacteria have the gene coding for cagA and express CagA and VacA. Type Ⅱ bacteia do not express either cagA or vacA[11]. Therefore, the difference between type Ⅰ and type Ⅱ bacteria is due to toxicity. In our study, the cagA gene was detected in only 3 HCC and 2 gastric cancer samples (Figure 4). None of the samples reacted with primers for vacA in the two groups. As for the genotype of *H pylori*, type Ⅱ bacteria have preference over type Ⅰ, which is consistent with the report of Avenaud *et al*[11].

To confirm the *Helicobacter* spp. distribution in HCC patients, *Helicobacter* spp. 16S rRNA-mRNA was detected by *in situ* hybridization with a positive rate of 60.7%, which was coincident with the result by PCR. Besides, the positive rate in gastric cancer patients was 68%. The positive granules residing within the epithelium plasma membrane were distributed symmetrically. *Helicobacter* spp. was negative in patients with colonic cancer except for one case and those with other liver diseases and myoma of uterus. The positive rate in HCC and gastric cancer patients had no statistical significance ($P > 0.05$). When HCC patients were compared with those with other liver diseases, there was no statistical significance ($P < 0.01$).

In conclusion, *Helicobacter* spp. infection is related with diseases of the digestive system, especially HCC and gastric cancer. Further studies are needed to establish the role of *H pylori* in HCC.

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Recombinant hybrid protein, Shiga toxin and granulocyte macrophage colony stimulating factor effectively induce apoptosis of colon cancer cells

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Abstract

AIM: To investigate the selective cytotoxic effect of constructed hybrid protein on cells expressing granulocyte macrophage colony stimulating factor (GM-CSF) receptor.

METHODS: HepG2 (human hepatoma) and LS174T (colon carcinoma) were used in this study. The fused gene was induced with 0.02% of arabinose for 4 h and the expressed protein was detected by Western blotting. The chimeric protein expressed in *E.coli* was checked for its cytotoxic activity on these cells and apoptosis was measured by comet assay and nuclear staining.

RESULTS: The chimeric protein was found to be cytotoxic to the colon cancer cell line expressing GM-CSFRs, but not to HepG2 lacking these receptors. Maximum activity was observed at the concentration of 40 ng/mL after 24 h incubation. The IC_{50} was 20 ± 3.5 ng/mL.

CONCLUSION: Selective cytotoxic effect of the hybrid protein on the colon cancer cell line expressing GM-CSF receptors (GM-CSFRs) receptor and apoptosis can be observed in this cell line. The hybrid protein can be considered as a therapeutic agent.

Key words: Shiga toxin; hGM-CSF; Apoptosis; Colon cancer; Cell lines

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INTRODUCTION

Although systemic treatment of colon cancer including combination of surgery, hormone therapy, chemotherapy is commonly believed to be effective in prolonging patient survival, colon cancer remains one of the principal cancer-related deaths[1]. Human cancer is becoming more definable by cell surface proteins. One of these molecules is GM-CSF receptor that expresses in several tumors including colon cancer[2].

GM-CSF is used to attenuate the myelosupressive effects of chemotherapy in the treatment of hematologic malignancies and solid tumors, but GM-CSF usually does not stimulate growth of solid tumors[2]. It was reported that GM-CSFR is absent on most immature hematopoietic progenitors but increases expression during maturation[3]. This finding strongly suggests that GM-CSFR may be a useful target for recombinant toxins or immunotoxins.

Immunotoxin comprises cells targeting and killing moieties. Therefore, bacterial or plant toxins are used as the killing moieties. Shiga and Shiga-like toxins (STX, SLTs) are ribosome-inactivating proteins (RIPs) produced by *Shigella* and *E.coli*, composed of an enzymatic A subunit non-covalently associated with a pentamer receptor-binding subunit. SLTs inhibit protein synthesis in eukaryotic cells by releasing adenine residue from the highly conserved aminoacyl-tRNA-binding site which exists on large subunit of ribosome [4]. In this study, the catalytic domain of Shiga toxin (StxA1) and hGM-CSF were genetically fused and expressed in *E.coli*. Then, the function of recombinant protein was assessed on colon cancer cell line.

MATERIALS AND METHODS

Preparation of purified recombinant chimeric protein (StxA1-GM-CSF)

The catalytic domain of Shiga toxin, StxA1, was fused

teria with 0.02% arabinose for 4 h incubation. The expressed protein was purified following the manufacturer's instructions (Invitrogen, USA). The amount of protein was estimated by Bio-Rad protein assay kit (Bio-Rad, USA).

Cell culture

HepG2 cell line derived from human hepatoma and LS174T cell line derived from colon carcinoma were provided by Cell Bank of Institute of Development, Aging and Cancer, Tohoku University, Japan. These cell lines were kept in RPMI-1640 medium (Gibco-BRL, Germany) supplemented with 10% fetal bovine serum (Gibco-BRL , Germany) at 37 ℃ in 50 mL /L CO_2 atmosphere.

Cytotoxicity assay

The cytotoxic effect of SltxA1-GM-CSF was determined by trypan blue exclusion assay and MTT[5] assay. For trypan blue dye exclusion assay, 2.5×10^4 cells were seeded in one well of 96-well plates and different concentration (10-160 ng) of SltxA1-GM-CSF was added to each well. After incubation for 24, 48, and 72 h, the number of viable cells was determined.

For MTT assay, HepG2 and LS174T cell lines were cultured and StlxA1-GM-CSF was added as described above. After 24, 48 or 72 h incubation, 10 μL of 0.5 mg/mL of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, Japan) was added to each well and further incubated at 37 ℃ in 50 mL/L CO_2 atmosphere for 4 h to allow MTT to be converted to formazon crystals by reacting with metabolically active cells. Reactions were stopped by addition of 10% SDS in 0.01 mol/L HCl and absorbance at 570 nm was determined.

Neutral comet assay

Neutral comet assay was carried out as previously described[6] with some modifications. Briefly, freshly prepared cell suspension (2×10^3 / 10 μL) was mixed with 150 μL of 1% low-melting agars. The mixture was layered on top of the microscopic slide coated by 1% agarose. After low melting agarose was solidified in a refrigerator for 10 min, the slide was gently immersed in a freshly prepared lysing solution (2% SDS, 0.03 mol/L EDTA) for 30 min protected from light. After the slides were washed with TBE buffer, electrophoresis was carried out at 25 V for 25 min. Then, comets were visualized with 1 mmol/L propodium iodide. One hundred and fifty cells per slide were analysed under fluorescent microscope.

Hoechst staining

After administration of SltxA1-GM-CSF, cells were fixed with 1% paraformaldehyde for 30 min, washed with PBS and stained with 1mmol/L Hoechst 33342 (Sigma, Japan) for 10 min. Nuclear morphology of at least 400 cells was randomly observed under fluorescent microscope.

Statistical analysis

The results were expressed as mean ± SD and each value represented the mean of three experiments. Differences between groups were compared using the Student's *t* test. $P < 0.05$ was taken as significant.

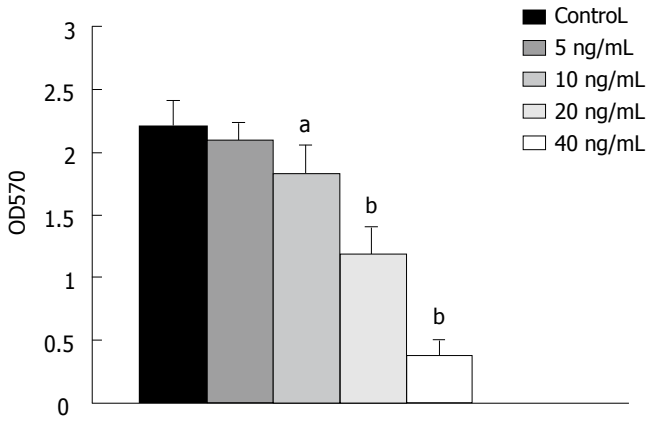

Figure 1 Effect of different concentrations of StxA1-GM-CSF on LS174T cell line by MTT assay. ($^aP < 0.01$, $^bP < 0.001$ *vs* trypan blue exclusion assay).

RESULTS

Expression and characterization of StxA1-GM-CSF

The fusion protein consisting of 1-255 a.a. of A1, the catalytic domain of Shiga-toxin and 1-127 a.a. of hGM-CSF was observed. The recombinant was analysed by SDS-PAGE and Western blot as previously described[7].

Cytotoxicity

Cytotoxicity of hGM-CSF to cells was analysed by both trypan blue exclusion assay and MTT assay. LS174T cells showed the extreme susceptibility to StxA1-GM-CSF (Figure 1), while HepG2 cells lacking GM-CSFR showed very poor susceptibility to StxA1-GM-CSF. Maximum activity was observed at the concentration of 40 ng/mL ($P < 0.001$) after 24 h incubation. Higher concentration and longer treatment time did not show any profound effect. The IC_{50} was 20 ± 3.5 ng/mL.

Apoptosis

We next examined death of LS174T cells induced by the StxA1-GM-CSF by comet assay. In comet assay apoptotic cells after treatment showed puffy tails and pin heads while normal cells had spherical heads but no tails (Figure 2). To verify the apoptotic results obtained by comet assay, cells were analyzed by Hoechst 33342 staining. Apoptotic cells with nuclear condensation and fragmentation were analyzed (Figure 3). The results indicated that the apoptotic cells were effectively induced by the toxin (Figure 4).

DISCUSSION

In this study we constructed the recombinant protein of StxA1-GM-CSF by overlap extension PCR, which was expressed in pBAD/gIIIA system[7]. Al-Jaufy *et al*[8] have also made the recombinant protein genetically. They fused StxA with HIV gp120-binding domain of CD4 by restriction enzyme sites. However, some limiting factors like incomplete digestion, absence of suitable restriction sites for genes of interest and appropriate vector have limited the

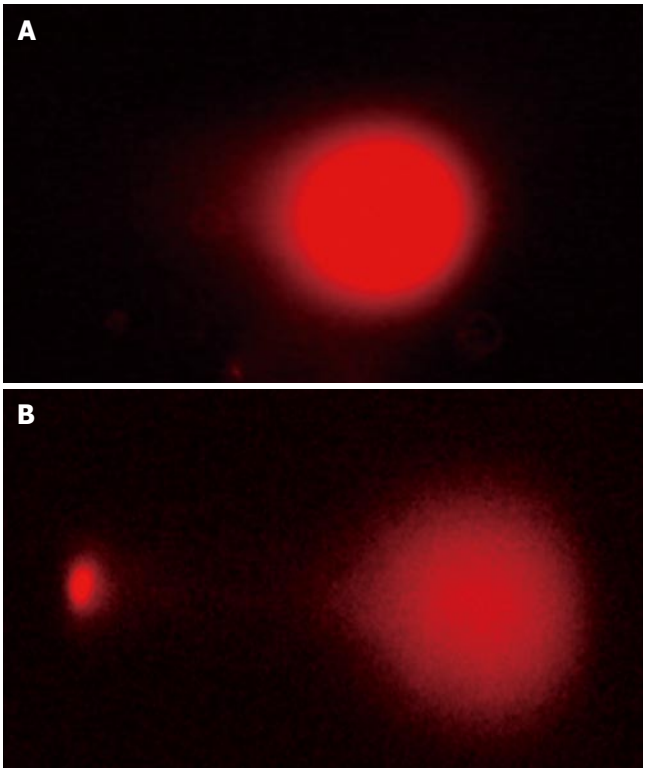

Figure 2 Morphology of normal cells (**A**) and apoptotic cells (**B**) after treatment with hybrid protein.

application of this method. Accuracy and ease of manipulation, are likely the advantages of PCR technique.

StxA is an established inhibitor of eukaryotic translation and more potent than ricin[9]. No reduction in its enzymatic inhibitory activity has been reported when it is genetically fused with HIV gp120-binding domain of CD4[8]. Similarly in our study, fusion of StlxA1 with hGM-CSF caused no reduction in its inhibitory effect. In addition, the enzymatic domain of diphtheria toxin has been fused to hGM-CSF using recombinant DNA techniques and is under clinical trial[10]. Our results suggest that StxA1-GM-CSF is more toxic than DT-GM-CSF, since IC_{50} is 20 ± 3.5 ng/mL for StxA1-GMCSF and 70 ± 18 ng/mL for DT-GM-CSF[8]. In this study, LS174T showed very obvious cytotoxic effect and changes characteristic of apoptosis were observed. A close correlation between protein synthesis inhibition and apoptosis induction has also been reported for DT-GM-CSF in which LS174T is not sensitive to apoptosis while other cell lines are more sensitive to cytotoxicity and apoptosis[11].

Agarose gel electrophoresis is one of the commonly used techniques for the detection of apoptotic cells. This technique usually involves a DNA isolation procedure from millions of cells and obtained results can not be quantified[12]. TUNEL assay is an established method for the detection of apoptotic cells, but this method is associated with a number of artefacts[13]. Annexin V labelled with fluorescent protein like FITC is used for rapid cytofluorometric analysis of apoptosis, but this method can produce false positive results when membrane is damaged[14]. Comet assay is sensitive, simple and fast. Usually this method is used to detect DNA damage like single or double strand breaks. On the other hand, this method also can detect single apoptotic cells in a large number of cells[15]. A few studies have tried to delineate the mechanism of apoptosis induction by Stx[16-18]. More knowledge about its mechanism can help us prevent shigellosis and enable us to tackle the problem of resistance to cancer therapy. Induction of apoptosis by hybrid protein indicates that catalytic domain of Shiga toxin in hybrid protein can not activate the cellular apoptosis machinery directly since the toxin inactivates ribosomes that inhibit protein synthesis.

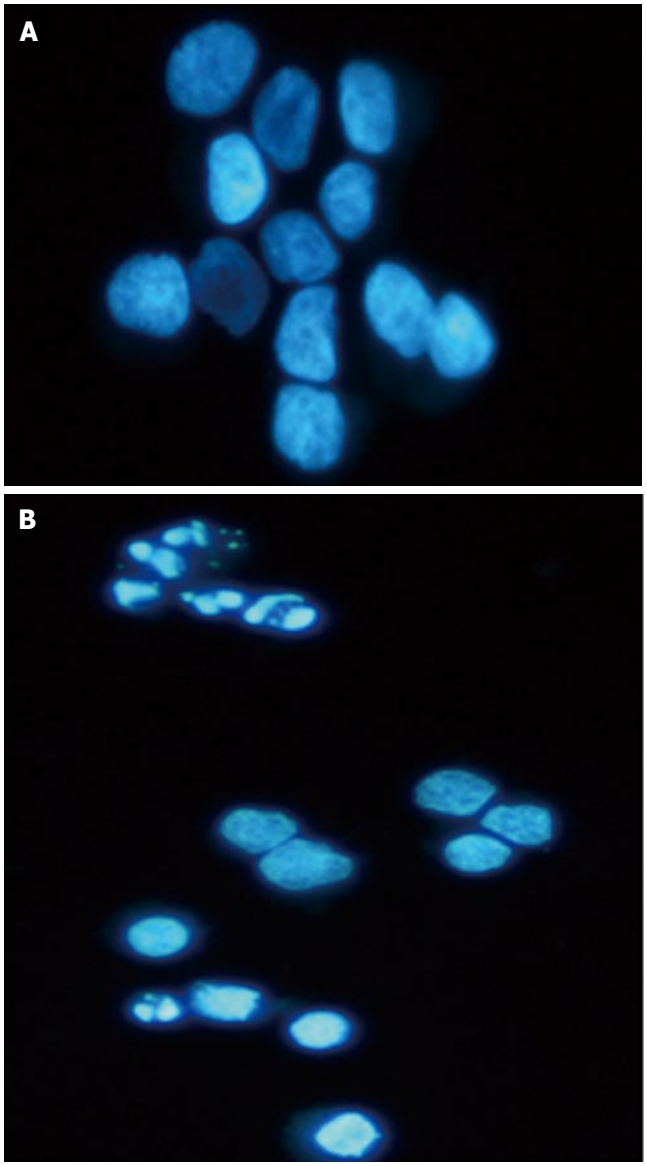

Figure 3 Hoechst 33342-staining of LS174T cells treated with StxA1-GM-CSF. **A**: control; **B**: cells after treatment.

In conclusion, more investigations are required to clarify how StxA1-GMCSF induces apoptosis. Our results reveal that the hybrid protein is toxic to the colon cancer cell line and can be considered as a therapeutic agent.

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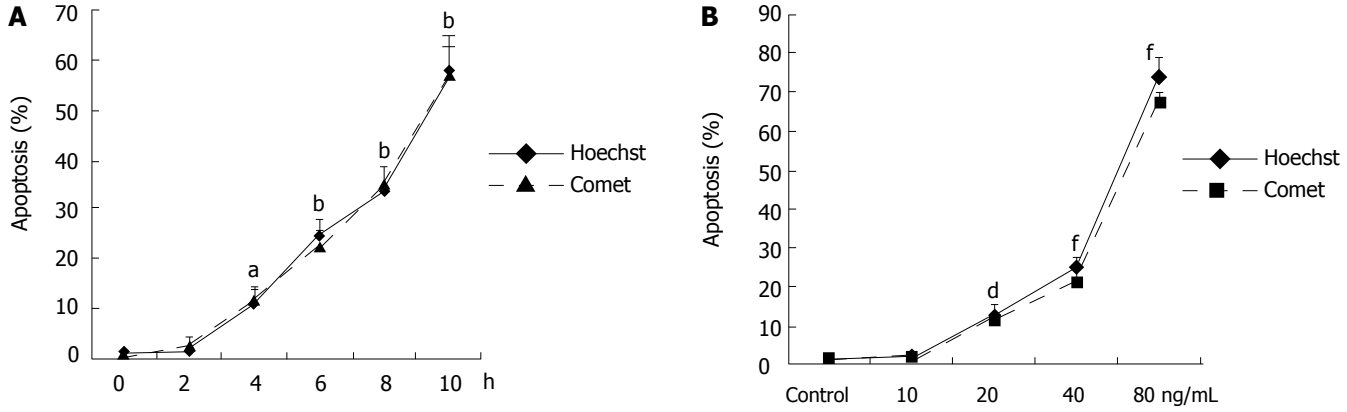

Figure 4 Apoptosis percentage in the presence of 40 ng/mL StxA1-GM-CSF at different time intervals (**A**), $^{a}P<0.05$, $^{b}P<0.001$. Apoptosis percentage in the presence of different concentration of StxA1-GMCSF for 6 h (B). $^{d}P < 0.01$, $^{f}P < 0.001$.

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BASIC RESEARCH

Three-dimensional MR and axial CT colonography *versus* conventional colonoscopy for detection of colon pathologies

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Abstract

AIM: To evaluate the sensitivity and specificity of MR colonography (MRC) and CT performance in detecting colon lesions, and to compare their sensitivity and specificity with that of conventional colonoscopy.

METHODS: Forty-two patients suspected of having colonic lesions, because of rectal bleeding, positive fecal occult blood test results or altered bowel habits, underwent the examinations. After insertion of a rectal tube, the colon was filled with 1000-1500 mL of a mixture of 9 g/L NaCl solution, 15-20 mL of 0.5 mmol/L gadopentetate dimeglumine and 100 mL of iodinized contrast material. Once colonic distension was achieved, three-dimensional gradient-echo (3D-GRE) sequences for MR colonography and complementary MR images were taken in all cases. Immediately after MR colonography, abdominal CT images were taken by spiral CT in the axial and supine position. Then all patients were examined by conventional colonoscopy (CC).

RESULTS: The sensitivity and specificity of MRC for colon pathologies were 96.4% and 100%, respectively. The percentage of correct diagnosis by MRC was 97.6%. The sensitivity and specificity of CT for colon pathologies were 92.8%, 100%, respectively. The percentage of correct diagnosis by CT was 95.2%.

CONCLUSION: In detecting colon lesions, MRC achieved a diagnostic accuracy similar to CC. However, MRC is minimally invasive, with no need for sedation or analgesics during investigation. There is a lower percentage of perforation risk, and all colon segments can be evaluated due to multi-sectional imaging availability; intramural, extra-intestinal components of colonic lesions, metastasis and any additional lesions can be evaluated easily. MRC and CT colonography are new radiological techniques that promise to be highly sensitive in the detection of colorectal mass and inflammatory bowel lesions.

Key words: MR colonography; CT colonography; Colorectal mass; Inflammatory bowel disease; Conventional colonoscopy

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INTRODUCTION

The colon is the end part of the gastrointestinal tract from the ileocecal valve to the anal region. For many years, barium examination and endoscopy were the only proven diagnostic methods for evaluating diseases of the colon. In the last 15-20 years, however, spiral computed tomography (CT) has also been shown to be an essential tool in radiological evaluation of the gastrointestinal tract. CT and MRI can be used to evaluate diseases of the colon. They can show the colon wall, colon lumen and the adjacent tissues and organs. CT and MRI are particularly useful in the initial staging of colon neoplasms, assessing the response of colon tumors to therapy and postoperative recurrence of gastrointestinal tumors, evaluating possible causes for gastrointestinal organ displacement and extrinsic impressions detected by barium studies or endoscopy. Moreover, CT and MRI are useful for the detection of inflammatory bowel disease[1-5]. Selective three-dimensional (3D) imaging of the colon was first described in 1994 by Vinning *et al*[6] as a method using spiral CT to provide a computer-simulated endoluminal perspective of the air distended colon. In 1997, Luboldt *et al*[8] first described 3D imaging of the colon filled with paramagnetic contrast as MR colonography[6-8].

Colorectal cancer is the third most common cancer and the second leading cause of cancer-related death in western countries. Most colorectal cancer evolves from pre-existing adenomatous polyps. The incidence of

colorectal cancer could be considerably reduced if polyps and small tumors were detected and eliminated prior to their malignant degeneration[9-11]. There is ongoing research for a colorectal cancer screening test that is cost-effective, safe, and acceptable to patients. Current screening methods for colorectal polyps and colonic cancer include fecal occult blood testing, sigmoidoscopy, colonoscopy and double contrast barium enema examination. The effectiveness of each modality as a screening tool remains controversial, and each method has inherent limitations. MR colonography based on MR imaging is a relatively new diagnostic modality for diagnosing colon pathology.

The purpose of this prospective study was to evaluate the sensitivity and specificity of MR colonography and CT in 42 patients who were suspected of having colonic lesions. Standard colonoscopy and histopathologic examination were accepted as the reference standard.

MATERIALS AND METHODS

Patients

A total of 42 patients (25 men, 17 women; mean age 59.3, range 2-85 years), who were suspected of having colonic lesions because of rectal bleeding, positive fecal occult blood test results or altered bowel habits, underwent MR colonography and CT examination, followed by a conventional colonoscopy (CC). All patients underwent standard bowel preparation 24 h before examination. All patients gave written informed consent, and the procedures were approved by the Local Ethics Committee.

Methods

MR colonography was performed on a 1.5T MR system (Edge, Picker, USA). No sedative or analgesic agents were used. Patients were placed in a supine position on the MR table. After insertion of a rectal tube, the colon was filled with 1000-1500 mL of a mixture of 0.9 g/L NaCl solution (1000 mL) and 0.5 mmol/L gadopentetate dimeglumine (15-20 mL) and 300 g/mL iodinized contrast material (100 mL). When the contrast material reached the cecum, the 3D colon imaging data were acquired using a T1W 3D Gradient-Echo Sequence (GRE) (TE: 2.49 ms, TR: 6 ms, flip angle: 10, thickness: 2.5 mm, FOV: 40 cm-43 cm, matrix: 128 × 192). Further sequences were performed on all patients of axial spin-echo (SE) T1W (TE: 10 ms, TR: 130 ms, flip angle: 90, thickness: 8 mm, FOV: 40 cm-43 cm matrix: 192 × 256), and fatsat SE T1W (TE: 20 ms, TR: 749 ms, flip angle: 90, thickness: 7 mm, FOV: 40 cm-43 cm, matrix: 192 × 256).

Immediately after the MR colonography, abdominal CT images were taken on the spiral CT (PQS, Picker, USA) in the axial planes (kV: 130, mA: 175, thickness: 5 mm, matrix: 512 × 512) and supine position. The three-dimensional MR data sets were analyzed in the multiplanar reformation and evaluated completely and separately and independently by two experienced radiologists. The CT axial plane images were evaluated by both the experienced radiologists. Each radiologist recorded the location and the size of colorectal masses or defined the colon lumen, wall and adjacent tissues to lesions of the large bowel, respectively. If their interpretation of the MR colonography and CT images differed, consensus was reached by review and discussion of the controversial images.

All patients were examined with the same endoscopist video (Pentax EC 38 40 TL, Tokyo, Japan) colonoscope. The location and size of any endoluminal lesions were identified and the colon wall pathologic appearance was recorded. All lesions found by CC were biopsied or removed by polypectomy. All specimens were examined histologically for differential diagnosis of inflammatory disease, hyperplastic polyps, adenomatous polyps, and cancers.

Standard colonoscopy and histopathologic examination were accepted as the references, so the MR colonography and CT were being evaluated for sensitivity, specificity and correct diagnosis ratio in the detection of colonic lesions. Each MRC examination lasted about 20-30 min, CT examination about 10-15 min and CC examination about 20-30 min. MR colonography and CT were well tolerated by all patients with no post-procedural complications after MR colonography, CT or CC.

RESULTS

A total of 42 patients suspected of having colonic lesions underwent MR colonography and abdominal spiral CT. Colonic lesions were identified by MR colonography in 26 patients. MR colonography was normal in 16 patients. On the basis of MR colonography and CT, 17 colon carcinoma (65.3%), 2 invasion to rectum (7.7%), 1 recurrent colon tumor (3.8%), 4 inflammatory bowel disease (15.3%), 1 hirschsprung disease (3.8%), and 1 diverticulosis (3.8%) were determined and these lesions were confirmed by conventional colonoscopy and histopathologic examination.

MR colonography and CT identified colorectal cancer in 17 patients. Malignant tumors of the colon were located in the rectum (6), rectosigmoid region (3), caecum (2), ascending colon (1) (Figure 1), descending colon (1) and sigmoid colon (4) (Figure 2). Malignant tumors of the colon appeared on MR colonography and CT as a tumor mass projecting into the lumen of the colon or as an asymmetrical or circumferential thickening of the bowel wall with deformation and narrowing of the lumen. Fourteen adenocarcinoma, two mucinous adenocarcinoma and one tubulovillous adenoma (carcinoma *in situ*) were confirmed by histopathologic examination. Seventeen patients with colon tumors underwent CC. A complete CC was achieved in 14 patients. In three patients, CC could not be evaluated completely due to occlusive carcinoma. However, in these patients, all of the colon segments were examined by MR colonography. The results of the MR colonography and CT were compared with the colonoscopy, histopathologic examination and surgery results. All patients with colon tumors had been correctly identified on MR colonography and CT.

Four patients with inflammatory bowel disease were correctly identified on MR colonography and CT. These were identified by histopathologic examination as ulcerative

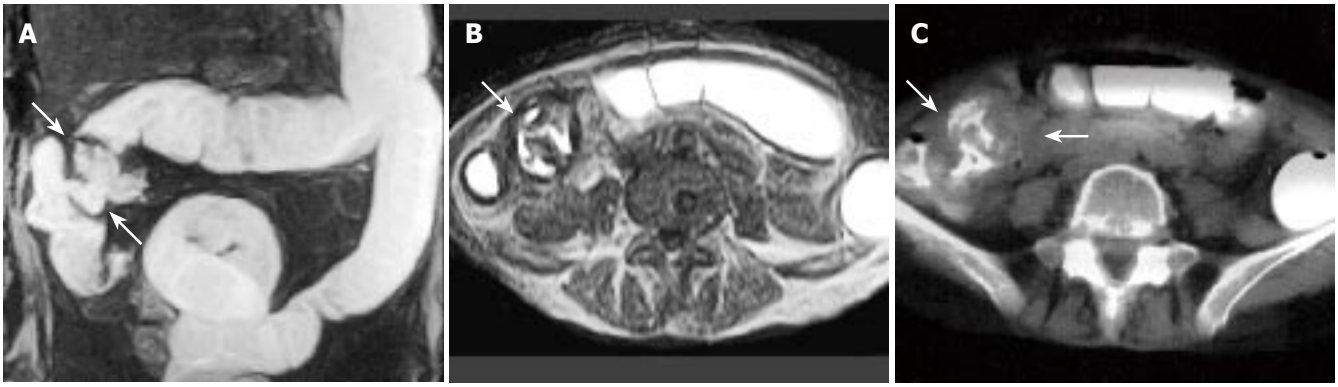

Figure 1 A 59-year-old woman with cecum and ascending colon carcinoma. **A**: MR colonography MIP (maximum intensity projection) image showing asymmetrical, irregular wall thickening at the cecum and ascending colon segments (arrows); **B**: SE T1W axial image; **C**: axial CT image showing asymmetrical, irregular wall thickening of the ascending colon (arrows).

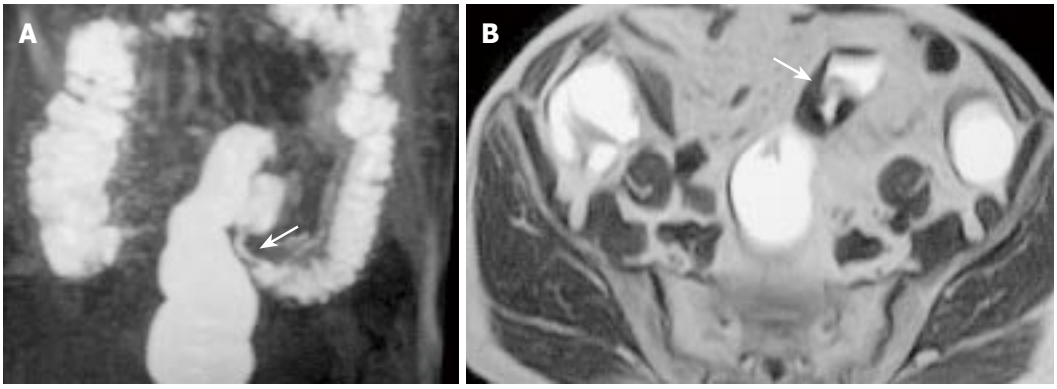

Figure 2 A 77-year-old man with sigmoid carcinoma. **A**: MR colonography MIP (maximum intensity projection) image showing asymmetrical, annular wall thickening at the sigmoid colon segment (arrow); **B**: SE T1W axial image showing asymmetrical, irregular wall thickening of the sigmoid colon (arrow).

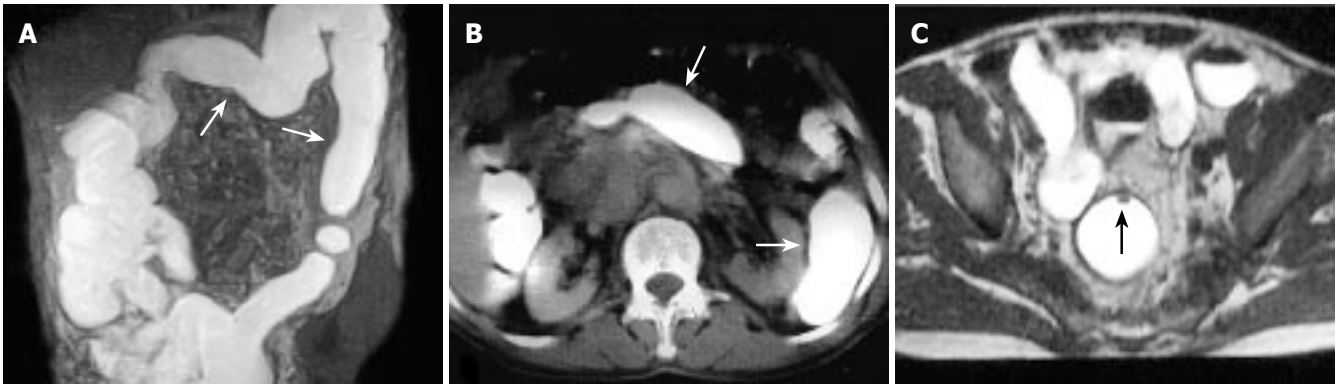

Figure 3 A 63-year-old man with ulcerative colitis. **A**: MR colonography MIP (maximum intensity projection) image; **B**: axial CT image showing haustral flattening at transverse and descending colon segments (arrows); **C**: SE T1W axial image showing a 0.5-cm polyp at the rectosigmoid junction (arrow).

colitis (Figure 3), non-specific colitis and ileocaecal region inflammatory disease (Figure 4). In one patient non-specific colitis was evaluated by histopathologic examination and colonoscopy, but it was not identified by MR colonography and CT. Thus MRC and CT failed to identify 20% (1/5) patients with inflammatory colon disease. In 2 patients with colon carcinoma, the colonoscopy detected polyps (size: 3 mm in one patient and 7 mm in the other) which had not been diagnosed with MR colonography. In one of them, the MR examination had been insufficient because of technical reasons. In the other patient, the small polyp was not identified from the adjacent mass. In the 2 patients with colon carcinoma and one patient with ulcerative colitis, colonoscopy detected polyps (size: 3 mm in 1 patient, 5 mm in 1 patient and 7 mm in the other) which had not been diagnosed with CT. In all three cases, the CT examination had been insufficient because of technical reasons and the small polyps had not been identified from the adjacent mass.

MR colonography and CT identified invasion to the rectum in two patients. In one case where there was bladder cancer invasion to the rectum, both the MR colonography and CT examinations identified invasion to the rectum and further MR colonography showed the

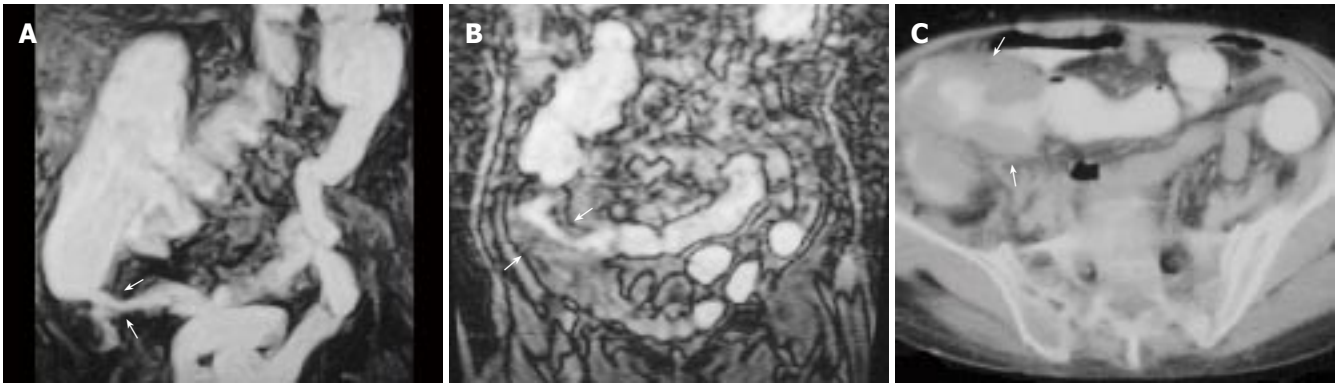

Figure 4 A 70-year-old man with inflammatory disease in the ileocaecal region. **A**: MR colonography MIP (maximum intensity projection) image; **B**: coronal plane MR colonography raw data image; **C**: axial CT image showing asymmetrical, irregular wall thickening of the ileocaecal region (arrows) and serpiginous strands extending into soft tissue due to inflammatory disease.

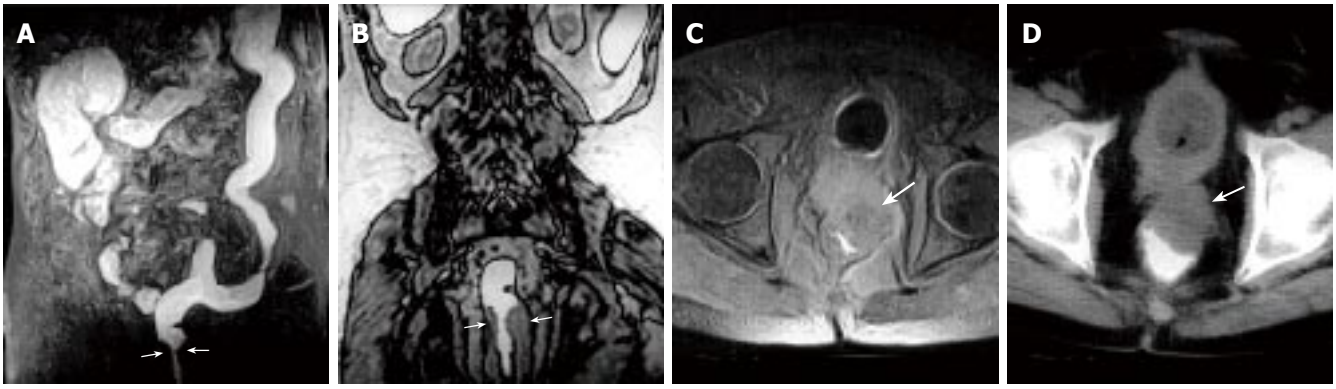

Figure 5 A 63-year-old man with rectum carcinoma invasion to prostate. **A**: MR colonography MIP (maximum intensity projection) image showing luminal narrowing at rectum (arrows); **B**: MR colonography raw data coronal image showing luminal narrowing due to annular thickening of the rectum wall (arrows); **C**: SE axial T1W image; **D**: axial CT image showing asymmetric wall thickening at rectum (arrows), and invasion to the fatty tissue arround the mass.

presence of rectovesical fistula due to bladder cancer. In the other patient, an invasion to the rectum due to prostate cancer was detected (Figure 5). The sensitivity and specificity of MR colonography for colon pathologies were 96.4% and 100%, respectively. The percentage of correct diagnosis of MR colonography was 97.6%. The sensitivity and specificity of CT for colon pathologies were 92.8% and 100%, respectively. The rate of correct diagnosis of CT was 95.2%. The MR colonography was well tolerated without sedation or analgesia and no complications were observed. MR colonography and CT identified 19 extracolonic lesions in 12 of 42 patients. These lesions were liver metastases, hydatic cyst of the liver, simple cyst of the liver, mesenteric cyst, gallbladder carcinoma, duodenum carcinoma, renal cyst, gastric tumor, multiple lymphadenopathy, intraperitoneal lipoma, hiatal hernia, subcapsular hematoma of the spleen and pleural effusion.

DISCUSSION

Colorectal cancer is the second leading cause of cancer-related death. Most colorectal cancer evolves from adenomatous polyps and screening for colorectal polyps with subsequent polypectomy has been shown to constitute an effective approach to decreasing its incidence[9,10]. However, as evidenced by disappointing participation in colorectal screening and the continuing high incidence of colorectal cancer, new screening strategies may prove beneficial. To prove effective in reducing mortality from colorectal cancer, new screening methods must demonstrate a high diagnostic accuracy at a low cost, and be proven safe and highly acceptable to patients[12].

Fecal occult blood testing has a sensitivity of less than 10% for adenomatous polyps, and a sensitivity of less than 15% for the detection of polyps under 2 cm in size[13]. In contrast, the promise of MR colonography is to detect malignant and premalignant polyps with a sensitivity rivaling colonoscopy.

Flexible sigmoidoscopy allows for the examination of only the distal 60 cm of the colon which limits evolution to the descending colon, sigmoid, and rectum; inevitably, lesions are missed in more than half the subjects who have advanced colonic adenoma located proximal to the splenic flexure but who do not have a distal index polyp[14,15]. Colonography, in contrast, images the entire colorectum and may be able to decrease the mortality by detecting more right-sided lesions. In retrospective evaluations of double-contrast barium enema examination, investigators have found sensitivities of 71%-95% for the detection of colorectal cancer[16]. However, in prospective studies of double-contrast barium enema examination, data have

shown the sensitivities as low as 50%-75% for colorectal cancer detection in asymptomatic patients with positive fecal occult blood test[17]. A recent study in which double-contrast barium enema examination was compared with colonoscopy for colonic surveillance after polypectomy found a poor detection rate of 48% for polyps ⩾10 mm in size, as well as a poor overall detection rate of only 39% for adenomas[18]. Unlike barium enema, MR colonography does not suffer from superimposition and can explain the attenuation characteristics of suspicious lesions, as well as allowing for the evaluation of pericolonic tissues.

In most centers, colonoscopy has emerged as the principal means of examining the colon. Although standard colonoscopy is a total colonic examination that allows lesion biopsy and resection, it fails to demonstrate the entire colon in up to 5% of cases examined by an experienced gastroenterologist[19], and up to 20% of all adenomas are missed[20].

Furthermore, there is a risk of complications associated with diagnostic and therapeutic colonoscopy, including perforation (1 in 1000), major haemorrhage (3 in 1000), and death (1 in 30 000)[12,20]. In addition, colonoscopy is limited by poor patient acceptance, which is a most important variable for a screening test[21,22]. Rex *et al*[23] have shown that even when it is offered free of charge, most patients refused to undergo colonoscopy for primary colorectal cancer screening.

In colorectal cancer screening, MR colonography can play an important role for patients who have undergone incomplete endoscopic colonoscopy. Common reasons for incomplete colonoscopy are redundant bowel loops and occlusive carcinoma. MR colonography can achieve a complete examination of the colon in these patients. In patients with occlusive carcinoma, the evaluation of the proximal colon is necessary to exclude a secondary neoplasia, which occurs in 5% of these cases[24]. MR colonography is available for detecting colorectal masses. Luboldt *et al*[25] performed MR colonography in 132 patients referred for CC, showing a sensitivity of 93% and specificity of 99% of MR colonography. A similar study by Pappalardo *et al*[26] compared MR colonography with conventional colonoscopy in 70 patients. All patients who underwent MR colonography had satisfactory studies and MR colonography achieved a diagnostic accuracy similar to that of conventional colonoscopy (sensitivity of 96%, specificity of 93%).

MR colonography techniques have recently been introduced as potential methods for colorectal screening. MR colonography may have a role in accurately staging colorectal cancers, in particular if combined with state-of-the-art MR imaging of the liver. In the same manner as for staging, MR colonography can also be used for post-operative surveillance[27]. Besides the detection and assessment of neoplastic disease, MR colonography can also be employed for the evaluation of inflammatory bowel disease. Over the past decade, several authors have found that MR imaging is a useful, non-invasive tool in patients with Crohn's disease, regardless of whether it is manifested in the small bowel, large bowel, or in the perianal region[28-30]. MR imaging can be used to assess disease activity and may help distinguish reversible inflammatory changes from irreversible fibrostenosis[31-33]. In contrast to double-contrast barium enema or conventional colonoscopy, MR colonography might be used to distinguish the Crohn's strictures that require surgery from those that might benefit from anti-inflammatory therapy, in addition to visualizing the colon proximal to a narrowing, and assessing extracolonic complications of the disease, including fistula and abscesses[34].

An enema with dilute iodinated contrast material can be administered well by the rectal tube in advance of the CT study, just prior to imaging. CT can also be used to evaluate the colon wall. In its characterization of colon neoplasms, CT is useful to categorize the extent of tumor as follows: intraluminal mass without wall thickening; wall thickening focal or diffuse with no extramural tumor extension; invasion of contiguous mesenchymal tissue; invasion of adjacent organs or other anatomic structures; involvement of regional lymph nodes; or metastatic spread to distant organs, lymph nodes or other structures[1-3]. CT colonography is available for detecting colorectal masses. Pickhardt *et al*[35] performed CT colonography in 1233 asymptomatic adults referred for CC, and found the sensitivity of virtual colonoscopy for adenomatous polyps was 93.8% for polyps at least 10 mm in diameter, 93.9% for polyps at least 8 mm in diameter, and 88.7% for polyps at least 6 mm in diameter. The specificity of virtual colonoscopy for adenomatous polyps was 96.0% for polyps at least 10 mm in diameter, 92.2% for polyps at least 8 mm in diameter, and 79.6% for polyps at least 6 mm in diameter. CT virtual colonoscopy with the use of a three-dimensional approach is an accurate screening method for the detection of colorectal neoplasia in asymptomatic average-risk adults and compares favorably with optical colonoscopy in terms of the detection of clinically relevant lesions[35].

CT can show diverticulosis and inflammatory bowel disease. CT findings in inflammatory disease include circumferential wall thickening, serpiginous soft-tissue strands extending into the mesenteric fat, and enlarged lymph nodes in the same region[5].

MR colonography and CT were used on 5 patients with inflammatory bowel disease, and 4 of 5 were correctly identified. MR colonography and CT failed to correctly identify in one patient who was diagnosed to have non-specific colitis by histopathologic examination. Currently, colonoscopy is generally reserved for patients with positive results from screening tests or those with a higher than average-risk of colorectal cancer, rather than applying it for routine screening. In the search for an adequate screening method, MR colonography has emerged relatively strongly. MR colonography possesses unique advantages over existing screening tests in that it is quick, less invasive, with no need for sedation or analgesics during investigation and with a lower percentage of perforation complications. Moreover, it enables evaluation of all colon segments because of multi-sectional imaging availability, thus enabling to evaluate intramural, extra-intestinal components of colonic lesions, metastasis and additional lesions. MR colonography is a fundamentally new imaging technique with the potential to alter current clinical approaches in the detection of colorectal neoplasms and

inflammatory bowel disease.

In conclusion, in the search for a rapid, less invasive, accurate, and well-tolerated colorectal examination method, magnetic resonance colonography can be an effective method.

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BASIC RESEARCH

Effect of verapamil on nitric oxide synthase in a portal vein-ligated rat model: Role of prostaglandin

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Abstract

AIM: To investigate the effects of verapamil on nitric oxide (NO) synthesis in a portal vein-ligated rat model.

METHODS: Systemic and splanchnic hemodynamics were measured by radiolabeled microspheres in portal hypertensive rats after acute administration of verapamil (2 mg/kg) on chronic treatment with N^{w}–nitro-L-arginine (NNA)(80 mg/kg) and/or indomethacin (2 mg/kg) .

RESULTS: Verapamil (2 mg/kg) caused a marked fall in both arterial pressure and cardiac output accompanied by an insignificant change in the portal pressure and no change in portal venous inflow. This result suggested that verapamil did not cause a reduction in portal vascular resistance of portal hypertensive rats, which was similar between N^{w}- nitro–L-arginine-treated and indomethacin-treated groups.

CONCLUSION: In portal hypertensive rats pretreated with NNA and/or indomethacin, acute verapamil administration can not reduce the portal pressure, suggesting that NO and prostaglandin play an important role in the pathogenesis of splanchnic arterial vasodilation in portal hypertension.

Key words: Verapamil; Nitric oxide synthase; Portal hypertension

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INTRODUCTION

Verapamil, a calcium entry blocker, in addition to its inhibiting action on the contraction of rat portal vein[1,2], can decrease hepatic venous pressure gradient[3,4] and is proposed as a drug in the treatment of variceal bleeding in patients with HBsAg-positive cirrhosis[4,5]. It was reported that verapamil reduces the intrahepatic shunting in cirrhotic rat- perfused liver[6]. Furthermore, verapamil can also reverse the altered microvascular exchange caused by sinusoidal capillarization in cirrhotic rats when given acutely or chronically[6,7]. However, studies testing verapamil, nifedipine and nicardipine in patients with cirrhosis have failed to show any reduction in hepatic vein pressure gradient, but increase in portal collateral blood flow, an effect that may be dangerous in patients with esophageal varices[8-10]. So far, the mechanism responsible for the effect of verapamil on splanchnic circulation in the rat model of pre-hepatic portal hypertension is still unknown.

Nitric oxide (NO) and prostacyclin (PGI2) are endogenous vasodilators synthesized by the vascular endothelium[11-13]. In fact, both can modulate the mesenteric vascular tone and are important contributors to splanchnic arterial vasodilation in portal hypertensive rats[12,13]. In addition, some investigators have reported that NO plays a major role in modulating collateral vascular resistance[14-16]. Increased PGI2 activities have been observed in the splanchnic vascular bed of portal hypertensive rat model[17-19]. However, it is unknown whether both vasodilators modify the response of splanchnic arterial vasodilation to verapamil in portal hypertensive rats. Thus, we aimed to identify any unique advantageous or deleterious haemodynamic effect of acute verapamil on nitric oxide synthese in a pre-hepatic portal hypertensive rat model. In addition, the influence of NO and prostaglandin on the responsiveness of splanchnic arterial vasodilation to verapamil was also evaluated.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 280-340 g at the time of surgery were used for experiments. The rats were housed in a plastic cage and allowed free access to food and water. All rats were fasted for 12 h before operation. In all experiments, the investigators followed the American Physiological Society Guiding Principles for the Care and Use of Laboratory Animals.

A prehepatic portal hypertensive animal model was induced by partial portal vein ligation (PVL) as previously described[20]. Anesthesia was performed with ketamine HCl (100 mg/kg body weight, intramuscularly). In brief, the portal vein was isolated and a 3-0 silk ligature was tied around the portal vein and a 20-gauge blunt-tipped needle. The needle was then removed and the vein allowed to reexpand. A second loose ligature was left around the portal vein with the 2 ends of the ligature placed on each side in the abdominal cavity. The abdomen was then closed and the animal was allowed to recover. Perfusion studies were performed on overnight-fasted rats 10-13 d after the operation, at which time an extensive collateralization of the portal system was fully established.

Experimental design

Rats with a ligated portal vein were divided into three groups. In each group of PVL rats, verapamil (Isoptin Knoll AG, Ludwigshafen, Germany) was given at a dose of 2 mg/kg parenterally, which decreased the mean arterial pressure (MAP) by more than 10%. One group of sham-operated rats received sterile saline (1 mL/kg) only. A group of PVL rats were also studied after the administration of sterile saline (1 mL/kg) alone to ensure that the hyperdynamic state could be achieved. Pressure dose-response curves were constructed for each group of rats 15 min after the administration of each dose. Seven doses were used at the concentration ranging from 0.02 to 2.0 mg/kg body weight. Verapamil was administered through a venous catheter. In the first group, sham-operated and PVL rats were treated daily following the ligation with one ip injection of N^{w}–nitro-L-arginine (NNA) (80 mg/kg). In the second group, sham-operated and PVL rats were treated daily following the ligation with one ip injection of indomethacin (INDO) (2 mg/kg). In the third group, sham-operated and PVL rats were treated daily following the ligation with ip injection of both NNA (80 mg/kg) and indomethacin (2 mg/kg).

Experiment preparation

All animals were anaesthetized with pentobarbital (50 mg/kg) and fastened on an animal board in dorsal recumbency. Both femoral vein and artery were cannulated with a polyethylene catheter (PE-50). The mean arterial blood pressure (MAP) was measured with a strain-gauge transducer (Statham P23 Db) connected to the femoral artery cannula and recorded continuously on a Grass polygraph (Grass Apparatus, Quincy, MA, USA). The skin over the left upper abdominal quandrant was shaved, a small (1.0 cm-2.0 cm) parasagittal incision was made at the level of the left anterior axillary line and the muscles were gently separated with forceps. The spleen was exposed by pulling it away from the perisplenic fat and then covered with a piece of warm Ringer-lactate moist gauze. Special care was taken to avoid any unnecessary manipulation of the spleen. The jejunal vein was cannulated with a PE-50 catheter for the continuous monitoring of portal pressure. Catheters were also placed in the left femoral artery and right carotid artery. The right carotid artery catheter was carefully advanced into the left ventricle under continuous pressure. Heart rate measurement was used to radiolabel the microsphere injection for the determination of portal venous inflow (PVI). All catheters were connected to Statham P-23-Db strain gauge transducers (Statham), and continuous pressure recordings of left ventricular and portal pressures (PP) were printed on a Grass model ID inscription recorder (Grass, Quincy, MA, USA). Baseline pressure and heart rate measurement were recorded in each animal.

Systemic and splanchnic hemodynamics

Splanchnic organ blood flow and cardiac output were determined twice (before and 15 min after administration of the drug) according to the reference sample method with intracardiac injection of isotope-labelled microspheres (15±3 μm in diameter)[21]. 「^{57}Co」 and 「^{113}Sn」-labelled microspheres (New England Nuclear, Boston, MA, USA) suspended in Tween 80 (0.05%) were used for the first or second injection. Approximately 180 000 「^{57}Co」 and 「^{113}Sn」-labelled microspheres were aspirated into plastic 1.0 mL syringes (volume 0.3-0.4 mL) and mixed in a vortex for 5 min. An additional 0.2 mL sample containing approximately 30 000 「^{46}Sc」-labelled microspheres was also placed into a 1.0 mL plastic syringe until injection.

Ten seconds after withdrawal of a blood sample from the left femoral artery for reference, microspheres were injected into the left ventricle over 10-15 s. Catheters were flushed with 0.2 Ml of 0.9% NaCl. Blood was withdrawn from the left femoral artery over 90 s at an approximate rate of 1 mL/min using a Harvard pump (Harvard Apparatus, Millis, MA, USA). Once withdrawal was complete, a volume of 0.9% NaC1 equal to the sample volume was withdrawn and reinfused, arterial blood pressure was monitored to ensure stability during microsphere distribution. As soon as the spleen was exposed, the 「^{46}Sc」-labelled microspheres were injected through a 23-gauge needle into the splenic pulp over a period of 20 s. The animals were then killed with an injection of bolus of saturated KC1 into the carotid artery catheter.

The liver, lungs, stomach, intestine, spleen, pancreas, mesentery and kidneys were dissected and weighed. The radioactivity of the organs was determined in a scintillation counter (Packard, Downers Grove, IL, USA) with an energy window set at 50-200 KeV for ^{57}Co, at 300-500 KeV for ^{113}Sn and at 800-1200 KeV for ^{46}Sc, respectively. At least 300 microspheres were trapped in the reference sample and organs to ensure validity of the measurement. The error in the measurement of radioactivity induced by spillover of each radioactive microsphere channel was corrected using ^{57}Co, ^{113}Sn and ^{46}Sc standards. Each standard was checked by a multichannel analyzer (Series

Table 1 Baseline characteristics in different study groups (mean ± SD)

Groups	*n*	Body weight (g)	Mean arterial pressure(mmHg)	Heart rate (beat/min)
Vehicle	8	326.7±10.6	98.6±3.6	296±18
Verapamil	8	332.6±11.8	96.5±4.8	320±16
Verapamil+vehicle	8	321.5±9.8	99.5±5.2	318±20
Verapamil+NNA	8	330.8±9.6	97.4±3.8	317±17
Verapamil+INDO	7	327.8±8.5	94.2±6.2	328±14
Verapamil+INDO+NNA	8	319.8±7.9	96.2±4.8	308±15

NNA: N^{ω}-nitro-L-arginine; INDO: indomethacin.

35 PLUS). Adequate microsphere mixing was assumed at a difference < 20% between the left and right kidneys. Any animal not meeting this requirement was rejected from the analysis. Cardiac output was calculated according to the following formula:

Cardiac output (mL/min) = (injected radioactivity (cpm) × reference sample blood flow (mL/min)) / reference sample radioactivity (cpm).

Injected radioactivity was calculated on the basis of the difference between the initial and residual radioactivity in the syringe. Organ blood flow was calculated as follows:

Organ blood flow (mL/min) = (organ radioactivity (cpm) × reference sample blood flow (mL/min)) / reference sample radioactivity (cpm).

Vascular resistance of different organs in the portal venous system was calculated according to the following formula:

Resistance (dyn.s.cm^{-5}×10^5) = (mean arterial pressure (mmHg) - portal venous pressure (mmHg)) × 80/organ blood flow (mL/min)

Portal tributary blood flow was calculated as the sum of the blood flow in the spleen, stomach, colon, mesentery and pancreas. The hepatic arterial blood flow was taken to be equal to the liver blood flow.

Portal systemic shunt (PSS) % was calculated as:

[ct/min in lung/ct/min in (lung + liver)] x 100

Drugs

Verapamil, NNA, INDO, and reagents for preparing Krebs' solution were purchased from Sigma. All solutions were freshly prepared on the day the experiment was conducted.

Statistical analysis

Results were expressed as mean ± SD. The concentration of verapamil at 50% of the maximal response (EC_{50}) in each preparation was calculated from sigmoid logistic curves and expressed as negative log molar (-log mol/L). Statistical analyses were performed by the paired or unpaired Student's *t* test and one-way analysis of variance with Tukey's test when appropriate. $P<0.05$ was considered statistically significant.

RESULTS

Systemic and splanchnic hemodynamics

No differences were observed in body weights, mean arterial pressures and heart rates among the three groups (Table 1). The PVL rats exhibited a hyperdynamic state of splanchnic circulation with a high cardiac index. The pressure-dose-response curves of the sham-operated and PVL rats after acute verapamil administration in different groups were similar to the curves previously reported by us[20](Figure 1).

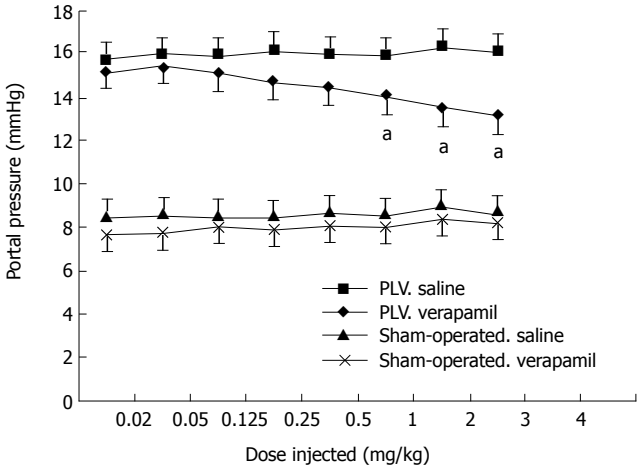

Figure 1 Portal pressure responses to verapamil in four groups of rats: $^aP<0.05$ *vs* sham-operated control. PVL: portal vein ligated.

No significant change of the portal pressure (PP) was noted when verapamil (2 mg/kg) was given to PVL rats by early chronic administration of NNA, INDO, or both in all three groups. The results of this study also indicated that acute verapamil administration could reduce the PP in different study groups of PVL rats in a hyperdynamic state. In all three groups of rats, the mean arterial pressure (MAP) significantly decreased following administration of verapamil compared to the control group. In contrast, the portal pressure, portal venous inflow (PVI) and hepatic arterial flow (HAF) were maintained, while cardiac output (CO) decreased significantly compared to the control group (Table 2, Figure 2). Thus, the effect of verapamil on chronic treatment with NNA (80 mg/kg), INDO (2 mg/kg), and both on splanchnic organ blood flow in sham-operated and PVL rats after 15 d from the control are shown in Figure 3. At a dose of 2 mg/mL verapamil, there was also no significant decrease in total peripheral resistance (TPR), splanchnic arterial resistance (SAR) and portal venous resistance (PVR) in all three groups of PVL rats (Table 3).

Portal-systemic shunting

After PVL rats were pretreated with either NNA or indomethacin for 15 d, acute verapamil (2 mg/kg) administration did not induce any significant reduction in the portal-systemic shunting. Furthermore, the portal pressure in both NNA- and indomethacin-pretreated PVL rats was also not significantly different from that in the control group. The portal pressure reduction was not correlated with portal-systemic shunting reduction in either NNA- or indomethacin-pretreated PVL rats (r = 0.05, NS; r = 0.03, NS).

Table 2 Effect of verapamil in combination with NNA, INDO, and NNA plus INDO on systemic and splanchnic haemodynamics in PVL rats (mean ± SE)

	Verapamil (n = 8)		Verapamil + NNA (n = 8)		Verapamil + INDO (n = 7)		Verapamil + NNA + INDO (n = 8)	
	Before	After	Before	After	Before	After	Before	After
CI[mL/(min.kg)]	423±38	298±42[a]	425±41	312±48[a]	424±39	332±51[a]	426±40	326±49[a]
MAP(mmHg)	105±5	96±6[a]	106±6	99±7[a]	107±4	98±7[a]	106±8	100±7[a]
PP(mmHg)	15.0±1.6	10.8±1.9[a]	14.3±1.8	13.8±2.1	15.2±1.9	14.5±2.4	15.1±1.6	14.6±1.4
PVI(mL/min)	27±2.8	26±3.2	26±2.7	25±3.4	28±3.1	27±3.5	27±3.2	26±3.9
HAF(mL/min)	7±1.6	8±1.8	8±1.8	9±2.2	7±1.9	8±2.2	9±2.0	9±2.8
PSS(%)	81.7±7.2	63.6±8.2[a]	80.2±8.3	76.5±9.2	81.2±6.2	77±7.8	80.5±6.6	76.2±9.5

CI: cardiac index; MAP: mean arterial pressure; PP: portal pressure; HAF: hepatic arterial flow; PSS:portalsystemic shunting; NNA: N^{w}-nitro-L-arginine; INDO: indomethacin; [a]$P < 0.05$ *vs* control group.

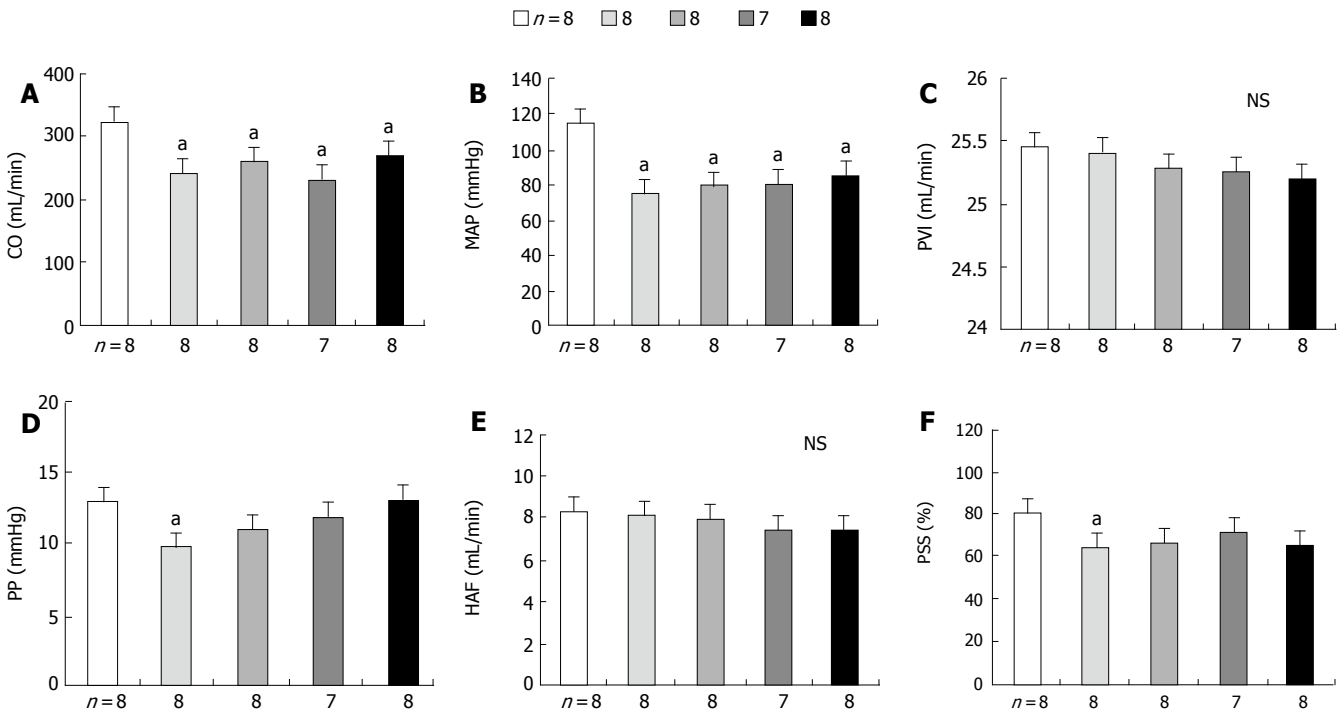

Figure 2 Effect of verapamil in combination with NNA (□ light grey), INDO (■ dark grey) and NNA plus INDO (■ black) on cardiac output (**A**), mean arterial pressure (**B**), portal venous inflow (**C**), portal pressure (**D**), hepatic arterial flow (**E**), and portal systemic shunting (**F**) in PVL rats. [a]$P < 0.05$ *vs* control group.

Table 3 Effect of verapamil in combination with NNA, INDO, and NNA plus INDO on systemic and splanchnic vascular resistance in PVL rats (mean ± SE)

	Verapamil (n = 8)		Verapamil + NNA (n = 8)		Verapamil + INDO (n = 7)		Verapamil + NNA + INDO (n = 8)	
	Before	After	Before	After	Before	After	Before	After
TPR (dyh.s.cm^{-5}×10^{4})	8.96±0.87	6.45±0.78[a]	8.84±0.72	8.47±0.98	8.76±0.88	8.52±0.91	8.78±0.93	8.58±0.89
SAR (dyh.s.cm^{-5}×10^{5})	4.38±0.72	3.16±0.62[a]	4.51±0.89	4.18±0.71	4.45±0.92	4.20±0.84	4.46±0.84	4.22±0.94
PVR (dyh.s.cm^{-5}×10^{4})	7.49±0.68	6.12±0.58[a]	7.46±0.72	7.23±0.67	7.54±0.97	7.41±0.95	7.46±0.65	6.95±0.87

TPR: total peripheral resistance; SAR: splanchnic arterial resistance; PVR: portal venous resistance; NNA: N^{w}-nitro-L-arginine; INDO: indomethacin. [a]$P < 0.05$ *vs* control group.

DISCUSSION

The results of the present study showed that verapamil administration did not favorably influence splanchnic arterial vasodilation in portal vein-ligated rats pretreated with NNA and indomethacin as expected.

These findings are not consistent with those of a previous study in rats with carbon tetrachloride-induced cirrhosis[6], in which rat model it was demonstrated that verapamil reduces hepatic resistance, exerts beneficial effect on the hepatic microvascular exchange, improves liver function and reduces portal pressure if chronically administered. It was reported that verapamil is a useful therapeutic agent for patients with cirrhosis[4]. In a small group of patients, Freeman *et al*[3] demonstrated that verapamil administered intravenously, decreases the hepatic venous pressure gradient (HVPG). Furthermore, we

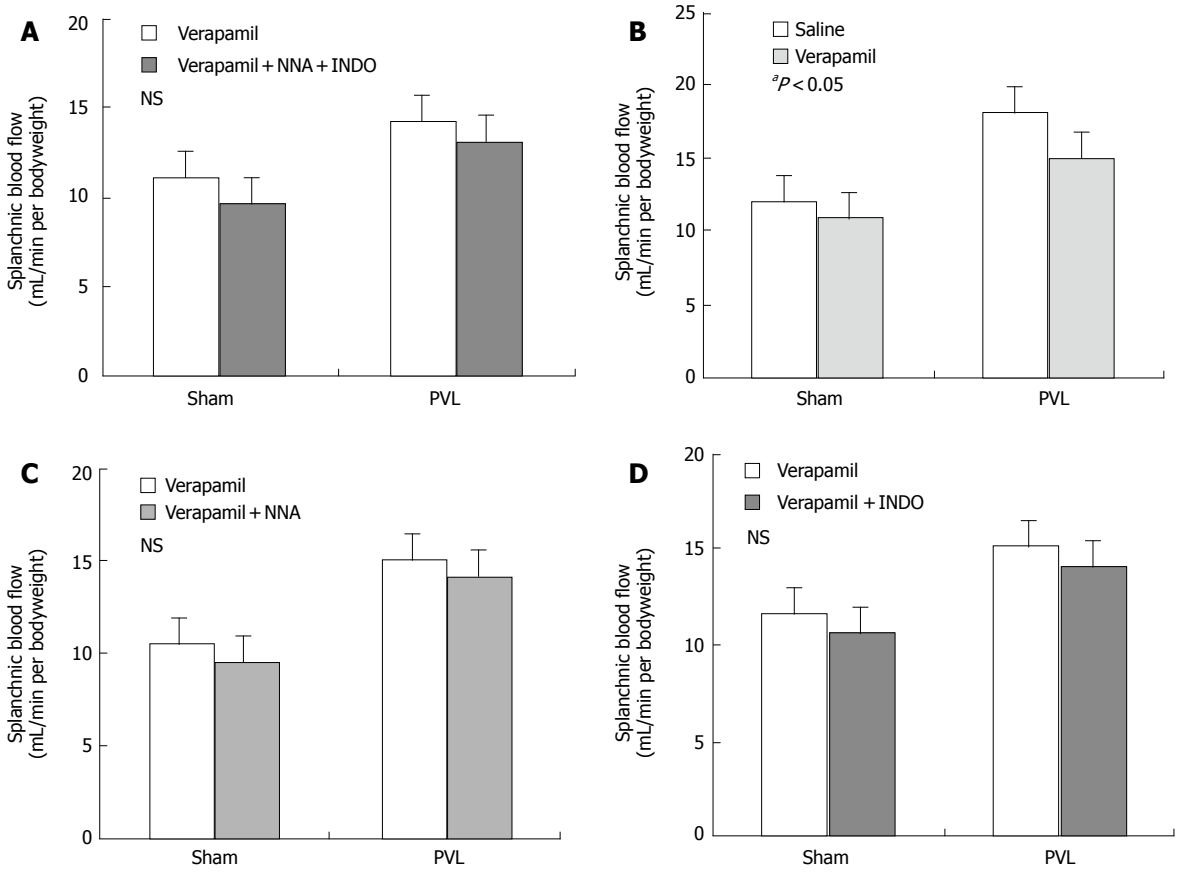

Figure 3 Effects of verapamil in combination with NNA (80 mg/Kg), INDO (2 mg/Kg) and NNA plus INDO on splanchnic blood flow in sham-operated and PVL rats after 15 days. **A**: verapamil plus NNA plus INDO; **B**: verapamil alone; **C**: verapamil plus NNA; **D**: verapamil plus INDO. $^aP < 0.05$ *vs* control group.

have obtained similar results in patients with postnecrotic cirrhosis after both acute and chronic administration of verapamil[4,5].

The present study failed to document any beneficial effect of verapamil in portal hypertensive rats pretreated with NNA and indomethacin. This might not be due to a low dose or a poor absorption of verapamil, as documented by the dose response curve which was well within the therapeutic range. Pharmacodynamic efficacy was also manifested by the observed systemic effects of verapamil, including increased HR and peripheral vasodilation as well as decreased arterial pressure and SVR. Verapamil did not exert any noticeable effect on portal pressure, PVI and HAF. Accordingly, the hepatic vascular resistance did not decrease in our study. Because of this lack of effect on the splanchnic and hepatic arterial vasodilation, verapamil failed to influence liver perfusion. Thus, our results do not support the use of verapamil in the treatment of cirrhosis and portal hypertension pretreated with NNA and indomethacin. Further evidence against the use of verapamil in portal hypertension has been demonstrated by Narasa *et al*[8] who in accordance with our findings have failed to document any decrease in portal pressure following verapamil administration in portal hypertensive patients.

The reason for the discrepancies between the current results and previous studies is not clear. It may be related to the fact that our rat model was produced by portal vein ligation, causing presinusoidal portal hypertension, and these rats were pretreated with NNA and indomethacin. It is also possible that in this kind of animal model, verapamil cannot reduce portal pressure by decreasing intrahepatic portal resistance, and improve hepatic function by affecting the apparent hepatic blood flow[22]. So far, the effect of verapamil on rat hepatic stellate cells is still unknown[23]. The structural changes have not been observed and within the liver the hepatic microcirculation cannot be pharmacologically altered because verapamil has a high first-pass effect[1,24]. However, we have previously documented in a similar model of portal hypertensive rats that the hepatic vascular resistance and portal pressure can be lowered by verapamil[4]. Although animal experiments have demonstrated that verapamil can produce a complex interplay of alterations in preload (splanchnic venodilatation), afterload and myocardial contractility by inhibiting the constrictor responses of the splanchnic capacitance vessels of small resistant arterioles and arteries to the sympathetic nervous outflow[1,6,7], this finding indicates that the effect of verapamil on splanchnic arterial vasodilation can be pharmacologically modified by pretreatment with NNA and indomethacin.

Nitric oxide and prostaglandin are endogenous vasodilators produced by vascular endothelial cells[11-15]. Non-specific inhibition of NO synthesis can restore mesenteric vascular responsiveness[25] and normalize splanchnic vascular resistance[26], thus ameliorating portal-systemic shunting and hyperdynamic circulation[27,28]. Both NO and prostaglandin participate in the regulation of

vascular tone in different vascular beds of normal and portal hypertensive animals[15-18]. It has been shown that in portal hypertensive rats, inhibition of nitric oxide synthesis increases not only intrahepatic and splanchnic vascular resistance but also portal-collateral resistance[11,15,29]. Recently, increased prostacyclin (PGI 2) activities have been observed in systemic circulation and portal vein segments of portal hypertensive rats[18]. In addition, prostaglandin is involved in the modulation of collateral vascular tone in portal hypertensive rats and can modify the vasoconstrictive effect of vasopressin[25]. Nitric oxide and PGI 2 produce relaxation of the vascular smooth muscle by different mechanisms. Nitric oxide works by activating guanylate cyclase which increases the intracellular levels of guanosine 3, 5-cyclic monophosphate[12].

In conclusion, acute verapamil administration does not increase the splanchnic arterial vasodilation in pre-hepatic portal hypertensive rats pretreated with NNA and /or indomethacin. In conjunction with our previous reports[20], these findings indicate that both NO and prostaglandin can modulate the splanchnic vascular response to verapamil and may therefore participate in the development and maintenance of portal hypertension.

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BASIC RESEARCH

Relationship between transforming growth factor β1 and anti-fibrotic effect of interleukin-10

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Abstract

AIM: To study the effect of interleukin-10 (IL-10) on the expression of transforming growth factor β1 (TGF-β1) in hepatic fibrosis rats and the anti-fibrotic role of exogenous IL-10.

METHODS: Hepatic fibrosis was induced by carbon tetrachloride administered (CCl_4) intraperitoneally. The experiment was performed in two stages. In the first stage, 60 SD rats were divided randomly into normal control group 1 (GN_1, n = 8), hepatic fibrosis group (GC, n = 28) and IL-10 intervened group (GI, n = 24). At the beginning of the 7th and 11th wk, hepatic stellate cells (HSCs) were isolated, reverse transcription-polymerase chain reation (RT-PCR) and immunocytochemistry were performed to detect the expression of TGF-β1 in HSCs. Histological examination was used to determine the degree of hepatic fibrosis. In the second stage, 47 SD rats were divided randomly into normal control group 2 (GN_2, n = 6) and CCl_4 group (GZ, n = 41). At the end of the 9th week, rats in GZ group were allocated randomly into model group (GM, n = 9), IL-10 treatment group (GT, n = 9) and recovered group (GR, n = 9). At the end of the 12th week, all rats were sacrificed. RT-PCR and immunohistochemistry were performed to detect the expression of TGF-β1 in liver tissue. ELISA was used to assay serum TGF-β1 levels.

RESULTS: Hepatic fibrosis developed in rats with the increase of the injection frequency of CCl_4. In the first stage, hepatic fibrosis developed and HSCs were isolated successfully. At the 7th and 11th week, TGF-β1 mRNA in GC group increased significantly compared with that in GN_1 (P = 0.001/0.042) and GI groups (P = 0.001/0.007), whereas there was no significant difference between the two groups. The levels of TGF-β1 at the beginning of the 7th wk was higher than that of the 11th wk (P = 0.049). Immunocytochemistry results of TGF-β1 were consistent with the above findings. In the second stage, TGF-β1 increased significantly in GM group compared to GN_2. After treatment with IL-10, TGF-β1 declined obviously. The expression of TGF-β1 decreased in GR group but was still higher than that in GT group.

CONCLUSION: The levels of TGF-β1 are increased in hepatic fibrosis rats and decreased after treatment with exogenous IL-10. IL-10 may play an anti-fibrotic role by suppressing TGF-β1 expression.

Key words: Hepatic fibrosis; Hepatic stellate cells; Interleukin-10; Transforming growth factor-β1

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INTRODUCTION

Hepatic fibrosis, which represents the wound healing response of the liver, is a common sequel of liver injury characterized by increased deposition and altered composition of extracellular matrix (ECM) [1-2]. Hepatic stellate cells (HSCs) are the major source of ECM and regarded as the principle cell type in the development of hepatic fibrosis [3-5]. TGF-β1 appears to be the main fibrogenic mediator and exerts its effects through autocrine or paracrine on HSCs [6]. Hepatocytes also express TGF-β1 when isolated and cultured *in vitro* as a response to an altered extracellular environment. It has been reported that interleukin-10 (IL-10) could relieve the degree of rat hepatic fibrosis induced by carbon tetrachloride (CCl_4) [7]. In the present study, we isolated HSCs and detected the expression of TGF-β1 in HSCs and liver tissues, and explored the relationship between TGF-β1 and hepatic fibrosis in an attempt to explain the possible mechanisms of the antifibrotic activities of exogenous IL-10 *in vivo*.

MATERIALS AND METHODS

Establishment of models

All rats were bred under routine conditions (at room temperature of 22 ℃ ± 2 ℃, a humidity of 55% ± 5%) with a light/dark cycle and free access to drinking water and food (BK Company in Shanghai, China). In the first stage, 60 clean male SD rats were divided randomly into normal control group 1 (GN_1, n = 8), hepatic fibrosis group (GC, n = 28) and IL-10 intervened group (GI, n = 24). Rats in GN_1 group were injected intraperitoneally with saline at a dose of 2 mL/kg twice a week. Rats in the other two groups received intraperitoneal injection of 50% CCl_4 dissolved in castor oil (2 mL/kg) twice a week as described previously[8]. From the third week, rats in GI group were injected intraperitoneally with IL-10 dissolved in saline (4 μg/kg) 20 min before CCl_4 administration as previously described[9]. The intervention lasted to the end of the experiment. In the second stage, 47 SD rats were divided randomly into normal control group 2 (GN_2, n = 6) and CCl_4 group (GZ, n = 41). Rats in the GN_2 group were injected intraperitoneally with saline at a dose of 2 mL/kg, twice a week. Rats in the GZ group received intraperitoneal injection of 50% CCl_4 dissolved in castor oil (2 mL/kg) twice a week. At the end of the 9th week, rats in the GZ group were divided randomly into model group (GM, n = 9), IL-10 treatment group (GT, n = 9) and recovered group (GR, n = 9). Rats in the GM group were injected with CCl_4 continuously: Rats in the GT group received intraperitoneal IL-10 dissolved in saline (4 μg /kg) after injection of CCl_4 for 9 weeks and rats in the GR group were not treated. Three rats in the GN_2 group were executed at the 9th week and the other rats were sacrificed at the 12th week.

Histological examination

In the first stage, at the beginning of the 7th and 11th week, 2 rats from each group were chosen randomly for histological examination. Liver tissues were fixed in 10% formalin and embedded with paraffin. Sections were stained with hematoxylin and eosin (HE) and examined under a light microscope.

Isolation, incubation and identification of HSCs

Isolation, incubation and identification of HSCs were performed as previously described[10]. Briefly, at the beginning of the 7th and 11th week, 5 rats from each group were chosen randomly to perfuse with 0.13% pronase E and 0.025% type-IV collagenase through a portal vein catheter. The liver tissue suspension was incubated with 0.02% pronase E and 0.025% type-IV collagenase under agitation. The suspension obtained from the digested liver tissue was centrifuged with 11% Nycodenz density gradient to purify HSCs. Then, the HSCs were seeded at the concentration of 1×10^6 cells /mL of Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum in 96-well plates and kept at 37 ℃ in a 50 mL/L CO_2 atmosphere for 72 h. The HSCs were identified by their typical phase-contrast microscopic appearance and immunocytochemistry using antibody directed against desmin. Cell vitality was checked by trypan-blue exclusion.

RNA extraction and RT-PCR

Total RNA was extracted from freshly isolated HSCs and liver tissues according to the instructions of the RNA isolation kit (Jingmei Biotechnology Company, Shenzhen). Its quantity and purity were assessed by measuring the optical density at 260 nm and 280 nm. After measurement of RNA amount, samples were either used immediately for RT or stored at -70 ℃.

For RT, 1 μg total RNA was transcribed following the instructions of the first strand cDNA synthesis kit (Jingmei Biotechnology Company, Shenzhen). Twenty μL reaction mixture was transcribed at 42 ℃ for 60 min, at 99 ℃ for 5 min and stored at -20 ℃.

For PCR, primers coding for the house-keeping gene-β-actin were added into the reaction mixture to standardize the results. Fifty μL aqua was added into the reactive system containing 2 μL cDNA, 5 μL 10×buffer, 5 μL 25 mmoL/L $MgCl_2$, 1 μL 10 mmoL/L dNTP, 1 μL 20 mmoL/L up stream and down stream primer of target gene, 1 μL 20 mmoL/L β-actin primer pairs, 3U Taq DNA polymerase. Then PCR was performed with predenaturation at 94 ℃ for 5 min followed by 30 cycles at 94 ℃ for 45 s, at 55 ℃ for 30 s, at 72 ℃ for 60 s and a final extension at 72 ℃ for 7 min. Primers were designed according to the reference of GenBank (Table 1). PCR products were immediately analyzed by 20 g/L agarose gel electrophoresis and the density of resultant bands was semi-quantified by scanning densitometry using the ratio of TGF-β1 to β-actin to assess the relative level.

Table 1 Primer sequences used in the study

mRNA	Upstream primer	Downstream primer	Product length (bp)
TGF-β1	CTC TGC AGG CGC AGC TCT G	GGA CTC TCC ACC TGC AAG AC	392
β-actin	CCA ACC GTG AAA AGA TGA CC	CAG GAG GAG CAA TGA TCT TG	660

Immunocytochemistry

Most of HSCs attached to the dishes after 72 h of primary culture. The 96-well plates were washed twice with 0.1 moL/L PBS and cells were fixed with poly-formaldehyde at 4 ℃ overnight. The following procedures were performed according to the instructions of streptavidin/peroxidase (S-P) kit (Beijing Zhongshan Company). Briefly, cells were washed with PBS, incubated with bovine serum albumin in PBS, reacted with primary antibody dissolved in PBS, washed, incubated with peroxidase-conjugated second antibody, washed again and reacted with S-P for 20 min. Color reaction was developed by incubation with DAB. In negative controls, the primary antibody was replaced by PBS.

Immunohistochemistry of hepatic TGF-β1

After dewaxed with xylene and rehydrated through a graded alcohol series, sections were digested with 4 g/L trypsin. The following procedures were similar to the steps of immunocytochemistry. Slides were then analyzed with an image analyzing system to obtain the relative contents of hepatic TGF-β1. The characteristics of the antibodies used in this study are summarized in Table 2.

Table 2 Antibodies used for immunocytochemistry and immunohistochemistry

Antibody	Type	Source	Working solution
Desmin	Polyclonal, mouse	Beijing Zhongshan Co.	1:100
TGF-β1	Polyclonal, rabbit	BOSTER Co.	1:20/1:25
Antibody to rabbit IgG	Goat	Beijing Zhongshan Co.	Ready to use
Antibody to mouse IgG	Goat	Beijing Zhongshan Co.	Ready to use

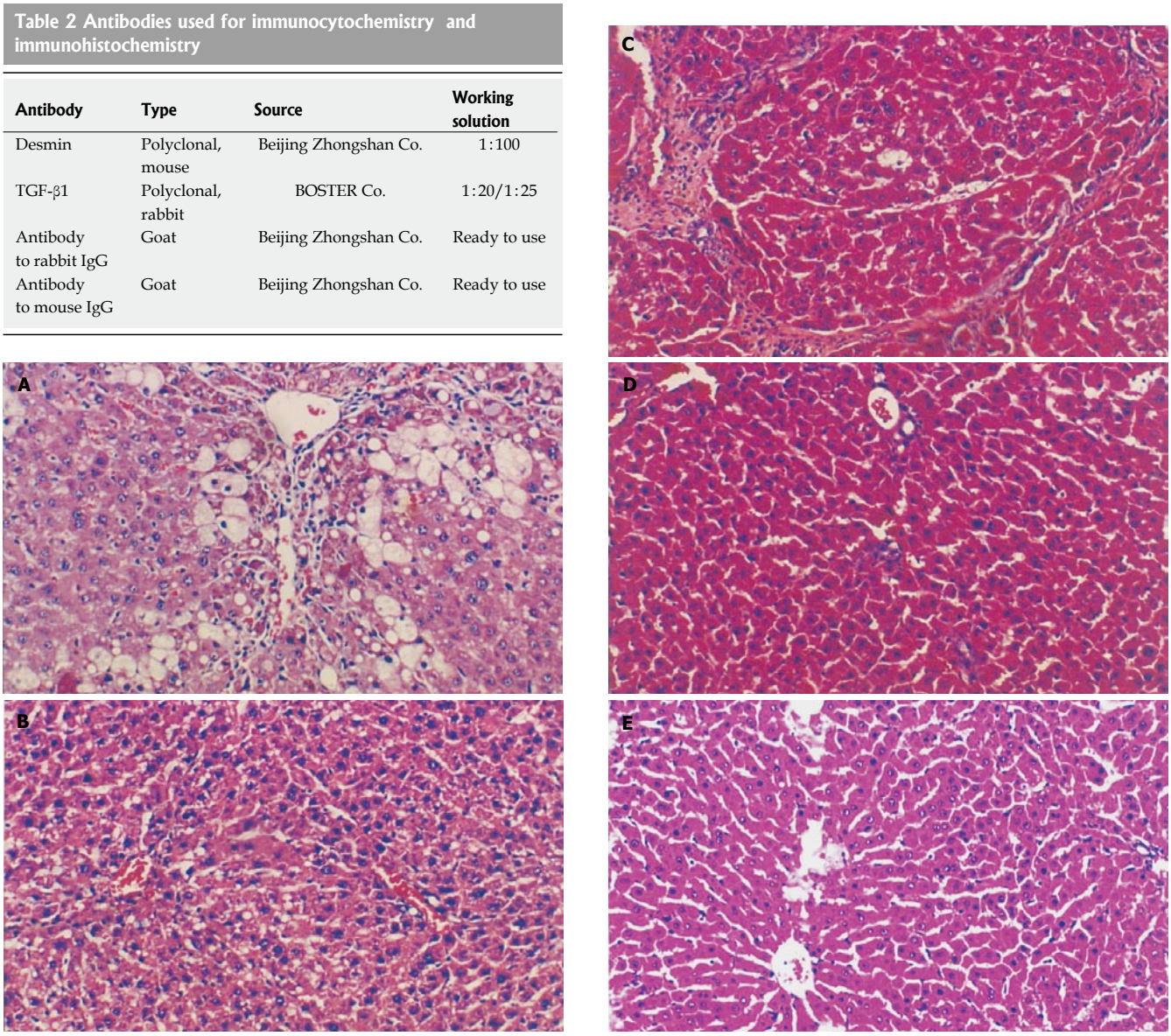

Figure 1 Effect of IL-10 on liver histological change in group C at the 7th wk (**A**), group I at the 7th wk (**B**), group C at the 11th wk (**C**), group I at the 11th wk (**D**), and group N (**E**).

ELISA of serum TGF-β1

Assay of serum TGF-β1 content was performed with double antibody ABC-ELISA method according to the ELISA kit instructions (Maoyuan Science Technology Limited Company, Shanghai).

Statistical analysis

All data were expressed as mean±SE. The significance for the difference between the groups was studied with SPSS11.0 by one-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

Histological examination

Hepatic fibrosis became remarkable after the treatment with CCl_4. At the 7th wk, specimens from the GC group showed steatosis and ballooning degeneration, the collagen fibers increased and began to extend to the parenchyma, many mononuclear cells and unusual neutrophils surrounded the centrilobular veins and fibrotic septa (Figure 1A), while only a few inflammatory cells infiltrated around the centrilobular veins without evident changes of lobular structure in the GI group (Figure 1B). At the 11th week, the reticular fiber extended into the hepatic plate and full delimitation was developed (Figure 1C), while less fibrosis septa and inflammatory infiltration were seen in the GI group (Figure 1D). Specimens from the GN_1 group showed normal lobular structure (Figure 1E). Due to the limit of samples, no statistical data presented disparity between the two groups, but fibrogenesis in the GI group was much less severe than that in the GC group.

Isolation and identification of HSCs

A total of $2–4.5\times10^7$ cells were harvested from each rat. HSCs were identified by immunoreaction with desmin (Figure 2). The mean purity of freshly isolated HSCs was 95% ± 5%. The cell vitality checked by trypan-

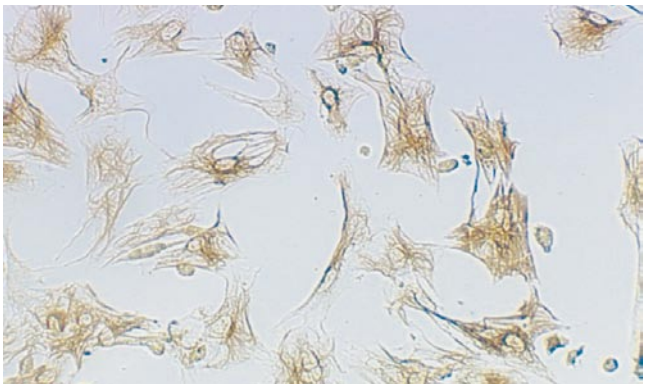

Figure 2 Desmin expression in freshly isolated HSCs (SP × 100).

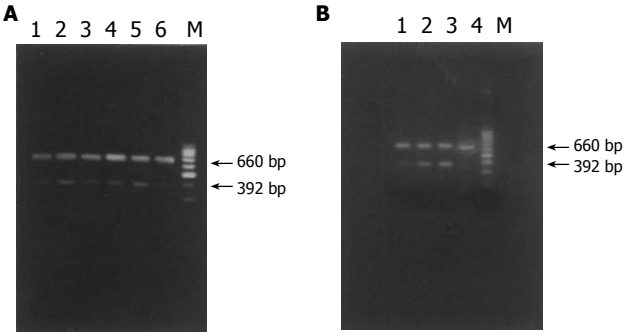

Figure 3 Expression of TGF-β1 in HSCs the first (**A**) and the second stage (**B**).

blue exclusion was higher than 95%.

Expression of mRNA

The purity of RNA was determined by the ratio of OD260 and OD280 ranging between 1.8-2.0. In the first stage, at the 7th and 11th wk, TGF-β1 mRNA in the GC group increased obviously compared to the GN_1 and GI groups ($P < 0.01$), but no difference was seen between the two groups. TGF-β1 expression at the 7th wk was higher than that at the 11th wk ($P < 0.05$, Table 3, Figure 3A). In the second stage, TGF-β1 mRNA in the GM group increased notably compared to the GN_2 group ($P < 0.01$) and declined obviously after treatment with IL-10 ($P < 0.01$). Its expression in the GR group was lower than that in the GM group ($P < 0.01$) but still higher than that in the GT group ($P < 0.05$, Table 4, Figure 3B).

Immunocytochemistry and immunohistochemistry

In the first stage, TGF-β1 positive expressions were localized in cytoplasm and nuclei of HSCs in all groups by immunocytochemistry after 72 h of primary culture. Most of the cells attached to and spread over the plastic substratum. At the 7th wk, the size of HSCs in the GC and GI groups was a little larger than that in the GN_1 group. At the 11th wk, cell phenotype in the GI group showed circle or ellipse and was a little smaller than that in the 7th wk, no obvious change of phenotype was seen in the GC group. Although the number of samples was limited, the expression of TGF-β1 in the GC group was higher than that in the GN_1 group and decreased after treatment with IL-10 (Figures 4A-4D). In the second stage, TGF-β1 positive expressions in the GM group were localized in most

Table 3 Expression of TGF-β1 mRNA in HSCs (mean ± SE)

Wk	*n*	Group N	Group C	Group I
7	5	0.143±0.009	0.267±0.025[b]	0.140±0.008[d]
11	5	0.141±0.004	0.207±0.029[ab]	0.123±0.009[d]

[b]$P<0.01$ *vs* group N; [d]$P<0.01$ *vs* group C; [a]$P<0.05$ *vs* wk 7.

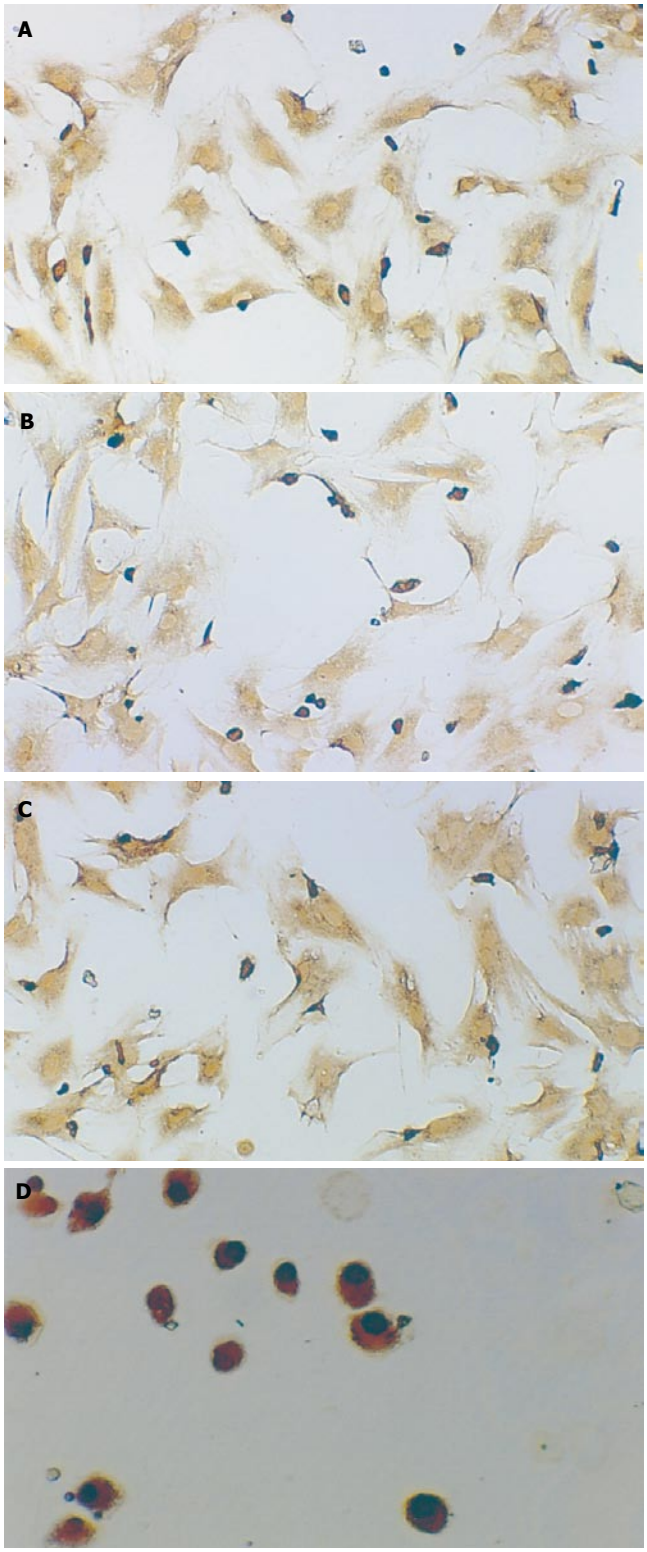

Figure 4 Expression of TGF-β1 protein in HSCs in group C (**A**) at the 7th wk, group I (**B**) at the 7th wk, group C at the 11th wk (**C**), and group I (**D**) at the 11th wk.

Table 4 Expression of TGF-β1 in liver tissues (mean ± SE)

GROUP	*n*	TGF-β1 content		
		Immunhistochemistry	RT-PCR	ELISA
N_2	6	0.810 ± 0.141	0.188 ± 0.014	95.442 ± 35.341
M	9	1.804 ± 0.382[a]	0.449 ± 0.040[b]	242.593 ± 53.902[a]
R	9	0.963 ± 0.121[c]	0.279 ± 0.015[bd]	165.514 ± 23.193[ac]
T	9	0.501 ± 0.250[ace]	0.182 ± 0.027[de]	113.472 ± 15.101[ce]

[a]*P* <0.05, [b]*P* <0.01 *vs* group N; [c]*P* <0.05,[d]*P* <0.01 *vs* group M; [e]*P* < 0.05 *vs* group R.

of the liver tissues, appearing brown. After treatment with IL-10, the distributing area was smaller and the color was lighter. TGF-β1 expression in the GR group was lower than that in the GM group but still higher than that in the GT group (Table 4, Figures 5A-5D).

Expression of serum TGF-β1

The expression of serum TGF-β1 detected by ELISA in different groups had the same tendency as that detected by RT-PCR and immunohistochemistry in the second stage (Table 4).

DISCUSSION

Although significant progress has been made in understanding the pathogenesis of hepatic fibrosis, a rational therapy that prevents the progression or even reverses fibrosis remains elusive[11-13]. IL-10 is produced mainly by TH_2 cells and inhibits the functions of TH_1 cells. It down-regulates pro-cytokines synthesis and is associated with amelioration of the inflammatory response[14-16] and fibrosis[17-18]. The present study also found the trend from the pathological sections that IL-10 could relieve the degree of inflammation. In this experiment, we detected TGF-β1 in hepatic fibrosis group as well as in IL-10 treatment group.

TGF-β1 is a multi-functional cytokine in the liver involved in regulation of liver growth and induction of hepatocyte apoptosis, and is the most important medium involved in fibrotic and cirrhotic liver[19-22]. It can promote the development of liver fibrosis by inducing the synthesis of ECM proteins and down-regulating the expression of matrix, thus degrading enzymes and stimulating synthesis of their respective inhibitors[23-26]. The present study showed that, with the development of hepatic fibrosis, TGF-β1 increased in hepatic fibrosis rats and decreased after treatment with IL-10. TGF-β1 mRNA level in HSCs did not run parallel with the progression of hepatic fibrosis in the first stage. TGF-β1 protein level detected by immunohistochemistry and ELISA in IL-10 treatment group was lower than that in normal control group in the second stage. These results suggest that TGF-β1 is closely correlated with hepatic fibrosis and that the improvement of hepatic fibrosis is related with the decreasing expression of TGF-β1. These results also suggest that the expression of TGF-β1 of HSCs cannot be regarded as a predictable factor for the degree of hepatic fibrosis and that the ability of HSCs to produce TGF-β1 is declined when fibrosis develops. While the results of ELISA proved that IL-10 exerted its inhibitory effects on the expression of TGF-β1 not only in liver but also in serum, suggesting that IL-10 can inhibit the expression of TGF-β1. However, further studies are needed to confirm whether the expression of TGF-β1 in normal liver inhibited by IL-10 has any adverse effects and whether the effect of IL-10 on TGF-β1 is modulated by weakening the signal transduction.

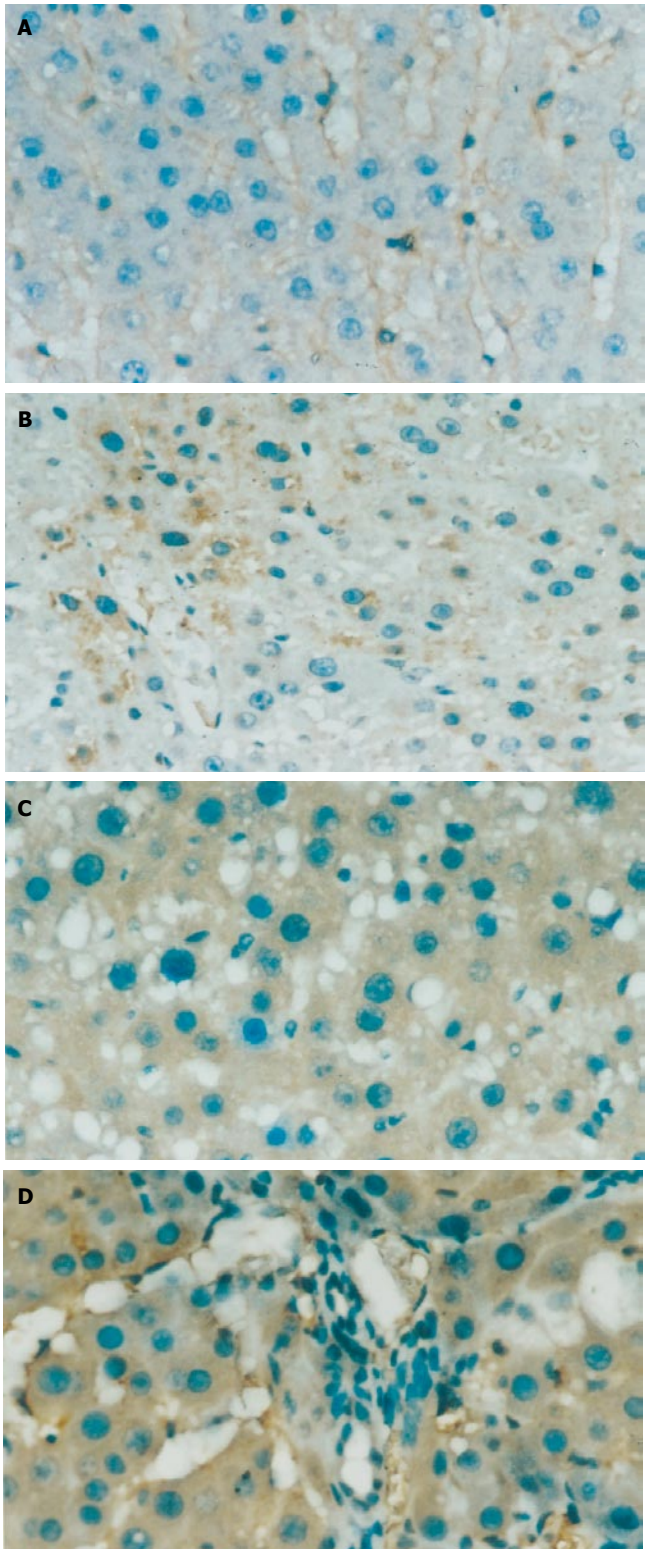

Figure 5 Expression of TGF-β1 in liver tissue in group N_2 (**A**), group T (**B**), group R (**C**), and group M (**D**).

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BASIC RESEARCH

Biological role of surface Toxoplasma gondii antigen in development of vaccine

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Abstract

AIM: To analyze the biological role of the surface antigen of *Toxoplasma gondii (T gondii)* in development of vaccine.

METHODS: The surface antigen of *T gondii* (SAG1) was expressed *in vitro*. The immune response of the host to the antigen was investigated by detection of specific antibody reaction to SAG1 and production of cytokines. Mice were immunized with recombinant SAG1 and challenged with lethal strain of *T gondii* RH. The monoclonal antibody to r-SAG1 was prepared and used to study the effects of SAG1 on *T gondii* tachyzoites under electromicroscope.

RESULTS: The mice immunized with recombinant SAG1 delayed death for 60 h compared to the control group. The recombinant SAG1 induced specific high titer of IgG and IgM antibodies as well as IFN-γ, IL-2 and IL-4 cytokines in mice. In contrast, IL-12, IL-6 and TNF-α were undetectable. When *T gondii* tachyzoites were treated with the monoclonal antibody to r-SAG1, the parasites were gathered together, destroyed, deformed, swollen, and holes and gaps formed on the surface.

CONCLUSION: SAG1 may be an excellent vaccine candidate against *T gondii*. The immune protection induced by SAG1 against *T gondii* may be regulated by both hormone- and cell-mediated immune response.

Key words: *Toxoplasma gondii;* Recombinant SAG1; Monoclonal antibody; Cytokines; Morphology change

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INTRODUCTION

Toxoplasma gondii (T gondii) is an intracellular coccidian parasite and causes the most common parasitic disease of animals and human beings[1]. The definitive hosts for the parasite are members of the Felidae family. The clinical manifestations associated with feline toxoplasmosis are anorexia, weight loss, lethargy, dyspnoea, ocular signs, pyrexia, vomiting and diarrhea, jaundice, myositis and abortion. Humans become infected when they ingest the Toxoplasma at infective stages (oocysts and tissue cysts) found in some cat feces and in raw meats. People weak in immune function may develop severe diseases such as encephalitis, pneumonia or other life-threatening conditions. Infants born with congenital toxoplasmosis may develop permanent diseases such as mental retardation or eye, liver and brain diseases. In cirrhotic patients, Toxoplasma IgG and IgM antibody positivity is as high as 68.5%[2]. In patients with AIDS, *T gondii* colitis can occur[3]. In veterinary medicine, *T gondii* infection may influence economics due to neonatal loss in sheep and goats[4], or as a source of transmission to humans[5]. Thus, it is of great value to develop an effective vaccine against *T gondii*.

The characterization of the molecules which play the role in the pathogenesis and immune protection is the important step in vaccine development. The surface of *T gondii* is the first component to contact with the host cells and the surface antigen of the parasite is recognized as the major study target. It was reported that there are 5 proteins in the superfamily of the surface antigens (SAG) of *T gondii*, including SAG1, SAG2 (22 Ku), p23, p35, and SAG3 (43 Ku). SAG1 is a 30 Ku glycoprotein and is therefore designated as SAG1/P30[6] and can be detected in the tachyzoite and sporozoite stages[7,8]. It was reported that SAG1 can elicit a lethal inflammatory process in mouse model of pathogen-driven ileitis[9]. However, the biological role of this surface protein remains unclear.

In the current study, the biological function of SAG1 was studied through the analysis of the induction of specific antibody, elicitation of specific cytokines by the recombinant protein for SAG1 (r-SAG1 or r-P30) of *T gondii*. The immune protection ability of SAG1 was studied by challenging experiments. The results show that SAG1 is a very important protein and its biological function is regulated by multiple mechanisms.

MATERIALS AND METHODS

Parasites and antigens

T gondii RH tachyzoites were maintained by two weekly passages of tachyzoites to peritoneum of BALB/C mouse. Four days later parasites in the peritoneal fluid were collected and the cavity was washed with 5 mL of phosphate buffered saline (PBS). The total tachyzoite crude antigen was obtained from washed and pelleted tachyzoites, resuspended in PBS and freeze-thawed three times, then subjected to 2 cycles of ultrasound disruption (UTR200) for 10 min and incubated at 37 ℃ for 2 h with 1% decanoyl-N-methylglucamide (MEGA 10, Sigma). After centrifugation at 36 000 r/min for 30 min, the pellet was discarded and the supernatant was aliquoted and stored at -70℃. The protein concentration was determined by BCA assay (Pierce) using BSA as standard.

Cloning and expression of SAG1 gene in E. coli

About 5×10^7 *T gondii* RH strain tachyzoites were concentrated by centrifugation, washed with PBS, then lysed in 0.1 mol/L Tris-HCl (pH 8.0) containing 1% sodium dodecyl sulphate (SDS), 0.1 mol/L NaCl and 10 mmol/L EDTA and then treated with proteinase K (100 μg/mL) at 55℃ for 2 h. The genomic DNA was extracted by phenol/chloroform method followed by ethanol precipitation. After centrifugation the pellet was dissolved in TE buffer (10 mmol/L Tris-HCl, pH 8.0 and 1 mmol/L EDTA) and used as a template for polymerase chain reaction (PCR) amplification, which used the primers (5' TGGtttcactcttaagtgccctaaaacagc-3'and 5' ctgcattaacctgc agccccggcaaactc-3') together with PCR buffer, dNTP and Taq polymerase. The amplified SAG1 gene was inserted into the *Nco*I and *Hind*III sites of the plasmid pET-30a, and expressed as a His-tag fusion protein in *E. coli* BL21 (DE3) strain according to the manufacturer's instructions. The transformed bacteria were centrifuged and lysed by a combination of detergent Triton X-100, lysozyme and ultrasonication. The suspension was centrifuged and the pellet was dissolved in 8 mol/L urea solution containing 50 mmol/L Tris-HCl (pH 8.0), 1 mmol/L dithiothreitol (DTT) and 1 mmol/L EDTA. One hour after incubation at room temperature (RT), the supernatant was contrifuged and dialyzed at 4℃ followed by 2 mol/L urea solution at 4℃ for 1 h each. Dialysis was done twice in 50 mmol/L Tris-HCl (pH 8.0) with 1 mmol/L DTT at 4℃ and each lasting for 1 h. This was followed by overnight dialysis at 4℃ in the same buffer. The dialyzed sample was centrifuged and the supernatant was recovered and used as antigen.

Immunization and challenge

Five to 7-wk-old female BALB/c mice (purchased from Shandong University) housed under approved conditions of the animal research facility, were used in this study. Twenty-one BALB/c mice were immunized at two locations at the base of the tail with 30 μg of *T gondii* recombinant SAG1 in 0.1 mL of saline, which was emulsified with an equal volume of complete Freund's adjuvant (CFA) (Sigma, USA). Sixteen mice were injected with PBS. Booster was done 2 wk after the first injection using Freund's incomplete adjuvant for the r-SAG1 group. Sera were collected at 3 wk post immunization for antibody test. For cytokine test, mice were killed at 6 wk post immunization. The mice inoculated with r-SAG1 of *T gondii* were challenged intraperitoneally with 1×10^5 tachyzoite forms of *T gondii* RH strain 9 wk after the first immunization.

ELISA

To measure antigen-specific antibodies, plates were coated overnight at 4℃ with 10 μg/mL solution of antigen in 0.05 mol/L potassium phosphate buffer pH 8 (50 μL per well). Blocking was carried out with 2% BSA in PBS (pH 7.2) for 2 h at RT. After washed with PBS containing 0.05% Tween 20 (PBST20), sera were diluted in 1% BSA-PBST20 (50 μL per well) and incubated for 1 h at RT. After washed, bound antibodies were detected by incubation at RT for 1 h with horseradish peroxidase-labeled goat anti-mouse immunoglobulins at 1:2 000 dilution in 1% BSA-PBST20 (50 μL per well). Peroxidase activity was revealed by adding 50 μL per well of a solution containing 12.5% H_2O_2, 0.1 mol/L citrate-phosphate pH 4 and 10 mg/mL of TMB. The reaction was stopped by adding 50 μL of 2 mol/L H_2SO_4 and the absorbance (A) was read at 450 nm in an ELISA microplate reader (Bio-Rad, USA). A sample was considered positive when A > A mean + 3 SD (cut off), where A is that of the tested sample, and A mean and SD are the mean and the standard deviation of the A of the sample from PBS, respectively.

SDS-PAGE and immunoblotting

SDS-PAGE was performed on 12% polyacrylamide gels according to the procedure of Laemmli[10] using a total tachyzoite extract or r-SAG1. Gels were transferred to nitrocellulose membranes as previously described[11] and blocked in PBS containing 5% nonfat dry milk. Membranes were incubated with primary Abs and then with anti-species alkaline phosphatase conjugates, both were diluted in PBS containing 1% nonfat dry milk. The alkaline phosphatase activity was detected with the ProtoBlot nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate color development system (Promega, Madison, WI).

Cytokine determination

At 6 wk post immunization, mice were sacrificed and single-cell suspension was prepared as previously described[12]. A volume of 10 μg/mL of r-SAG1 of *T gondii* was added and the supernatants were collected from cultures after 72 h and assayed by ELISA for cytokines.

The monoclonal antibody (mAb) was used for assay of IL-6 (MP5-20F3), IL-2 (JES6-1A12), IL-12 (9A5), IL-4 (BVD4-1D11), TNF-α (TN3-19.12), IFN-γ (AN-18) (BD Pharmingen).

Preparation of monoclonal antibodies

Monoclonal antibodies against recombinant SAG1 of *T gondii* were obtained by fusion of SP2/0 myeloma cells with the spleen cells of BALB/c mice immunized with r-SAG1 in the presence of polyethylene-glycol (Sigma) according to standard protocols. Hybrid cells were cultured in hypoxantine, aminopterin and thymidine (HAT) in DMEM/20% FBS medium. Clones growing in selected media were expanded and tested for antibody production. Positive clones were cloned twice by limiting dilution. Hybridomas were screened for specific antibody production by ELISA and immunoblot against the *T gondii* antigen. The identification of mAb isotype was made by ELISA using goat anti-mouse IgG subclasses (Nordic) appropriately diluted in PBS containing 0.1% Tween-20, 2% normal goat serum, followed by incubation with alkaline phosphatase conjugated rabbit anti-goat IgG (Sigma). Hybrid cells producing mAbs were injected to mouse intraperitoneally and after 2 wk ascites fluid was collected.

Electron microscopy

T gondii strains were harvested from BALB/C mice and washed twice with 0.05 mol/L pH7.2 PBS. The ascites fluid mAb against r-SAG1 was added (1:100) to the parasites. After incubation at 37℃ for 1h, the parasites were washed 3 times with PBS and fixed in 4% paraformaldehyde, 0.05% glutaraldehyde in cacodylate buffer+3% sucrose for 2 h at RT. After dehydration and embedding, the slides were observed under electron microscope (Hitachi 600, Hitachi, Japan).

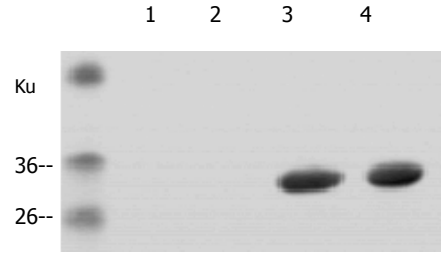

Figure 1 A strong band at 30 ku position after reaction with serum from mice infected with T. r-SAG1 of *Toxoplasma gondii* tachyzoites. The band was electrophoresed on SDS-12% polyacrylamide gel and then transferred onto a nitrocellulose membrane probed with sera (diluted at 1:100) from PBS-inoculated mice (lanes 1 and 2) or from mice infected with crude antigens of *T. gondii* (lanes 3 and 4). Bound antibodies were detected using anti-mouse-alkaline phosphatase conjugate. The positions of molecular masses are shown on the left.

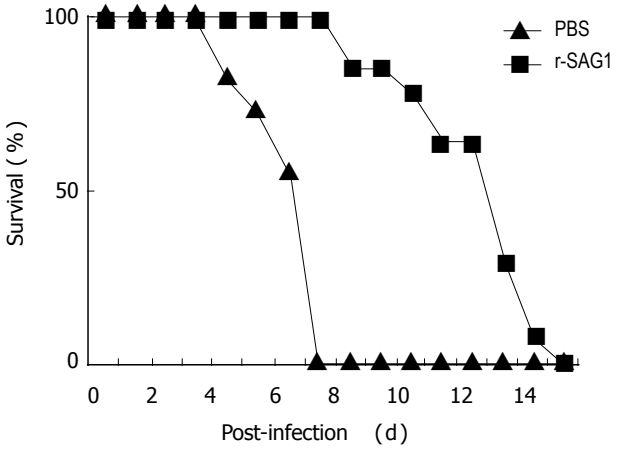

Figure 2 Protection of r-SAG1 against infection with tachyzoites of *Toxoplasma gondii*. Sixteen BALB/C mice were immunized with r-SAG1 of *T. gondii* and 11 mice were injected with PBS as a control. The mice were challenged with $1X10^5$ tachyzoites of *Toxoplasma gondii* RH at 9 wk post immunization. The number of the dead mice was counted.

RESULTS

Characterization of SAG1 recombinant protein

To study the biological function of the *P30* gene encoding SAG1, the *P30* gene of *T gondii* RH strain was amplified by PCR, the PCR product was subcloned into bacterial expression vector pET-30a and SAG1 was expressed as Histidin-fusion protein in *E. coli*. The gene was sequenced and the recombinant SAG1 was analyzed by Western blotting. A strong band at 30 Ku position was found after reaction with serum from mice infected with *T gondii* (Figure 1), indicating that the *P30* gene could encode a functional protein.

Immunization with r-SAG1 protein could protect mice from a lethal T gondii challenge

To test whether r-SAG1 could afford protection against a lethal *T gondii* RH strain infection, BALB/c mice were immunized with r-SAG1 as described in the methods. Nine weeks after the first injection, the animals were intraperitoneally infected with 1×10^5 tachyzoites of the highly virulent RH strain. The death number of the mice was counted after challenge. It was found that after challenge with RH tachyzoites, mice started to die on day 4 in PBS-inoculated control group. On day 7, all the mice in control group were dead. However, mice immunized with r-SAG1 delayed death for 60 h compared to those in the control group (Figure 2).

r-SAG1 induced specific antibody response

To investigate whether r-SAG1 could induce humoral immune response, the sera from mice were collected 3 wk after the last immunization. The titer and classes of immunoglobulin were tested by ELISA. It was obvious that vaccination of the mice with r-SAG1 induced a strong antibody response. The highest reaction antibody was immunoglobulin G (IgG). IgM was detected with a lower titer than that of IgG. IgA antibody response to r-SAG1 was very weak (Figure 3).

To test the specificity of the reaction of the antigen and antibody, the crude antigen of *T gondii* tachyzoites was separated on 12% SDS-PAGE and reacted with one of the mouse sera immunized with r-SAG1 by Western blotting.

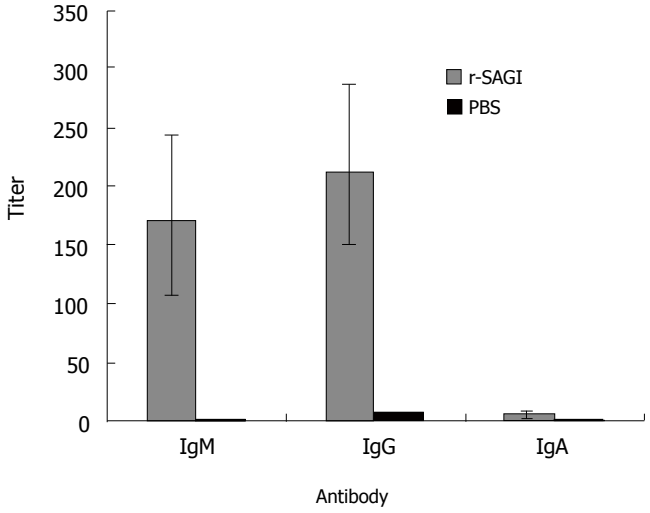

Figure 3 Antibody responses to r-SAG1. BALB/C mice were immunized with 30 μg r-SAG1 of *T gondii* or with PBS as a control. Booster was done with Freund's incomplete adjuvant 2 wk after the first injection. Three weeks post-immunization, sera were collected from 5 mice of each group. Antibodies were tested individually by ELISA.

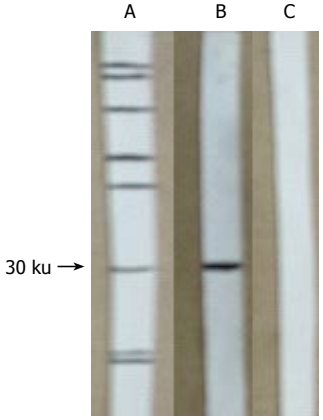

Figure 4 Profile of cytokines induced by r-SAG1 in PBS-inoculated mice (**A**) or r-SAG1 of *T gondii* immunized mice (**B**). Crude antigens of Toxoplasma gondii tachyzoites were electrophoresed on SDS-12% polyacrylamide gel and then transferred onto a nitrocellulose membrane probed with sera (diluted at 1:100). Bound antibodies were detected using anti-mouse-alkaline phosphatase conjugate. The positions of molecular masses are shown on the left.

A strong band was present on the 30 Ku position (Figure 3), corresponding to the predicted size from amino acid sequence.

Profile of cytokines induced by r-SAG1

To investigate the possibility of cell immune response induced by r-SAG1, the spleens of the mice immunized by r-SAG1 were isolated and the cytokines in the spleen cell suspensions were detected by ELISA. Compared to the control group, the spleen cells from r-SAG1-vaccinated BALB/c mice produced significant high levels of IFN-γ, IL-2 and IL-4, while IL-12, IL-6 and TNF-α were undetectable in splenocyte supernatants from all experimental and control animals analyzed (Figure 4).

Morphology change of T. gonddi tachyzoites caused by r-SAG1 antibody

To further elucidate the biological function of r-SAG1, monoclonal antibodies against r-SAG1 were prepared. Two specific clones against r-SAG1 were obtained and

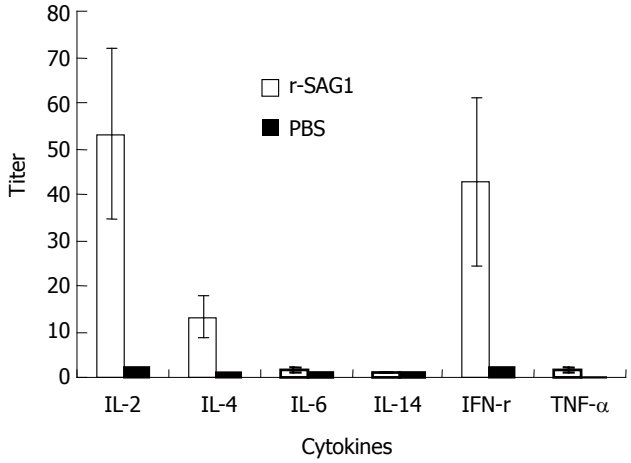

Figure 5 Production of cytokines induced by r-SAG1. BALB/C mice were immunized with r-SAG1 of *T gondii* or with PBS as a control. Six weeks after immunization, cytokines were tested individually by ELISA in 5 mice of each group.

designated as mAb2B3 and mAb1H6, respectively. Both clones were IgM isotype. The antibody produced a specific band at 30 Ku in Western blotting with *T gondii* crude antigens and r-SAG1 antigen.

To study the morphology effects of r-SAG1 antibody on *T gondii*, *T gonddi* tachyzoites were treated with specific antibody mAb2B3 and the morphology was observed under electron microscope. Compared to the PBS-treated control group, *T gondii* tachyzoites were gathered together and some tachyzoites were destroyed, some parasites were deformed(Figure 5). The holes and gaps on the surface of tachyzoites as well as the swollen parasites were observed (Figure 6).

DISCUSSION

In the current study, the recombinant protein for SAG1 of *T gondii* was prepared and the biological function of SAG1 was studied through the investigation of the lethal protection of mice. The possible mechanism was studied by the observation of the induction of specific antibody, elicitation of specific cytokines and the reaction of SAG1 specific antibody with *T gondii* tachyzoites. The study showed that SAG1 elicited a strong immune protective reaction which might be controlled by multiple mechanisms.

This study showed that the mice immunized with r-SAG1 delayed death for 60 h when challenged with *T gondii* RH tachyzoites, demonstrating that SAG1 is an important antigen of *T gondii*. The protection of the host against organism infection may involve many factors including specific antibody. It was reported that antibodies can regulate toxoplasma tachyzoites and thereby promote their killing by normal macrophages[13]. Kang *et al*[14] have proved that antibodies can block infection of host cells. In addition, antibodies in the presence of complement can kill extracellular toxoplasmas[15]. In the present study, the morphology changes of the parasite, such as agglutination, deformation, swelling hole and gap formation on the

Figure 6 Effects of monoclonal antibody to r-SAG1 on tachyzoites of *Toxoplasma gondii*. **A:** Agglutination of the parasites, hole and gap formation on the surface, destroyed parasites and membrane thickness; **B:** Deformed parasite; **C:** Swollen parasites. *Toxoplasma gondii* tachyzoites were collected from mice and washed with PBS. Monoclonal antibody mAb2B3 to r-SAG1 from mouse ascites fluid was diluted and reacted with the parasites and observed under electro-microscope at 6800 x.

surface, corresponding to SAG1 antibody treatment indicated that antibody might play an important role in the protection. The inference was verified further by the specific antibody production following the immunization of r-SAG1. After three weeks of vaccination with r-SAG1, high titer specific IgG and IgM were detected and the high titer of the antibodies was detected (data not shown). IgM antibodies could persist for at least 50 d and IgG could persist for an even longer time[16]. Therefore, when the mice were challenged at 9 wk, both IgG and IgM could contact with parasites to destroy the parasites, inhibit the infection of host cells, and thereby protect the mice from death.

In this study, the expression of cytokines such as IFN-γ, IL-4 and IL-2 was up-regulated in SAG1 immunized mice compared with control group. It was reported that IFN-γ induces inflammatory responses, thus limiting parasite proliferation[17]. In patients with alveolar echinococcosis, IFN-γ is higher, which might play a role in immunological defense against the parasite infection[18]. IL-2 (-/-) mice are unable to generate a protective IFN-γ response following infection with *T gondii* while IL-2 (-/-) mice have an intrinsic defect in their ability to activate and expand IFN-γ, producing T cells required for resistance to *T gondii*[19]. The production of IL-4 and IFN-γ allows identification of the Th0, Th1, or Th2 orientation of helper T cell clones. The production of antigen-specific cytokines suggests that SAG1 is able to elicit anti-SAG1 specific CD4+T-cells. Cell immune response is involved in the protection of the parasites. In addition, IFN-γ producs CD8+CTL which plays a prominent role in controlling *T gondii* infection[20]. It is possible that at least part of the detected IFN-γ can lead to CD8+CTL activation. However, further experiments are required to elucidate this question.

In conclusion, SAG1 induces dominant antibody response and a strong Th1-like T cell response characterized by high titer IFN-γ production during infection. The protective role of SAG1 may be controlled cooperatively by humoral and cellular immune response. The evidence of SAG1 protection against challenge of the parasite proves that SAG1 is a good vaccine candidate for the control of toxoplasmosis.

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BASIC RESEARCH

Filtrate of fermented mycelia from *Antrodia camphorata* reduces liver fibrosis induced by carbon tetrachloride in rats

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Abstract

AIM: To investigate the effects of filtrate of fermented mycelia from *Antrodia camphorata* (FMAC) on liver fibrosis induced by carbon tetrachloride (CCl_4) in rats.

METHODS: Forty Wistar rats were divided randomly into control group and model group. All model rats were given 200 mL/L CCl_4 (2 mL/Kg, po) twice a week for 8 wk. Four weeks after CCl_4 treatment, thirty model rats were further divided randomly into 3 subgroups: CCl_4 and two FMAC subgroups. Rats in CCl_4 and 2 FMAC subgroups were treated with FMAC 0, 0.5 and 1.0 g/kg, daily via gastrogavage beginning at the fifth week and the end of the eighth week. Spleen weight, blood synthetic markers (albumin and prothrombin time) and hepatic malondialdehyde (MDA) and hydroxyproline (HP) concentrations were determined. Expression of collagen I, tissue inhibitor of metalloproteinases (TIMP)-1 and transforming growth factor β1 (TGF-β1) mRNA were detected by RT-PCR. Histochemical staining of Masson's trichrome was performed.

RESULTS: CCl_4 caused liver fibrosis, featuring increased prothrombin time, hepatic MDA and HP contents, and spleen weight and decreased plasma albumin level. Compared with CCl_4 subgroup, FMAC subgroup (1 g/kg) significantly decreased the prothrombin time (36.7 ± 7.2 and 25.1 ± 10.2 in CCl_4 and FMAC groups, respectively, $P < 0.05$) and increased plasma albumin concentration (22.7 ± 1.0 and 30.7 ± 2.5 in CCl_4 and FMAC groups, respectively, $P < 0.05$). Spleen weight was significantly lower in rats treated with CCl_4 and FMAC (1 g/kg) compared to CCl_4 treated rats only (2.7 ± 0.1 and 2.4 ± 0.2 in CCl_4 and FMAC groups, respectively, $P < 0.05$). The amounts of hepatic MDA and HP in CCl_4 ± FAMC (1 g/kg) subgroup were also lower than those in CCl_4 subgroup (MDA: 3.9 ± 0.1 and 2.4 ± 0.6 in CCl_4 and CCl_4 + FMAC groups, respectively, $P < 0.01$; HP: 1730.7 ± 258.0 and 1311.5 ± 238.8 in CCl_4 and CCl_4 + FMAC groups, respectively, $P < 0.01$). Histologic examinations showed that CCl_4 + FMAC subgroups had thinner or less fibrotic septa than CCl_4 group. RT-PCR analysis indicated that FMAC (1 g/kg) reduced mRNA levels of collagen I, TIMP-1 and TGF-β1 (collagen I: 5.63 ± 2.08 and 1.78 ± 0.48 in CCl_4 and CCl_4 + FMAC groups, respectively, $P < 0.01$; TIMP-1: 1.70 ± 0.82 and 0.34 ± 0.02 in CCl_4 and CCl_4 + FMAC groups, respectively, $P < 0.01$; TGF-β1:38.03 ± 11.9 and 4.26 ± 2.17 in CCl_4 and CCl_4 + FMAC groups, respectively, $P < 0.01$) in the CCl_4-treated liver.

CONCLUSION: It demonstrates that FMAC can retard the progression of liver fibrosis induced by CCl_4 in rats.

Key words: *Antrodia camphorata*; Liver fibrosis; Carbon tetrachloride

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INTRODUCTION

Antrodia camphorata is a new species of the genus *Antrodia* (*Polyporaceae*) parasitic in the inner cavity of the endemic species *Cinnamomum kanehirai* Hay[1]. Traditionally, it has been used as a remedy for food-, alcohol-, drug-intoxication, diarrhea, abdominal pain, hypertension, skin itching, and liver cancer among Chinese. The growth rate of natural *A. camphorata* in the wild is very slow, and it is difficult to cultivate in a green house, thus, it is expensive to obtain fruiting bodies. Therefore, using a submerged culture method to obtain useful cellular materials, or to produce effective substances from cultured mycelia might be a possible way to overcome the disadvantage of the retarded growth of fruiting bodies. In Taiwan, several biotechnology companies have developed the submerged culture method for *A. camphorata*. In the market of Taiwan, the yield of mycelia or culture filtrate of fermented mycelia

is dependent on the different biotechnology companies. Preliminary pharmacological studies revealed that the antioxidant abilities of the culture filtrate of fermented mycelia from *Antrodia camphorata* (FMAC) were correlated with their total polyphenols content based on the evaluation of different antioxidant system[2].

Liver fibrosis is the common end stage of most chronic liver disease regardless of the etiology[3], and its progression leads to cirrhosis and liver cancer. Although the exact mechanisms of pathogenesis in liver cirrhosis are still obscure, the role of the free radical and lipid peroxides has attracted considerable attention[4]. It has been found that the metabolism of CCl_4 involves the production of free radicals through its activation by drug metabolizing enzymes located in the endoplasmic reticulum[5]. CCl_4 is capable of causing liver lipid peroxidation, resulting in liver fibrosis[6]. Hsiao *et al*[7] reported that *A. camphorata* extract exerted protection against chronic chemical-induced hepatic injury in mice. In addition, Song *et al*[8] showed that FMAC possessed a protective activity against acute liver injury induced by CCl_4. However, the effect of FMAC in chronic liver disease is still unknown. In the present study, we attempted to assess the effect of FMAC on chronic CCl_4-induced liver fibrosis in rats.

MATERIALS AND METHODS

Preparation of test substance

FMAC was provided by Food Industry Research & Development Institute, Hsinchu, Taiwan. Culture of *Antrodia camphorata* BCRC 930032 was inoculated onto potato dextrose agar (PDA) and incubated at 30 ℃ for 15 to 20 d. The whole colony was then cut and put into the bottle with 50 mL sterile water. After homogenization, the fragmentated mycelia suspension was used as inoculum. The seed culture was prepared in a 20 L fermentor (BioTop) agitated at 150 r/min with aeration rate of 0.2 vvm and temperature of 30 ℃. A 5-d culture of 15 L mycelia inoculum was inoculated into a 250 L agitated fermentor (BioTop). The fermentation condition was the same as the seed fermentation but operating with an aeration rate of 0.075 vvm. The deep red culture filtrate was separated from the broth harvested at the 331st hour and poured through the non-woven fabric on a 20-mesh sieve. FMAC was concentrated about 20 fold (450 g/L) under reduced pressure at 50 ℃, and stored at -30 ℃ until use. FMAC was suspended in distilled water and administered orally to each rat at a volume of 10 mL/kg body weight.

Since antioxidant and anti-radical properties of plant extracts have been attributed to most phenolic compounds, it is expected that the effectiveness of the extracts is related to their phenolic content[9]. To guarantee the reproducibility of pharmacological experiments, the phenolic compounds in FMAC were determined by a modification of the method of Barness *et al*[10] using catechin as the standard. The concentration of phenolic groups in FMAC was 39.71 μg/mg.

Animals

Male Wistar rats were obtained from the National Laboratory of Animal Breeding and Research Center, National Science Council, and fed with a standard laboratory chow and tap water *ad libitum*. The experimental animals were housed in air-conditioned room of 21-24 ℃ with 12 h of light. The rats were allowed free access to powdered feed, and main water that was supplied through an automatic watering system. When they reached 250-300 g, the rats were used for experiments. Rats were divided randomly into control and model groups according to the body weight in proper range one day before administration of the test substance. All animals received humane care and the study protocols were in compliance with our institution's guidelines for use of laboratory animals.

CCl_4-induced liver fibrosis

Fibrosis was induced in thirty rats by an oral administration of 2 mL/kg body weight of 200 mL/L CCl_4 (diluted in olive oil) twice a week for 8 wk. At the end of 4 th wk after CCl_4 treatment, the CCl_4-treated rats were further divided into 3 subgroups based on the plasma alanine aminotransferase (ALT) level, since the plasma ALT is the major parameter for liver injury. The plasma ALT levels for normal control and 3 CCl_4-treated subgroups were 675 ± 62, 8856 ± 1321, 9005 ± 1659 and 8208 ± 1324 (nkat/L), respectively. The animals received CCl_4 with distilled water or FMAC (0.5, 1.0 g/kg; *po*, daily) which was added at the last four wk of the treatment. The time interval between CCl_4 and FMAC administrations were 5 h to avoid the disturbance of absorption of each other. After blood was drawn from rats at the eighth week, the animals were sacrificed at the same time and the liver and spleen were quickly taken off. They were then weighed after being clearly washed with cold normal saline and sucked up of the moisture. The largest lobe of liver was divided into four parts, and the same parts were 1) submerged in 40 g/L neutral formaldehyde for the preparation of pathological section; 2) after weighed, the liver was completely dried at 100 ℃ for the determination of collagen content; 3) the samples for RT-PCR analysis were kept in liquid nitrogen; 4) other sample was stored at -80 ℃ until assay.

Assessment of liver functions

The blood was centrifuged at 4700 r/min (Jouan BR4i, France) at 4 ℃ for 15 min to separate the plasma. The levels of plasma ALT and albumin were assayed using clinical test kits (Roche Diagnostics) spectrophotometrically (Cobas Mira; Roche, Rotkreuz, Switzerland). Prothrombin time was measured using a coagulation analyzer (Sysmex-CA1000) and reagent (Dade thromboplastin C plus).

Assays of hepatic lipid peroxidation and hydroxyproline

Livers were homogenized in nine volumes of ice-cold 0.15 mol/L KCl, 1.9 mmol/L ethylenediaminetetraacetic acid. The homogenate was used for the determination of lipid peroxidation. Lipid peroxidation was measured by the methods of Ohkawa *et al*[11] using 2-thiobarbituric acid. The lipid peroxidation was expressed as malondialdehyde (MDA) μmol/g protein. Protein was measured by the method of Lowry *et al*[12] using bovine serum albumin as the standard. Hydroxyproline (HP) determination fol-

Table 1 Primer sequences for PCR amplification

mRNA		Primer sequence	Length (bp)
Collagen I	Sense	5' CGA CTA AGT TGG AGG GAA CGG TC 3'	182
	Antisense	5' TGG CAT GTT GCT AGG CAC GAC 3'	
TIMP-1	Sense	5' TCC CTT GCA AAC TGG AGA GT 3'	140
	Antisense	5' GTC ATC GAG ACC CCA AGG TA 3'	
TGF-b1	Sense	5' TAT AGC AAC AAT TCC TGC CG 3'	162
	Antisense	5' TGC TGT CAC AGG AGC AGTG 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 2 Effect of FMAC on plasma albumin concentration and prothrombin time in CCl_4-treated rats

Group	Dose (g/kg per d)	Albumin (g/L)	Prothrombin time (sec)
Control	–	36.0 ± 1.3	17.7 ± 0.9
CCl_4 + H_2O	–	22.7 ± 1.0[b]	36.7 ± 7.2[b]
CCl_4 + FMAC	0.5	23.1 ± 5.1	28.5 ± 9.9
	1	30 .7± 2.5[a]	25.1 ± 10.2[a]

[a]$P < 0.05$ *vs* CCl_4 + H_2O group; [b]$P < 0.01$ *vs* control group.

Table 3 Effect of FMAC on spleen weight, hepatic malondialdehyde and hydroxyproline contents in CCl_4-treated rats

Group	Dose (g/kg per d)	Spleen (g)	Malondialdehyde (μmol/g protein)	Hydroxyproline (μg/g tissue)
Control	–	1.1 ± 0.1	1.9 ± 0.1	645.0 ± 64.5
CCl_4 + H_2O	–	2.7 ± 0.1[d]	3.9 ± 0.1[d]	1730.7 ± 258.0[d]
CCl_4 + FMAC	0.5	2.8 ± 0.2	2.7 ± 0.1	1741.5 ± 257.1
	1	2.4 ± 0.2[a]	2.4 ± 0.6[b]	1311.5 ± 238.8[b]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* CCl_4 + H_2O group; [d]$P < 0.001$ *vs* control group.

lowed a method designed by Neuman *et al*[13]. Dried liver tissue after hydrolysis was oxidized by H_2O_2 and colored by p-dimethylaminobenzoaldehyde and absorbance was determined at 540 nm. The amount of HP is expressed in mg/g wet tissue.

RNA extraction and RT-PCR analysis

Total RNA was isolated from livers of the rats using the acid guanidum thiocyanate-phenol-chloroform extraction methods as described by Chomczynski *et al*[14]. Five micrograms of total RNA from each liver sample were subjected to reverse transcription (RT) by MMuLV reverse transcriptase in a 50 μL reaction volume. Aliquots of the reverse transcription mix were used for amplification by polymerase chain reaction (PCR) of fragments specific to collagen I, transforming growth factor (TGF)-β1 and tissue inhibitor of matrix metalloproteinase (TIMP)-1 using the primer pairs listed in Table 1. The levels of expression of all the transcripts were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in the same tissue sample. PCR product was run on a 20 g/L agarose gel recorded by polarid film; bands were quantitated by densitometer.

Pathological examinations

For histopathological examination, the formalin-fixed liver was embedded in paraffin, cut into 4-5 μm thick sections, stained with Masson's trichrome. Fibrosis was graded according to the method of Ruwart *et al*[15], 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; 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 without knowledge of sample treatment.

Statistical analysis

Data were presented as mean ± SD. All other experimental data, except the pathological findings, were treated by one-way analysis of variance using the Dunnett's test. Liver histopathological examination data were analyzed by the Kruskall-Wallis non-parametric test, followed by a Mann–Whitney *U*-test. The significance level was set at $P < 0.05$.

RESULTS

Concentrations of plasma albumin and prothrombin time

The plasma albumin concentrations were lower in rats given CCl_4 than that in the control group (Table 2). While in the rats treated by FMAC (1 g/kg), the levels of plasma albumin was markedly higher than that in the CCl_4 model group. The prothrombin time in the CCl_4 model group was much longer than that in control group. FMAC (1 g/kg) significantly shortened the prothrombin time.

Weights of spleen

Marked splenomegaly was caused by CCl_4 treatment; the weight of spleen in the CCl_4-treated group was about 245% of the control group (Table 3). The increase of spleen weight by CCl_4 treatment was significantly reduced by FMAC (1 g/kg).

Liver MDA and HP contents

CCl_4 induced liver fibrosis to the rats resulting in a marked increase of hepatic MDA and HP contents (Table 3). FMAC (1 g/kg) treatment significantly reduced the increase of hepatic MDA and HP contents caused by CCl_4.

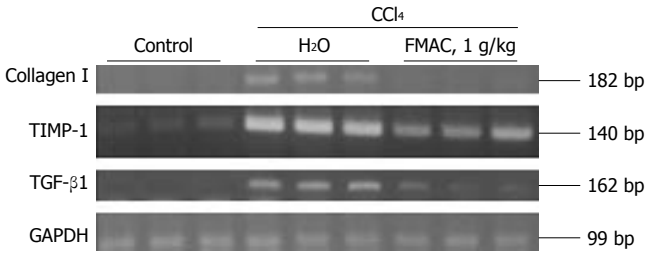

Figure 1 Effect of FMAC on the hepatic mRNA expressions of collagen I, TIMP-1 and TGF-β1 in CCl_4-treated rats.

Table 4 Effect of FMAC on hepatic mRNA expressions of collagen I, TIMP-1 and TGF-β1 in CCl_4-treated rats

Group	Dose (g/kg per d)	Collagen I: GAPDH ratio	TGF-β1: GAPDH ratio	TIMP-1: GAPDH ratio
Control	–	0.68 ± 0.55	0.38 ± 0.18	0.14 ± 0.08
CCl_4 + H_2O	–	5.63 ± 2.08[b]	38.03 ± 11.9[b]	1.70 ± 0.82[b]
CCl_4 + FMAC	0.5	3.94 ± 1.18	28.90 ± 7.22	0.58 ± 0.02
	1	1.78 ± 0.48[d]	4.26 ± 2.17[d]	0.34 ± 0.02[d]

[b]$P < 0.001$ *vs* Control group; [d]$P < 0.01$ *vs* CCl_4 + H_2O group.

RT-PCR analysis of liver tissue

Fragments specific to collagen I, TIMP-1 and TGF-β1 were amplified by RT-PCR (Figure 1). The values from densitometric analysis, after normalization against the corresponding GAPDH transcript were expressed as the collagen I/GAPDH, TIMP-1/GAPDH and TGF-β1/GAPDH ratios. The levels of collagen I, TIMP-1 and TGF-β1 mRNA in rat liver were significantly increased by CCl_4 treatment (Table 4), while the administration of FMAC (1 g/kg) significantly decreased the levels of collagen I, TIMP-1 and TGF-β1 mRNA.

Pathological examination

CCl_4 induced liver damage of the rats. Masson's stain showed clear nodular fibrosis at the central vein and the portal vein area (Figure 2B). Treatment of FMAC (1 g/kg) showed marked improvement of these pathological changes of the tissues (Figure 2C and Table 5).

DISCUSSION

The results of the present study indicate that even after the initiation of hepatic fibrosis in a rat model of CCl_4-induced liver damage, FMAC administration reduced liver fibrosis, as demonstrated by smaller increases in hepatic collagen and lower mRNA expression of collagen I compared with CCl_4 model group. These effects were mainly observed when FMAC was administered from wk 5 to wk 8 of CCl_4 treatment. Both plasma albumin and blood clotting factors were mainly synthesized in the liver. When the chronic liver damage led to fibrosis, the albumin contents dropped and prothrombin time prolonged[16,17]. In this experiment, CCl_4 induced chronic liver lesions in rats and there appeared a decrease of plasma albumin and an increase of prothrombin time. FMAC clearly counteracted both the decrease of albumin content in the plasma and the prolongation of prothrombin time. These results showed that FMAC ameliorated the decline of liver synthetic functions caused by chronic liver injuries.

Liver fibrosis or cirrhosis leads to blockage of blood flow into the liver and causes portal hypertension and it also influences the blood flow of spleen and gives rise to splenomegalia[18]. CCl_4 in this experiment induced chronic

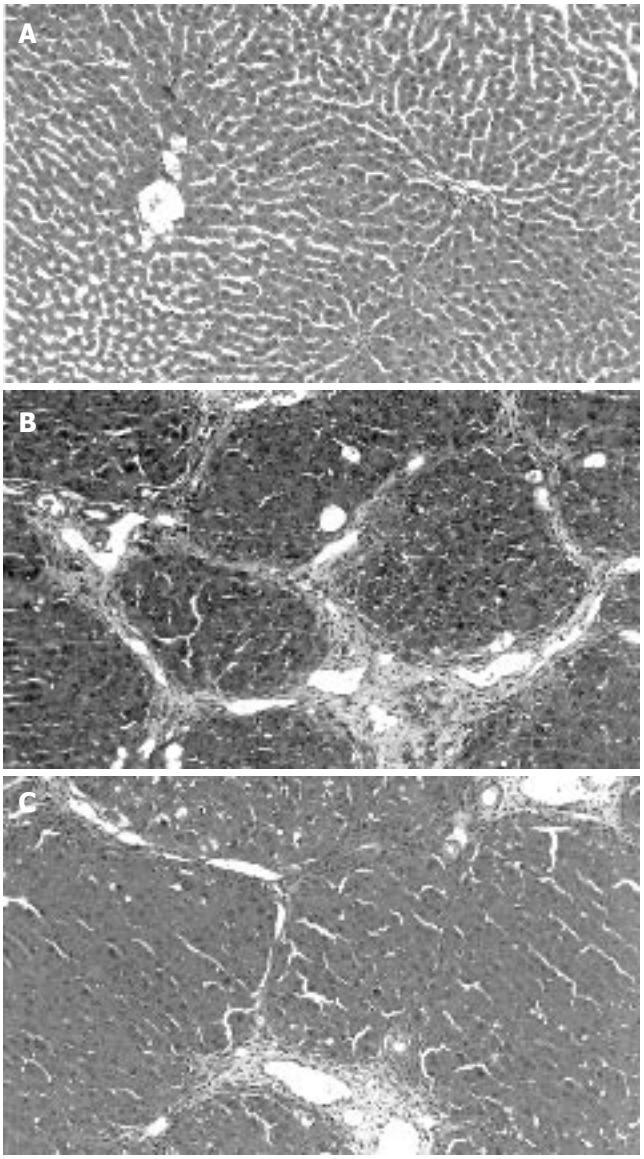

Figure 2 Liver histopathology of rats (Masson's stain). **A**: control group; **B**: CCl_4 + H_2O group, showing micronodular formation and complete septa interconnection with each other; **C**: CCl_4 + FMAC (1 g/kg) group, showing a marked reduction in fiber deposition. Scale bar = 50 μm.

Table 5 Effect of FMAC on CCl4-induced liver fibrosis in rats

Group	Dose (g/kg per d)	Score of hepatic fibrosis 0	1	2	3	4	Average
Control	–	10	0	0	0	0	0
CCl_4 + H_2O	–	0	0	3	6	1	2.8 ± 0.6
CCl_4 + FMAC	0.5	0	1	3	5	1	2.7 ± 0.8
	1.0	0	4	5	1	0	1.7 ± 0.7[b]

[b]$P < 0.01$ *vs* CCl_4 + H_2O group.

hepatic fibrosis as well as splenomegalia. FMAC could improve splenomegalia, indicating that it might ameliorate portal hypertension.

It is well known that liver fibrosis is a result of increased collagen synthesis[19], and HP is the unique component in collagen[19]. The amount of collagen can be reflected by the contents of HP and can be used to express the extent of fibrosis[19]. When CCl_4 was applied in this experiment to induce liver fibrosis, the content of HP in liver obviously increased. FMAC could reduce the content of HP, which was confirmed by the histopathological examinations. Many studies have shown that level of collagen I increases during liver fibrosis[20]. Therefore, we also investigated the effect of FMAC on the mRNA expression of collagen I. Treatment with FMAC was effective in reducing the amount of collagen I mRNA expression. This result further confirmed that FMAC could remit hepatic fibrosis.

Regardless of the etiologic factors, gross remodeling of extracellular matrix in the fibrotic liver is regulated by a balance of synthesis and enzymatic degradation of extracellular matrix[21]. Matrix degradation is catalyzed by the activity of matrix metalloproteinases. The activities of matrix metalloproteinases are inhibited by tissue inhibitors of metalloproteinases (TIMPs). The expression of TIMPs drastically increased or decreased with time during liver fibrogenesis and fibrosis resolution, respectively[22]. Four members of the TIMP family have been characterized so far and designated as TIMP-1 to TIMP-4[23]. It has been suggested that TIMP-1 plays an important role in the pathogenesis of liver fibrosis[24]. Consistent with previously published work[25], we observed elevated levels of TIMP-1 upon treatment with CCl_4. Treatment with FMAC was effective in reducing the level of TIMP-1 expression, indicating liver fibrosis resolution might be enhanced. This result supported that FMAC could suppress liver fibrotic progression caused by CCl_4.

TGF-β1 is a profibrogenic cytokine, because it directly stimulates extracellular matrix production by both Kupffer cells and stellate cells[26,27]. Increased levels of TGF-β1 mRNA expression have been found in patients with liver fibrosis as well as in experimental models of liver fibrosis[28,29]. 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[30]. In this experiment, CCl_4 treatment increased, while FMAC significantly reduced TGF-β1 mRNA expression. This result suggested that FMAC ameliorated liver fibrosis perhaps by reducing TGF-β1 secretion.

Increased free radical production and lipid peroxidation have been proposed as a major cellular mechanism involved in CCl_4 hepatotoxicity[5]. Furthermore, a close relationship has been reported between lipid peroxidation and fibrogenesis in rats, in which fibrosis was induced by CCl_4 administration[6]. Our results confirmed these findings that hepatic lipid peroxidation is increased during hepatic fibrogenesis. We also found that FMAC inhibited CCl_4-induced hepatic lipid peroxidation. These results indicated that FMAC might inhibit lipid peroxidation, and consequently attenuate the development of liver fibrosis. A large number of studies indicated that FMAC is a good free radical scavenger[3, 31].

In conclusion, the present study has demonstrated that FMAC retards the progression of liver fibrosis in CCl_4-treated rats possibly by scavenging free radicals formed in the liver. It may be expected that FMAC has preventive potentials in liver fibrosis.

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BASIC RESEARCH

Expression and activity of inducible nitric oxide synthase and endothelial nitric oxide synthase correlate with ethanol-induced liver injury

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Abstract

AIM: To study the expression and activity of inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) in rats with ethanol-induced liver injury and their relation with liver damage, activation of nuclear factor-κB (NF-κB) and tumor necrosis factor-α (TNF-α) expression in the liver.

METHODS: Female Sprague-Dawley rats were given fish oil (0.5 mL) along with ethanol or isocaloric dextrose daily via gastrogavage for 4 or 6 wk. Liver injury was assessed using serum alanine aminotransferase (ALT) activity and pathological analysis. Liver malondialdehyde (MDA), nitric oxide contents, iNOS and eNOS activity were determined. NF-κB p65, iNOS, eNOS and TNF-α protein or mRNA expression in the liver were detected by immunohistochemistry or reverse transcriptase-polymerase chain reaction (RT-PCR).

RESULTS: Chronic ethanol gavage for 4 wk caused steatosis, inflammation and necrosis in the liver, and elevated serum ALT activity. Prolonged ethanol administration (6 wk) enhanced the liver damage. These responses were accompanied with increased lipid peroxidation, NO contents, iNOS activity and reduced eNOS activity. NF-κB p65, iNOS and TNF-α protein or mRNA expression were markedly induced after chronic ethanol gavage, whereas eNOS mRNA expression remained unchanged. The enhanced iNOS activity and expression were positively correlated with the liver damage, especially the necro-inflammation, activation of NF-κB, and TNF-α mRNA expression.

CONCLUSION: iNOS expression and activity are induced in the liver after chronic ethanol exposure in rats, which are correlated with the liver damage, especially the necro-inflammation, activation of NF-κB and TNF-α expression. eNOS activity is reduced, but its mRNA expression is not affected.

Key words: Alcoholic liver disease; Inducible nitric oxide synthase; Endothelial nitric oxide synthase; Nuclear factor-κB

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INTRODUCTION

Nitric oxide (NO) has been recognized as an important mediator of physiological and pathophysiological processes. It is produced by at least two isoforms of nitric oxide synthase (NOS) in the liver, such as eNOS and iNOS. eNOS is a Ca^{2+}- and calmodulin-dependent constitutive isoform and plays an important role in vasorelaxation, whereas iNOS is not a constitutive enzyme and its expression may be induced by stimuli such as lipopolysaccharide or proinflammatory cytokines[1,2].

The role of NO in alcohol-induced liver injury still remains controversial. Nanji *et al*[3] reported that arginine, a substrate for NO, can significantly attenuate ethanol-induced liver injury. Treatment with N-nitro-L-arginine methyl ester (L-NAME), a nonselective NOS inhibitor, enhances alcohol-induced liver injury in the Tsukamoto-French enteral rat model[4]. However, iNOS knockout mice or wild-type mice treated with N-(3-aminomethyl) benzyl-acetamindine (1400W), a highly selective iNOS inhibitor, are protected against liver damage caused by alcohol[5]. Uzun *et al*[6] showed that L-NAME might produce a restorative effect on ethanol-induced liver damage.

Nuclear factor-κB (NF-κB) is a ubiquitous transcription factor that plays an important role in regulation of inflammatory responses. NF-κB is composed of homo- and hetero-dimers of five members of the Rel family, including NF-κB1 (p50), NF-κB2 (p52), Rel A (p65), Rel B, and Rel C. The most prevalent activated form of NF-

κB is a heterodimer consisting of a p50 or a p52 subunit and p65. NF-κB exists in cytoplasm in an inactive form associated with regulatory proteins called IκB. After stimulation, it is translocated to the nuclei and bound to decameric DNA sequences, and activates transcription of target genes[7]. NF-κB has been shown to be functionally important for iNOS induction[8]. In the present study, we used fish oil plus ethanol gavage model of alcoholic liver disease, and examined the expression and activity of iNOS and eNOS in the liver, and their relation with liver damage, activation of NF-κB and TNF-α expression.

MATERIALS AND METHODS

Chemicals and reagents

Polyclonal rabbit anti-iNOS and anti-NF-κB p65 were obtained from Santa Cruz Biotechnology, Inc. Biotinylated goat-anti-rabbit IgG was purchased from Beijing Zhongshan Reagent Corp. TRIzol reagent was purchased from Invitrogen. DL2000 DNA ladder marker was from TaKaRa Biotech Co., Ltd. M-MLV reverse transcriptase and its buffer, deoxyribonucleotide (dNTP, 10 mmol/L) and oligo(dT)$_{15}$ primer were from Promega Corp. Taq DNA polymerase and its buffer, rRNasin ribonuclease inhibitor was from Biostar. Polymerase chain reaction (PCR) primers for eNOS, iNOS, TNF-α and GAPDH, were synthesized by Sai-Bai-Sheng Biocompany (Shanghai, China). Malondialdehyde (MDA), nitric oxide (NO) and nitric oxide synthase (NOS) activity assay kits were purchased from Nanjing Jiancheng Bioengineering Co.Ltd, China.

Animal model

Female Sprague-Dawley rats weighing 200-250 g, were obtained from the Experimental Animal Center of Wuhan University. After acclimation for 6-7 d, animals were randomly divided into 4-wk dextrose group (n = 5), 6-wk dextrose group (n = 5), 4-wk ethanol group (n = 8), and 6-wk ethanol group (n = 8). Rats were given 0.5 mL fish oil along with ethanol or isocaloric dextrose intragastrically by gavage. The initial dose of ethanol was 6 g/kg per day (solutions maximally containing 56 mL/100 mL alcohol), and the dose was progressively increased during wk 1 to a maintenance dose of 8 g/kg per day that was continued for another 3 or 5 wk. All rats had free access to regular standard rat chow throughout the experiment. The animals were weighed three times per wk. At the end of the experiment, the animals were anaesthetized with urethane (20%, 1.0 g/kg) and sacrificed by bleeding from femoral arteries and veins. Blood samples were collected. Immediately after exsanguination, the livers were harvested. Small portions of the liver were kept at -70 ℃ for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, whereas another portion was separated and immersed in 10% buffered formalin solution for histological and immunohistochemical examination. All animals were given humane care in compliance with the institutional guidelines.

Pathological evaluation

Liver specimens, 1.0 cm×0.5 cm×0.3 cm in size, were processed for light microscopy. This processing consisted of fixing the specimens in 10% formaldehyde for 12-24 h, embedding them in paraffin, slicing sections of 5 μm in thickness and staining the sections with hematoxylin and eosin. Histological assessment was performed by a pathologist unaware of the study. The severity of liver pathology was assessed as follows[9]: steatosis (the percentage of liver cells containing fat), 1+, <25% of cells containing fat; 2+, 26%-50% of cells containing fat; 3+, 51%-75% of cells containing fat; and 4+, >75% of cells containing fat. Necrosis was evaluated as the number of necrotic foci/mm^2 and inflammation was scored as the number of inflammatory cells/mm^2.

Serum alanine aminotransferase assay

Blood samples were allowed to clot, and the sera were isolated by centrifugation at 1000 r/min for 10 min and kept at -20 ℃ before determination. Enzymatic activity of alanine aminotransferase (ALT) was measured using a commercial kit by an RA 1000 automatic biochemical analyzer (Japan).

Liver MDA contents and NOS activity assay

Liver samples were thawed, weighed and homogenized 1 : 9 w:v in 0.9% saline. Then the homogenates were centrifuged at 3 000 r/min for 10 min at 4 ℃ and the supernatant was taken for the assays of MDA contents, NOS activity and total protein.

MDA was assayed by measuring the thiobarbituric acid-reactive substances (TBARS) levels spectrophotometrically at 532 nm. Results were expressed as nmol.mg^{-1} protein.

NOS catalyzed the formation of NO and L-citrulline from L-arginine and molecular oxygen, and NO reacted with a nucleophile to generate color compounds. The absorbance at 530 nm NOS activity was calculated and expressed as U/mg protein. One unit of NOS activity was defined as the production of 1 nmol nitric oxide per second per mg tissue protein. Total NOS activity was measured as follows: 10% tissue homogenate (100 μL) was incubated with 200 μL substrate buffer, 10 μL reaction accelerator and 100 μL color development reagent at 37 ℃ for 15 min after mixing. Then 100 μL clearing reagent and 2 mL stop solution were added, mixed and absorbances were read at 530 nm. For measuring iNOS activity, an inhibitor was added before incubation according to the manufacturer's instructions.

Total protein concentration was determined using the Coomassie blue method with bovine serum albumin as standard.

Liver NO assay

Liver samples were thawed, weighed and homogenized 1 : 9 w:v in 0.9% saline. The homogenates were then centrifuged at 1 000 r/min for 5 min at 4 ℃, the supernatant was taken for NO assay and total protein determination.

NO was assayed spectrophotometrically by measuring total nitrate plus nitrite (NO_3^- plus NO_2^-) and the stable end products of NO metabolism. In the procedure nitrate was enzymatically converted into nitrite by the enzyme nitrate reductase, followed by quantitation of nitrite using

Table 1 PCR primers used for iNOS, eNOS, TNF-α and GAPDH

Name	Sense	Antisense	Product length (bp)
iNOS	TTCTTTGCTTCTGTGCTAATGCG	GTTGTTGCTGAACTTCCAATCGT	1061
eNOS	TGGGCAGCATCACCTACGATA	GGAACCACTCCTTTTGATCGAGTTAT	202
TNF-α	GCCAATGGCATGGATCTCAAAG	CAGAGCAATGACTCCAAAGT	357
GAPDH	TCCCTCAAGATTGTCAGCAA	AGATCCACAACGGATACATT	309

Griess reagent at the absorbance of 550 nm as previously described[10]. Results were expressed as μmol/g protein.

Immunohistochemical detection of iNOS and NF-kB p65 in liver

Five μm thick sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 3% H_2O_2 in deionised water for 5-10 min. Nonspecific binding sites were blocked by incubating the sections in 10% normal rabbit serum for 10-15 min. The sections were then incubated with polyclonal rabbit anti-iNOS (dilution 1:25) or anti-NF-kB p65 (dilution 1:100) overnight at 4 ℃, followed by incubation with biotinylated goat-anti-rabbit IgG at room temperature for 10-15 min. After 3×3 min PBS rinses, the horseradish-peroxidase-conjugated streptavidin solution was added and incubated at room temperature for 10-15 min. The antibody binding sites were visualized by incubation with a diaminobenzidine-H_2O_2 solution. The sections incubated with PBS instead of the primary antibody were used as negative controls. Brown-yellow granules in cytoplasm or nuclei were recognized as positive staining for iNOS or NF-kB p65 respectively. NF-kB immunoreactivity was expressed as the number of positive cells/high-power field (× 400).

RT-PCR analysis of iNOS, eNOS and TNF-α mRNA expression in liver

Total RNA was isolated from approximatively 50-100 mg snap-frozen liver tissue using the TRIzol protocol as suggested by the supplier. Following precipitation, the RNA was resuspended in RNAse-free buffer, the concentration was assayed by measuring ultra-violet light absorbance at 260 nm and purity was estimated from the ratio of A_{260}/A_{280}.

Single-stranded complementary DNA (cDNA) was synthesized from the total RNA using the following method. In brief, 2 μg RNA was preincubated with 0.5 μg oligo(dT)$_{15}$ primer and diethylpyrocarbonate (DEPC)-treated water was added to a total volume of 15 μL at 70 ℃ for 5 min, then rapidly chilled on ice. To the annealed primer/template 5 μL M-MLV 5×reaction buffer, 1.25 μL dNTP (10 mmol/L, each), 25 units of rRNasin ribonuclease inhibitor, 200 units of M-MLV RT and DEPC-treated water were added to a final volume of 25 μL. The reaction was incubated at 42 ℃ for 60 min and terminated by placing it on ice after deactivation at 85 ℃ for 5 min. The resulting cDNA was used as a template for subsequent PCR.

The PCR mixture contained 5 μL of 10×Taq buffer, 1 μL of dNTP (10 mmol/L, each), 1 μL of gene specific primers (Table 1, sense and anti-sense primers, 25 pmol/μL, each), 2.0 units of Taq DNA polymerase and 1 μL of cDNA in a total volume of 50 μL. Thirty-five cycles of amplification were performed with initial incubation at 94 ℃ for 3 min and a final extension at 72 ℃ for 7 min, each cycle consisted of denaturation at 94 ℃ for 45 s, annealing at 54 ℃ for 45 s and extension at 72 ℃ for 1 min. To ensure the use of equal amounts of cDNA from each group samples in PCR, the aliquots of the reverse transcription products were used in PCR with the primers for house-keeping gene GAPDH. The quantities of cDNA producing equal amounts of GAPDH-PCR-product were used in PCR with the primers for iNOS, eNOS and TNF-α. Following RT-PCR, 5 μL samples of amplified products was resolved by electrophoresis on 2% agarose gel and stained with ethidium bromide. The level of each PCR product was semi-quantitatively evaluated using a digital camera and an image analysis system (Vilber Lourmat, France), and normalized to GAPDH.

Statistical analysis

Results were presented as mean ± SD unless otherwise indicated. Differences between groups were analyzed using analysis of variance with *post hoc* analysis using LSD test. The correlation was analyzed with Spearman's correlation coefficients. $P < 0.05$ was considered statistically significant.

RESULTS

In each of the four groups, the rats increased their weight at a constant rate. There was no difference in weight gain among the groups.

Pathological changes and serum aminotransferase activity

The animals given fish oil plus dextrose developed slight steatosis in the liver, but no obvious inflammation or necrosis was observed (Figure 1A). However, chronic ethanol gavage for 4 wk caused steatosis, minimal to mild inflammation and necrosis in the liver (Figure 1B). Prolonged ethanol administration (6 wk) enhanced the liver damage. Pronounced macrovesicular and microvesicular steatosis as well as spotty necrosis and mild inflammation were observed (Figure 1C, Table 2).

Consistent with the histological changes, serum ALT levels, an index of liver cell injury, were significantly increased in 4-wk ethanol group, and further increased in 6-wk ethanol group as compared with dextrose groups (Table 2).

Liver MDA and NO contents

Liver contents of MDA, a marker of lipid peroxidation, were significantly increased after 4 wk ethanol gavage

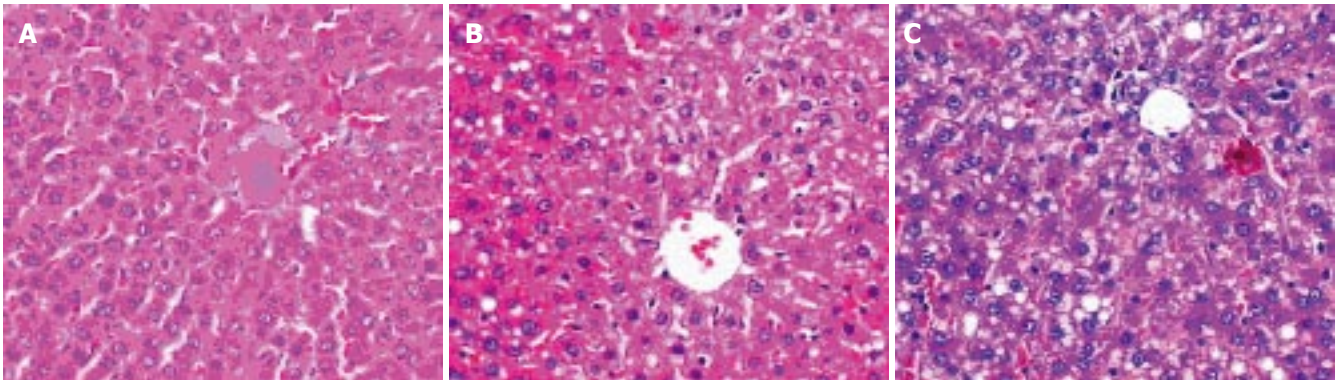

Figure 1 Representative pathologic changes in 4-wk dextrose group (**A**), 4-wk ethanol group (**B**), and 6-wk ethanol group (**C**). Original magnification, ×200.

Table 2 Pathological scores, serum ALT levels and liver MDA contents in different experimental groups (mean ± SD)

Groups	Fatty liver	Necroinflammation	ALT(U/L^{-1})	MDA(nmol/mg^{-1} protein)
4-wk dextrose	0.2±0.25	1.0±1.0	43.4±3.05	4.83±0.70
4-wk ethanol	2.1±0.64[a]	16.1±2.17[a]	117.3±6.92[a]	8.64±0.62[a]
6-wk dextrose	0.6±0.35	1.6±1.52	46.0±3.16	5.36±0.31
6-wk ethanol	2.9±0.64[b]	19.9±4.29[b]	124.1±7.28[b]	9.38±0.39[b]

[a]$P<0.05$ *vs* 4-wk dextrose group; [b]$P<0.05$ *vs* 6-wk dextrose group.

Table 3 Liver NO contents, iNOS and eNOS activity in different experimental groups (mean ± SD)

Groups	NO (μmol/g^{-1} protein)	iNOS (U/mg^{-1} protein)	eNOS(U/mg^{-1} protein)
4-wk dextrose	0.75±0.14	0.27±0.07	0.64±0.06
4-wk ethanol	1.67±0.15[a]	0.60±0.07[a]	0.48±0.03[a]
6-wk dextrose	0.87±0.07	0.35±0.06	0.58±0.04
6-wk ethanol	1.84±0.12[b]	0.70±0.09[b]	0.43±0.05[b]

[a]$P<0.05$ *vs* 4-wk dextrose group; [b]$P<0.05$ *vs* 6-wk dextrose group.

compared with dextrose groups. Prolonged ethanol exposure (6 wk) led to a further increase in MDA contents (Table 2).

Levels of NO in the liver of two dextrose groups were 0.75 ± 0.14 and 0.87 ± 0.07 μmol/g protein, respectively. Chronic ethanol gavage-induced NO level was two-fold higher in 4-wk ethanol group and further higher in 6-wk ethanol group (Table 3).

Liver NOS activity

The isoforms of NOS present in the liver were mainly eNOS and iNOS as previously reported[2]. The amount of total NOS activity minus iNOS activity might represent the activity of eNOS. Chronic fish oil plus ethanol gavage led to a marked elevation in iNOS activity with further elevation in 6-wk ethanol group compared with 4-wk ethanol group. In contrast, the eNOS activity was significantly reduced compared with dextrose groups (Table 3).

Expression of NF-κB p65 in liver

NF-κB p65 staining was present in cytoplasm and nuclei, but only nuclear staining was considered positive. There was no positive staining in two dextrose groups, whereas following chronic ethanol administration, remarkable enhancement in the positive staining was observed (Figures 2A and 2B). The number of positive cells in 4-wk and 6-wk ethanol groups was 8.0 ± 1.1 and 10.0 ± 1.9 /high-power field, respectively (Figure 2C). The NF-κB p65 positive cells were primarily Kupffer cells and hepatocytes.

iNOS, eNOS and TNF-α expression in liver

Only faint immunoreactive staining of iNOS was detected in the liver from dextrose groups (Figure 3A). However, intense staining of iNOS was observed in 4-wk ethanol group (Figure 3B), and more intense staining was found in 6-wk ethanol group (Figure 3C). The staining was mainly present in both severely damaged and perivascular areas.

iNOS mRNA was barely detectable in the liver of dextrose groups, but after chronic ethanol gavage (4 wk), iNOS mRNA was markedly induced. Prolonged ethanol gavage (6 wk) was associated with more intense bands (Figure 4). In dextrose groups only faint bands for TNF-α were detected. After chronic ethanol gavage, similar

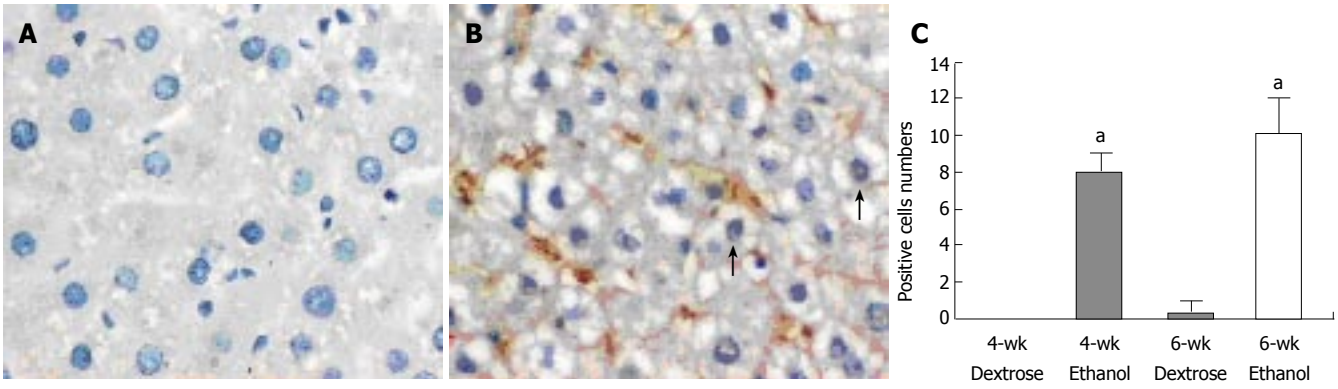

Figure 2 Expression of NF-κB p65 in 6-wk dextrose group (**A**), 6-wk ethanol group (**B**) and the number of positive cells in high-power fields (**C**). [a] $P < 0.01$ *vs* dextrose group. Original magnification, ×400.

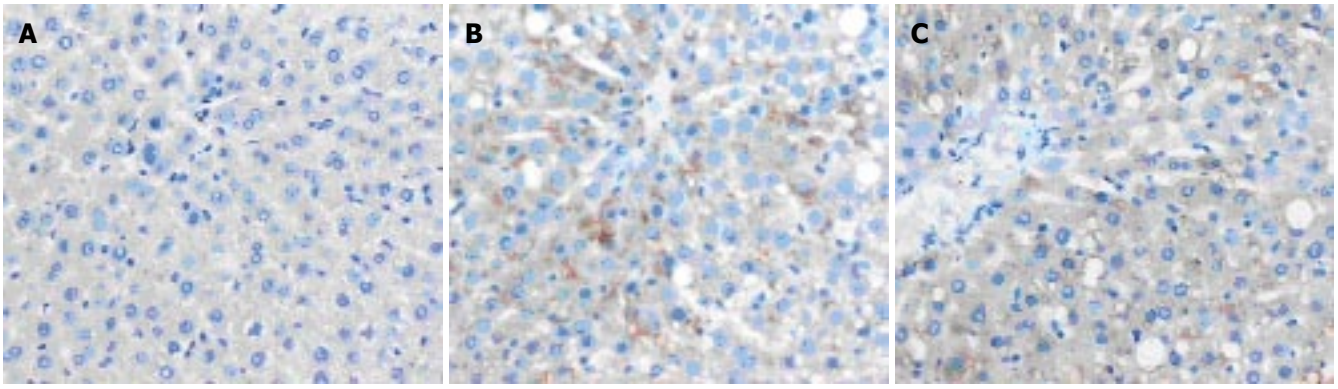

Figure 3 Immunohistochemical detection of iNOS in 6-wk dextrose group (**A**), 4-wk ethanol group (**B**), and 6-wk ethanol group (**C**). Original magnification, ×200.

expression pattern of iNOS was also observed in TNF-α mRNA expression (Figure 4).

In contrast, there was no significant change in eNOS mRNA expression between dextrose and ethanol groups (Figure 4).

Relationship between liver iNOS activity, expression and other parameters

Correlation analysis showed that liver iNOS activity was positively correlated with the severity of liver damage (steatosis, necroinflammation) ($r = 0.71$ and 0.93, respectively, $P < 0.05$), especially the necroinflammation. iNOS expression was only detected in rats with liver damage, activation of NF-κB and intense TNF-α mRNA expression. The intensity of the former paralleled that of the latter.

DISCUSSION

It has been reported that dietary fatty acids play an important role in the pathogenesis of alcoholic liver disease[11,12]. Polyunsaturated fatty acids enriched in fish oil promote alcoholic liver injury and pathological changes occur only in rats fed with ethanol containing polyunsaturated fatty acids[13]. Alcoholic liver injury is more severe and develops rapidly in women than in men[14]. Our study employed female rats and used fish oil plus ethanol gavage to make an animal model of alcoholic liver injury. The rats developed pathological changes in the liver after 4 or 6 wk, such as steatosis, spotty necrosis and inflammation, all of which resemble alterations found in clinical alcoholic liver disease. This chronic gavage of alcohol in rats is a simple experimental model that mimics key aspects of alcoholic liver disease in humans, and is useful for exploring the mechanism and treatment of alcoholic liver disease.

NO is an important biological mediator and has been shown to be involved in diverse physiological as well as pathological processes[1]. In our study, chronic ethanol gavage led to a significant elevation of liver NO contents compared to dextrose groups. NO is generated by NOS. We assayed the activity of total NOS and iNOS in liver. Because the main isoforms of NOS in the liver are eNOS and iNOS[2], the amount of total NOS activity minus iNOS activity may represent the activity of eNOS. Our study showed that iNOS activity was significantly elevated after chronic ethanol consumption in rats, whereas eNOS activity was markedly reduced as compared with dextrose groups. Accompanying the enhanced activity, iNOS expression detected by immunohistochemistry and RT-PCR in the liver was significantly increased in ethanol groups compared to dextrose groups. However, the eNOS mRNA expression was comparable between these groups. The results suggest that the elevated NO release in the liver is attributable to the enhanced activity and expression of iNOS. Relationship analysis showed that enhanced

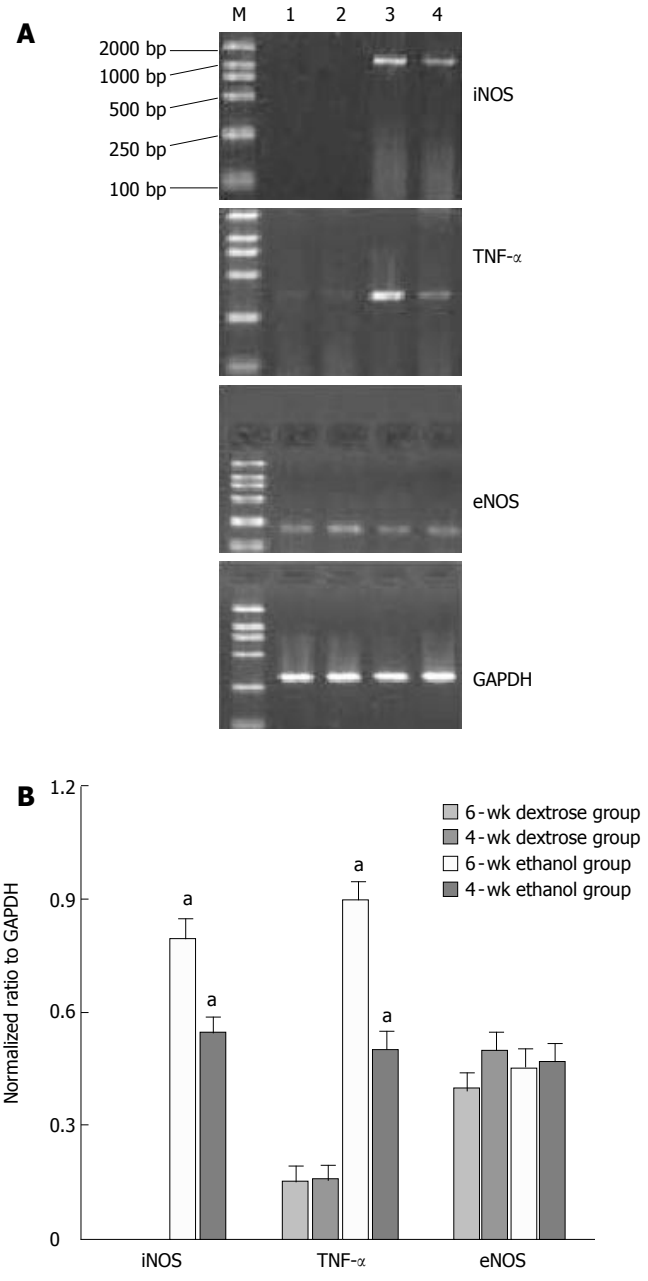

Figure 4 RT-PCR analysis of mRNAs for iNOS, eNOS and TNF-α in the liver. **A**: representative bands for iNOS, TNF-α, eNOS and GAPDH transcripts (lane 1: 6-wk dextrose group, lane 2: 4-wk dextrose group, lane 3: 6-wk ethanol group, lane 4: 4-wk ethanol group); **B**: normalized densitometric ratios of iNOS, TNF-α and eNOS transcripts to GAPDH. [a] $P<0.01$ *vs* dextrose group.

iNOS activity and expression were associated with the severity of liver damage, especially the necroinflammation, suggesting that iNOS contributes to alcohol-induced liver injury. It was reported that iNOS knockout mice or wild-type mice treated with 1400W, a highly selective iNOS inhibitor, are protected against alcohol-induced liver injury[5]. In contrast with iNOS, eNOS activity is reduced after treatment with ethanol. A recent report showed that chronic alcohol intake attenuates hepatic eNOS activity by increasing the expression of the inhibitory protein caveolin-1 and enhancing its binding to eNOS[15]. Treatment with L-NAME, the stronger eNOS inhibitor, exacerbates alcohol-induced liver injury[4]. These facts suggest that the role of NO in ethanol-induced liver injury may be dependent on the isoforms of NOS.

Oxidant stress has been reported to play a role in the pathogenesis of alcohol-induced liver injury[16]. In this study, liver contents of MDA, a marker of lipid peroxidation, were significantly elevated in the ethanol groups as compared with dextrose groups. Oxidant stress can result in degradation of the cytoplasmic NF-κB inhibitor, IκB, allowing translocation of NF-κB to nuclei[17]. Our study showed that chronic ethanol gavage significantly enhanced the expression of active NF-κB in the liver, as evidenced by the increased number of NF-κB p65 positively stained (in nuclei) cells. Furthermore, TNF-α mRNA expression in the liver was markedly increased. These results are consistent with the report of Nanji *et al*[18], who showed that NF-κB is activated during alcoholic liver disease in the presence of pro-inflammatory stimuli, resulting in increased expression of pro-inflammatory cytokines and chemokines. In our study, correlation analysis showed that iNOS expression was positively associated with NF-κB p65 expression, suggesting that increased iNOS expression may be caused by the activation of NF-κB. Activated NF-κB, a transcription factor, may bind to the specific DNA sequence of iNOS to promote its expression[19].

In conclusion, iNOS expression and activity induced in ethanol-induced liver injury are responsible for the elevated NO production. The induction of iNOS is associated with liver damage, especially necroinflammation, activation of NF-κB and elevated TNF-α mRNA expression in the liver.

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CLINICAL RESEARCH

Food intolerance and skin prick test in treated and untreated irritable bowel syndrome

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Abstract

AIM: To correlate the clinical features of treated and untreated patients with irritable bowel syndrome (IBS) to the results of skin prick test (SPT) for food and inhalant allergens.

METHODS: We recruited 105 subjects to form three different target groups: treated group ($n = 44$) undergoing treatment for IBS, untreated group ($n = 31$) meeting the Rome II criteria without treatment for IBS, control group ($n = 30$) with no IBS symptoms.

RESULTS: SPT results were different among the three groups in which SPT was positive in 17 (38.6%) treated patients, in 5 (16.1%) untreated patients and in 1 (3.3%) control ($P < 0.01$). The number of positive SPTs was greater in the IBS group than in the control group ($P < 0.001$). The number of positive food SPTs was higher in the treated IBS group than in the untreated IBS group ($P = 0.03$).

CONCLUSION: Positive food SPT is higher in IBS patients than in controls.

Key words: Irritable bowel syndrome; Skin prick test; Food allergy

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INTRODUCTION

Irritable bowel syndrome (IBS) is common. Depending on the studies, the prevalence (using Rome II criteria) varies between 9%-22% among Caucasians[1-3]. In Korea, the prevalence of IBS has been reported to be 6.6% and there is no difference in the prevalence between men and women[4].

In patients with IBS, postprandial worsening of symptoms as well as food-related reactions to one or more foods is very common[5]. In published data, speculation of an immunological reaction to foods in IBS has been reported[6,7]. Data from dietary elimination and food challenge studies support the role of diet in the pathogenesis of IBS[8-10]. This hypothesis is supported by the response to disodium cromoglycate in such patients[11,12]. Although well designed randomized controlled studies are scarce, milk, wheat, egg and foods high in salicylates or amines are consistently a problem among the studies[13,14]. Recently, Dainese *et al*[7] have indicated discrepancies between reported food intolerance and skin prick test (SPT) findings in IBS patients, suggesting that the SPT used to identify food sensitization mediated by the IgE mechanism is inappropriate. IBS is a heterogeneous disease having various symptoms and severity. So in our study, IBS patients were divided into treatment and untreated groups. Moreover, to clarify the possible role of food hypersensitivity in IBS, we used both common food and inhalant antigens. The results of SPT for food allergens were compared to the SPT for inhalant allergens.

The aim of this study was to correlate the clinical features of treated and untreated IBS patients to the results of the skin prick test for food and inhalant allergens, total IgE level, and radio- allegro-sorbent test (RAST)

MATERIALS AND METHODS

Subjects

The study protocol was approved by the Institutional Review Board of the Hanyang University. Among the 480 medical students, 35 meeting the Rome II but not treated

Table 1 Characteristics of studied subjects, *n* (%)

	Treated (*n* = 44)	Untreated (*n* = 31)	Control (*n* = 30)
Age (years)	37.9+13.2	27.2+1.9	25.8+1.7
Gender			
Male	19 (43.2)	16 (51.6)	22 (73.3)
Female	25 (56.8)	15 (48.4)	8 (26.7)
IBS subgroups			
C-IBS	10 (22.7)	12 (38.7)	-
D-IBS	15 (34.1)	9 (29.0)	-
A-IBS	19 (43.2)	10 (32.3)	-
Type of initial health care system		-	-
Tertiary center	13 (29.5)	-	-
Primary care	25 (56.8)	-	-
OB/Gyn [3]	1 (2.3)	-	-
Oriental-medicine	5 (11.4)	-	-
Reason for seeking for health care			
Abdominal pain	17 (38.7)	-	-
Discomfort, bloating	14 (31.8)	-	-
Altered stool pattern	13 (29.5)	-	-

C-IBS = Constipation-predominant IBS; D-IBS = Diarrhea-predominant IBS; A-IBS = Alternating constipation and diarrhea IBS.

for IBS and 35 randomly selected healthy undergraduate students were recruited in the study. After giving their informed consent, all volunteers underwent a physical examination and laboratory screening (upper gastrointestinal endoscopy, colonoscopy, routine red and white blood cell count, and biochemical examinations). Subjects receiving any medication (antihistamines, steroids, H_2 receptor antagonist, anti-inflammatory drugs, and herb medications) over a two-week period prior to study and those with current severe allergic disease identified by interview were excluded from the study (n = 9). Therefore, out of 70 students, 31 untreated IBS students and 30 healthy students participated in the trial. Forty-four consecutive patients with IBS referred to our institution for evaluation and treatment served as positive controls. Standard workup included upper gastrointestinal endoscopy, laboratory testing and colonoscopy.

The enrolled patients were divided into three groups after clinical evaluation and the application of Rome II criteria. Group I consisted of 44 patients with IBS referred to our institution for evaluation and treatment. All treated patients were selected from a single tertiary outpatient clinic (treatment group). Group II consisted of 31 untreated IBS patients meeting the Rome Ⅱ criteria but not treated for IBS (untreated group). Group III consisted of 30 healthy undergraduate students (control group).

Questionnaire and clinical measurements

To assess abdominal symptoms and intensity, all study participants were given a questionnaire. A modified WHO-QOL (WHOQOL-BREEF) method validated by Min *et al*[15] was used to measure the quality of life in the IBS and control groups. The questionnaire consisted of 26 items measuring four dimensions of health: physical health, psychological health, social relationships and environmental health. Each item asked the respondents to indicate the extent to which their IBS interfered with their health during the previous four weeks.

Skin prick test and RAST

SPT was carried out on all subjects on the upper back involving the application of 70 fresh food extracts using the prick-by-prick method. Some of the fresh food extracts used were saury, mackerel, beef, pork, chicken, milk, egg white, egg yolk, wheat flour, buckwheat, rice, beans, apples, peaches, tomatoes, celery, carrots, onions, peanuts, chocolate, and coffee. A doctor carried out an additional SPT involving the application of inhalant allergens. The 11 commercial allergens tested for D-pteronyssinus, D-farinae, alternaria alternata, grass pollen, tree pollen, mugwort pollen, willow pollen, ragwort, dog hair, cat fur, and cockroaches mix. In both SPTs, one drop of the extract was placed on the skin, and a disposable syringe needle was placed into the drop and then into the skin until a small puncture was visible. A histamine control was employed. SPT was considered positive if the net wheal diameter was significantly greater than the net wheal diameter of the histamine reaction. The reaction to the extract was measured 15-20 min after application. Discomfort and bleeding were not evident.

Serum samples for total IgE and total eosinophil count were collected by trained phlebotomists. Two 10 mL blood samples from an antecubital vein were taken. IgE measurement was determined by the paper radioimmunosorbent test (PRIST, Pharmacia Laboratories, USA) using a Phadebas IgE kit. The total IgE test provided evidence for IgE sensitivity to antigens because the IgE antibody resulted in mast cell activation and release of histamine. Also, total eosinophils were stained with a Hinkelman solution, and the eosinophil count provided immediate hypersensitivity to IgE antibody.

Statistical analysis

Tables were constructed for frequency and percentage. Categorical data were analyzed using the chi-square test or Fisher's exact test. Continuous data were analyzed using the Student's t-test and one-way ANOVA or Scheffe's test. To minimize type I errors, $P < 0.05$ was considered statistically significant. Exact P-values are listed in the tables and text. All statistical analyses were performed using the SPSS 11.0 statistical package.

RESULTS

Patient population

In the study population, 44 treated IBS patients had a mean age of 38 years (37.9±13.2), 31 untreated IBS patients had a mean age of 27 years (27.2±1.9), and 30 controls had a mean age of 26 years (25.8±1.7). There were no statistically significant differences in gender ratio, height, weight, alcohol and coffee intake, and smoking habits among the three groups (not shown). In the treated group, the number of females (56.8%, n = 25) was greater than that of males (43.2%, n = 19). In this group, the most subtype IBS was found in 19 patients. The subtype constipation dominant IBS was found in 12 patients of untreated group. Subjects in the treated group mainly sought for a primary care setting as the first visit due to

Table 2 Symptoms, duration of symptoms, and onset of IBS, *n* (%)

	Treated (*n* = 44)	Untreated (*n* = 31)	*P*
Duration of symptom			0.035
Less than 1 h	5 (11.4)	7 (22.6)	
1-24 h	13 (29.5)	15 (48.4)	
More than 1 day	26 (59.1)	9 (29.0)	
Severity of subjective symptoms			0.001
Extremely severe	6 (13.6)	-	
Very	22 (50.0)	5 (15.6)	
Somewhat	14 (31.8)	19 (62.5)	
Not very	1 (2.3)	6 (18.8)	
Not at all severe	1 (2.3)	1 (3.1)	
Onset of IBS symptoms			0.065
Recent 6 mo	3 (6.8)	2 (6.3)	
1 year ago	8 (18.2)	1 (3.1)	
1-2 years ago	6 (13.6)	4 (12.5)	
2-5 years ago	13 (29.5)	12 (40.6)	
5-10 years ago	7 (15.9)	11 (34.4)	
10-20 years ago	5 (11.4)	1 (3.1)	
More than 20 years ago	2 (4.5)	-	

abdominal pain (15 patients), stool pattern change (13 patients) and abdominal bloating (13 patients) (Table 1).

Subgroup analysis and scores of quality of life (WHOQOL-BREEF)

We utilized a questionnaire to compare and estimate the onset, duration and severity of symptoms between the two IBS groups. More than half of the treated IBS patients (59.1%) reported that the symptoms lasted for more than 24 h, but only 29.0% of untreated IBS patients reported that the symptoms lasted for more than 24 h. Five IBS patients (11.4%) in the treated group complained of abdominal symptoms almost all day for more than one month. Subjective severity score of symptoms was also higher in the treated IBS patients than that of the untreated IBS patients ($P = 0.001$). But there was no significant difference in the onset of the disease between the two groups ($P = 0.065$) (Table 2).

Modified WHOQOL (WHOQOL-BREEF) questionnaire validated by Min *et al*[15] was used. It consisted of 26 items measuring four dimensions of health. All dimensions including overall quality of life scores were significantly lower in IBS patients than in controls (Table 3).

History of allergies and food intolerance

There was no significant difference in the reported food intolerance among the three groups. However, among the treated and untreated IBS patients, dairy products were reported to cause most of the intolerance. Of the treated and untreated IBS patients, 25 (56.8%) and 21 (67.7%) had an allergic condition and only 9 (30%) of the controls had an allergic condition ($P = 0.008$). However, no difference in the number of allergic conditions was found between the treated and untreated IBS patients. Rhinitis was the most common condition, and five patients had a history of asthma in the IBS patients (Table 4).

Skin prick test and IgE level

Seventy fresh food extract allergens were used to identify the cause of food hypersensitivity. SPT results were different among the three groups. SPT was positive in 17 (38.6%) treated IBS patients, in 5 (16.1%) untreated IBS patients, and in 1 (3.3%) control ($P < 0.01$). The number of positive SPTs was greater in the IBS patients than in the controls ($P < 0.001$). The number of positive food SPTs was greater in the treated patients than in the untreated patients ($P = 0.03$). The most frequent SPT positive food allergen was saury among the different groups. Five IBS patients had positive SPT for rice (Table 5). Eleven treated and untreated IBS patients and four controls had positive SPT for inhalant allergens. No significant difference in SPT inhalant allergens was noted among the three groups. The most common reactive inhalant allergens were D-farinae and D-pteronyssinus.

There were no significant differences in IgE and total eosinophil counts among the three groups. Also, no significant differences in IgE and total eosinophils were noted between the treated and untreated IBS patients (Table 6).

Table 3 Score of quality of life (mean ± SD)

Domain	Treated (*n* = 44)	Untreated (*n* = 31)	Control (*n* = 30)	*P* value[a]	*P* value[c]
Physical health	52.47±15.06	59.09±13.17	75.74±16.25	0.001	0.001
Psychological health	52.98±17.00	56.06±13.71	68.75±13.79	0.001	0.014
Social relationships	53.77±13.92	59.47±11.59	61.81±16.47	0.067	0.005
Environmental health	48.44±15.19	54.69±11.19	63.15±12.70	0.001	0.005
Overall QOL	5.79± 1.09	6.36± 1.33	8.21± 0.93	0.001	0.002

QOL: quality of life. [a]$P < 0.05$ by χ^2-test; [c]$P < 0.05$ by χ^2-test between IBS patients and control.

Characteristics according to the food SPT in treated and untreated groups

When SPT for food allergen and gender ratio were compared between those with a positive and negative SPT, no difference was noted. Also, there were no significant differences in positive SPT between IBS subtype and current allergy history. However, food intolerance and positive inhalant allergen were much higher in SPT positive food allergens ($P = 0.013$, $P = 0.006$). Twenty-two IBS patients had one or more skin positive tests. Serum samples were taken for RAST in order to compare the SPT results. Only three treated IBS patients had a positive RAST for beans, pork, and beef (Table 7).

DISCUSSION

The term "adverse food reaction" or "food-related symptoms" encompasses immunological responses and non-immunological responses to food. Food hypersensitivity/ allergy is used to describe conditions in which an immunological mechanism may be demonstrable[16,17]. In contrast, food intolerance is a non-immunological response to proteins that may result from particular constituents of foods such as toxins (e.g., food poisoning) or pharmacological agents (e.g., caffeine or tyramine) or from host factors such as lactase deficiency. However, food hypersensitivity

Table 4 History of allergies and food intolerance, *n* (%)

	Treated (*n* = 44)	Untreated (*n* = 31)	Control (*n* = 30)	*P* value [a]	*P* value [c]	*P* value [e]
History of allergies	25 (56.8)	21 (67.7)	9 (30.0)	0.017	NS	0.008
Atrophy	5 (11.4)	5 (16.1)	2 (22.2)			
Asthma	3 (6.8)	2 (6.5)	-			
Rhinitis	10 (22.7)	7 (22.6)	5 (55.6)			
Eczema	4 (9.1)	5 (16.1)	3 (33.3)			
Hives	4 (9.1)	7 (22.6)	-			
Reported food intolerance	36 (81.8)	27 (87.1)	23 (76.7)	NS	NS	NS
Dairy products	24 (54.5)	17 (54.8)	11 (36.7)			
Coffee	13 (29.5)	7 (22.6)	6 (20.0)			
Alcohol	16 (36.4)	15 (48.4)	15 (50.0)			
Cold, raw foods	15 (34.1)	10 (32.3)	8 (26.7)			
Spicy foods	17 (38.6)	6 (19.4)	8 (26.7)			
Others	10 (22.7)	3 (9.7)	1 (3.3)			

[a]$P < 0.05$ by χ^2-test; [c]$P < 0.05$ by χ^2-test between treated and untreated patients; [e]$P < 0.05$ by χ^2-test between IBS patients and controls.

Table 5 Skin prick test results using food allergens and inhalant allergens, *n* (%)

	Treated (*n* = 44)	Untreated (*n* = 31)	Control (*n* = 30)	*P* value [a]	*P* value [c]	*P* value [e]
Positive food SPT	17/44(38.6)	5/31 (16.1)	1/30 (3.3)	<0.001	0.03	<0.001
Saury	13 (76.5)	2 (40.0)	1 (100.0)			
Mackerel	3 (17.6)	-	-			
Beef	1 (5.9)	2 (40.0)	-			
Pork	2 (11.8)	1 (20.0)	-			
Buckwheat	3 (17.6)	-	-			
Rice	4 (23.5)	1 (20.0)	-			
Arrowroot	3 (17.6)	-	-			
Sweet potatoes	3 (17.6)	-	-			
Beans	2 (11.8)	1 (20.0)	-			
Cabbages	3 (17.6)	-	-			
Celery	3 (17.6)	-	-			
Onions	3 (17.6)	-	-			
Peach	1 (5.9)	1 (20.0)	-			
Tomato	1 (5.9)	-	-			
Melon	2 (11.8)	-	-			
Squid	1 (5.9)	1 (20.0)	-			
Trumpet shell	3 (17.6)	-	-			
Curry	2 (11.8)	-	-			
Positive inhalant SPT	11 (25.0)	11 (35.5)	13.3 (4)	NS	NS	NS
D-farinae	7 (63.6)	7 (63.6)	50.0 (2)			
D-pteronyssinus	5 (45.5)	8 (72.7)	50.0 (2)			
Alternaria alternate	2 (18.2)	-	25.0 (1)			
Grass pollen	27.3 (3)	1 (9.1)	-			
Tree pollen	36.4 (4)	1 (9.1)	1 (25.0)			
Mugwort pollen	-	2 (18.2)	-			
Willow pollen	9.1 (1)	-	-			
Ragweed	36.4 (4)	3 (27.3)	-			
Dog hair	45.5 (5)	1 (9.1)	1 (25.0)			
Cat fur	18.2 (2)	-	-			
Cockroach mix	9.1 (1)	-	-			

[a]$P < 0.05$ by χ^2-test; [c]$P < 0.05$ by χ^2-test between treated and untreated patients; [e]$P < 0.05$ by χ^2-test between IBS patients and controls.

resulting in food intolerance in the cause of gastrointestinal problems is much harder. Niec *et al*[18] reported that milk, wheat, eggs, potatoes, and celery are the most commonly identified factors causing gastrointestinal symptoms. Locke *et al*[19] reported that beans and legumes, chocolate, dairy products, and nuts are the most common foods causing hypersensitivity. It was reported that patients with IBS reveal intolerance to foods such as milk, bread, pizza, apple, hazelnut, tomato, egg, peach, and greens[7]. In our study, dairy products, coffee, alcohol, raw food, and spicy food were the most common foods causing problems. However, SPT was positive for saury, rice, mackerel, buck-

Table 6 IgE and total eosinophil count (mean ± SD)

	Treated (n = 44)	Untreated (n = 31)	Control (n = 30)	P value
IgE (IU/mL)	252.36±558.94	402.34±765.00	320.53±659.07	NS
Eosinophil (/μL)	153.74±149.68	181.72±126.04	166.42±139.10	NS

Table 7 Characteristics of treated and untreated patients with IBS, n (%)

Characteristics	Positive food SPT (n = 22)	Negative food SPT (n = 53)	P value
Gender			NS
Male	7 (31.8)	28 (52.8)	
Female	15 (68.2)	25 (47.2)	
Subtypes			NS
A-IBS	8 (36.4)	21 (39.6)	
C-IBS	9 (40.9)	13 (24.5)	
D-IBS	5 (22.7)	19 (35.8)	
Current history of allergies	13 (59.1)	33 (62.3)	NS
Reported food intolerance	9 (40.9)	7 (13.2)	0.013
Inhalant positive SPT	11 (50.0)	11 (20.8)	0.006
Positive RAST	3 (13.6)	-	

wheat, sweet potatoes, celery, onions, and trumpet shell. The results of different studies are inconsistent, which poses the question of the population specificity of such studies.

No correlation between SPT and patient's intolerance to certain foods was noted. There are several explanations for the discrepancies between reported food intolerance and SPT results. First, SPT is generally considered the most convenient and least expensive screening method for detecting allergic reactions in most patients. However, until the diagnostic efficacy of SPT is fully established with standardized allergens and methods, a positive skin test alone cannot confirm a definite clinical sensitivity to an allergen. Second, clinicians consider cross-reactions among various plants and animal allergens[20, 21]. The conservation of these proteins across biologic substances affects cross-reactivity in several ways. Certain foods (e.g., peanut) are able to sensitize and elicit reactions after oral exposure (type 1 allergy) and can trigger responses to related foods (e.g. legumes). Other foods (e.g., apples) containing labile proteins are not strong oral sensitizers. Helbling *et al*[22] have reported the clinical cross-reactivity to most fish species and several *in vitro* studies have demonstrated the existence of common allergens between different fish species[23-26]. The third explanation is that food allergies are localized at the intestinal mucosa and specific IgEs are primarily present in intestinal mucosa but not systemically. For this reason, Andre *et al*[27] have indicated the importance of stool IgE rather than serum IgE. Bischoff *et al*[28] have also stressed the importance of stool eosinophil counts and mast cell mediators.

Both SPT and RAST are for IgE-mediated disease, but there is no correlation between the two methods. However, RAST is not as sensitive as the skin test. As a result, in patients with a history of reactions to foods, insect stings, drugs, or latex, skin testing is still required because of its higher sensitivity even if the RAST is negative. The primary advantage of RAST over SPT is safety with the results not influenced by skin disease or medication[29].

In our study, the IBS patients showed much higher positive SPT rates than the controls ($P < 0.001$). The treated IBS patients were more likely to have a positive SPT than the untreated IBS patients ($P = 0.03$). The untreated IBS patients defined by fulfillment of the Rome IBS criteria complained of severe symptoms ($P < 0.05$) (Table 2), suggesting a possible relationship exists between IBS and allergic diathesis. Further studies are needed to explore this relationship. In addition, we also used common inhalant allergens to compare with the food allergens and did not find any difference in positive SPT between inhalant allergens among the three groups (25%, 35% and 13% respectively). White *et al*[30] reported that increased airway responsiveness to inhaled methacholine can be demonstrated in irritable bowel syndrome patients with no clinical evidence for atopic disease[31]. These findings are in contrast to the previous hypothesis that IBS is a generalized immune hypersensitivity state. These findings also suggest that food allergens may play a certain role in IBS patients. However, well designed dietary elimination and food challenge studies are needed to document the food hypersensitivity in IBS patients.

In this study, the untreated IBS patients were older than the treated patients ($P = 0.005$). Because age could affect SPT, it may confound our results. But the number and size of prick skin reactions increase throughout childhood until twenty years of age and then gradually decline until age fifty[32]. Even if age is considered, more treated patients were positive for SPT in our study.

In conclusion, more IBS patients are positive for SPT and food allergens than healthy controls. However, the IBS patients present more severe symptoms, lower overall quality of life, and higher positive SPT compared to the untreated IBS patients even though the history of allergies is not different.

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RAPID COMMUNICATION

Accuracy of combined PET/CT in image-guided interventions of liver lesions: An *ex-vivo* study

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Abstract

AIM: Positioning of interventional devices in liver lesions is a challenging task if only CT is available. We investigated the potential benefit of combined PET/CT images for localization of interventional devices in interventional liver studies.

METHODS: Thirty lesions each of hyperdense, isodense and hypodense attenuation compared to normal liver parenchyma were injected into 15 *ex-vivo* pig livers. All lesions were composed of the same amounts of gelatine containing 0.5 MBq of ^{18}F-FDG. Following lesion insertion, an interventional needle was placed in each lesion under CT-guidance solely. After that, a PET/CT study was performed. The localization of the needle within the lesion was assessed for CT alone and PET/CT and the root mean square (RMS) was calculated. Results were compared with macroscopic measurements after lesion dissection serving as the standard of reference.

RESULTS: In hypo- and isodense lesions PET/CT proved more accurate in defining the position of the interventional device when compared with CT alone. The mean RMS for CT and PET/CT differed significantly in isodense and hypodense lesions. No significant difference was found for hyperdense lesions.

CONCLUSION: Combined FDG-PET/CT imaging provides more accurate information than CT alone concerning the needle position in FDG-PET positive liver lesions. Therefore combined PET/CT might be potentially beneficial not only for localization of an interventional device, but may also be beneficial for guidance in interventional liver procedures.

INTRODUCTION

Treatment of liver lesions may be performed either surgically or interventionally. Surgery still represents the treatment of choice in solitary liver lesions or in patients with a limited number of lesions confined to a specific liver segment. However, certain cardiovascular or pulmonary risk factors may prevent patients from undergoing surgery with a curative intent [1,2]. In some patients minimally invasive interventional approaches may serve as an option for patient treatment. Interventional procedures for treatment of liver lesions include procedures such as radiofrequency ablation, laser induced ablation or chemoembolization [3-5]. Except for chemoembolization, all other therapeutic interventional procedures include placement of an interventional needle transcutaneously within the liver lesion. In most procedures, the needle is placed under CT, MRI, or ultrasound guidance. However, localization and guidance of an interventional device on morphological imaging can be challenging, particularly in selected cases where differentiation of the tumor from adjacent liver parenchyma may be difficult. These circumstances occur especially in tumors with only little or no contrast enhancement [6,7]. On the other hand, functional imaging has proved to be superior to morphological imaging in detection and characterization of these tumors, but is known for only limited anatomical information.

In any case accurate placement of an interventional device is indispensable for complete tumor treatment in every interventional method. In cases of equivocal findings on morphological imaging procedures, additional functional data can provide information about viable tumor parts. Combined PET/CT imaging systems provide accurate morphological and functional data sets within a single operation [8].

The aim of this study was to evaluate a potential benefit of combined PET/CT imaging concerning mor-

phology and complementary functional information when assessing the position of an interventional needle within an isolated liver without breathing. This might potentially improve the accuracy of localization of interventional devices compared with morphological imaging alone.

MATERIALS AND METHODS

Lesion model

Ninety artificial lesions, two hypodense, two isodense, and two hyperdense were placed in each of 15 *ex-vivo* pig livers by needle injection. The injected substances consisted of the following ingredients: Hypodense lesions: gelatine (9 g), sodium chloride (NaCl: 20 mL). Isodense lesions: gelatine (9 g), NaCl (20 mL), iodine-based contrast agent (0.2 mL/300 mg/mL Xenetix 300, Guerbet GmbH, Sulzbach, Germany). Hyperdense lesions: gelatine (9 g), NaCl (20 mL), iodine-based contrast agent (0.4 mL/300 mg/mL). Additionally 0.5 MBq ^{18}F in form of [^{18}F]-2-Fluoro-2-deoxy-D-glucose (FDG) were administered to all mixtures, thus 0.5 MBq FDG, 20 mL sodium chloride, 9 g of gelatine and the different amounts of contrast agents were used per lesion.

The concentration of FDG was chosen based on previous measurements of differences between tumor and liver background in 75 patients with liver malignancies. A ratio using the differences of Standard Uptake Values (SUV) between the tumor and the liver background was established. Based on this ratio, the amount of FDG was chosen to establish the same ratio between the artificial lesions and the liver background. Seventy-five consecutive patients with metastases of colorectal carcinoma, 25 patients with metastases of NSCLC (non small cell lung cancer) and 10 patients with hepatocellular carcinomas (HCC) were chosen from the local PACS. Patients with HCC were only partly PET-positive tumors or had only low values (SUV), but were chosen to define a broad spectrum of liver malignancies.

Lesion injection and interventional procedure

First, the artificial lesions were injected into the liver with a 7-gauge needle (Figure 1). After injection of the artificial lesion into the *ex-vivo* pig livers, the gelatine mixture hardened as it cooled. Lesion injection and the subsequent interventional device placement were carried out by different physicians. Thus, one physician injected the artificial lesions, while another physician inserted the biopsy needles in the following step.

Second, 18 gauge biopsy needles were inserted (by the second physician) in each liver, one needle in each lesion for a total of 6 needles per *ex-vivo* liver under strict CT-guidance. The needles were only inserted in lesions with favourable shape (round, oval or elliptic) to guarantee reproducible measurements. Lesions which partly dropped away through bile ducts and veins were excluded from the measurements. Additionally, before needle placement, each liver was covered by a thin, non-transparent sheet before needle placement to hide the injection sites.

CT and PET/CT imaging

CT and PET/CT imaging were performed with a Biograph™ system (Siemens Medical Solutions, Hoffman Estates, IL).

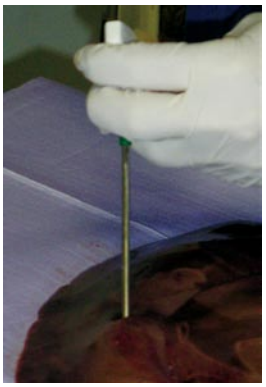

Figure 1 A 7-gauge biopsy needle and a syringe were used for insertion of the gelatine/FDG mixture into the liver. The mixture hardened as it cooled in the *ex-vivo*-liver.

The system consists of two components: a dual-slice CT scanner (Somatom Emotion, Siemens Medical Solutions, Erlangen, Germany) with a minimum gantry rotation time of 800 ms and a full ring PET tomograph (ECAT HR+, Siemens Medical Solutions, Hoffman Estates, IL). The PET system has an axial field of view of 15.5 cm per bed position and an in-plane spatial resolution of 4.6 mm. CT and PET images were acquired consecutively. Repetitive CT scans were carried out for needle positioning with a field-of-view focused on the interventional region. Acquisition parameters for the interventional CT were 120 mAs, 130 kV, 1 mm slice thickness and 1.5 mm table feed.

Following needle placement under strict CT guidance (without using any PET information) a combined PET/CT study was conducted with all needles in position covering the whole *ex-vivo* liver (CT: 120 mAs, 130 kV, 1 mm slice thickness with a 0.5 incremental reconstruction, 1.5 mm table feed, 1 mm collimation, PET: scan time 4 min, 3D data acquisition). CT and PET data sets from the combined imaging approach can be viewed separately or in fused mode on a commercially available computer workstation, which also allows distance measurements on fused images in all three dimensions. Thus, CT images and combined PET/CT images were evaluated separately while all needles inserted within the artificial lesions.

Image evaluation

CT images, either viewed alone or in fused mode (PET/CT), were adjusted to the soft tissue window (center: 50 Hounsfield Units (HU), width: 350 HU). PET images were adjusted to Full Width at Half Maximum (FWHM).

The lesions' width and height were measured in the coronal plane on the commercially available computer workstation and the length was measured in the same way using MPR (multi planar reformatting) in the sagittal direction. The accuracy of CT data alone and combined PET/CT data for determining the needles' position was assessed by measuring the distance (in the horizontal direction) of the needle tip to the lesions' margins on both imaging procedures (CT alone and combined PET/CT) with an electronic calliper in the coronal, transverse and sagittal planes. Imaging measurements from the workstation were correlated with macroscopic measurements after liver lesion dissection, which served as the standard of reference. For this purpose, the liver, with all needles still in place, was finely sliced to measure the distance from the needles' tip to the lesion's margins macroscopically.

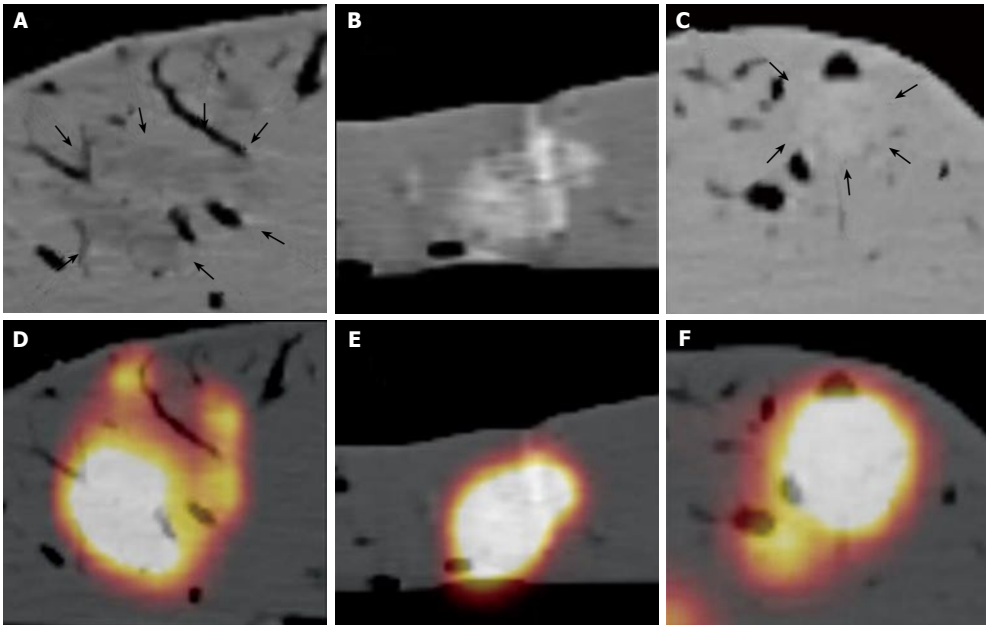

Figure 2 **A**: This shows a hypodense lesion. The margins of the hypodense lesion are barely seen (fine black arrows). In comparison, the combined PET/CT image of the same lesion shows exactly the margins of the lesion (Figure 2D). Furthermore, the combined PET/CT suggests a different location and extent of the lesion, which was confirmed by the standard of reference. **B**: This shows a hyperdense lesion. The margins of the lesions are well seen. The combined PET/CT shows the same location and extent of the lesion within the liver tissue (Figure 2E). Thus, in hyperdense lesions, a high correlation of CT and combined PET/CT measurements was found. **C**: This shows a nearly isodense lesion. The margins of the lesions can only be anticipated (fine black arrows). The corresponding PET/CT (Figure 2 F) of the same lesion shows a different extent of the same lesion with well defined and sharp margins.

All measurements concerning the position of the needle within a single lesion (distances to lesion margin) were compared separately, and the root mean square (RMS) was calculated. The RMS was chosen as a standard description to compare the mean values of different group measurements. The Root Mean Square is calculated as follows:

$$RMS = \sqrt{(x-x_0)^2+(y-y_0)^2+(z-z_0)^2}$$

In this formula, x, y and z are defined as the values derived from the macroscopic measurements, serving as the gold standard. In comparison, x_0, y_0 and z_0 are the values from CT measurement or PET/CT measurement. Therefore, in this formula, the values of the gold standard and the values of CT and PET/CT are already compared by calculating the deviation of measurements from CT or PET/CT compared with the macroscopic standard of re-ference. Thus, greater deviations (meaning less exact definition of the needles' tip on CT or combined PET/CT compared to the standard of reference) will be represented by higher RMS values. In comparison, lower RMS values are calculated based on less deviation of the needles' tip compared to the standard of reference. Values (mean values) were calculated for every lesion based on CT and PET/CT measurements.

Additionally, Hounsfield Units (HU) of every lesion and their surrounding liver tissue was measured and mean values for surrounding tissue and every lesion type were calculated to compare the lesions densities in relation to the liver background. Wilcoxon test for comparing two paired groups was used for statistical analysis.

RESULTS

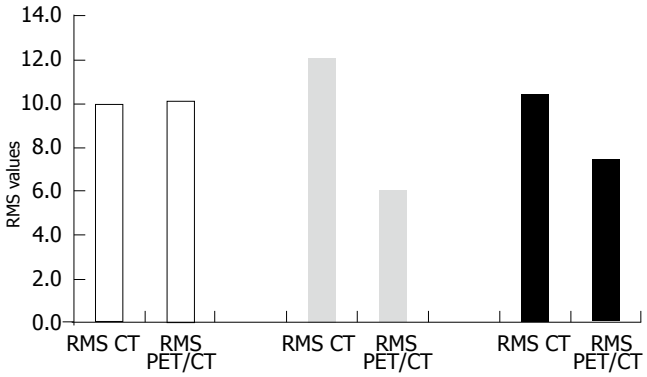

Figure 3 This shows the different mean RMS values for hyper-, iso-, and hypodens lesions. A statistically significant difference was found between the mean RMS values in CT and PET/CT in isodense lesions ($P < 0.05$, grey bars). Additionally, a statistically significant difference was found between the mean RMS values of CT and PET/CT in hypodense lesions ($P < 0.05$, black bars). No difference was found in hyperdense lesions (white bars).

In 90 lesions placed in 15 ex-vivo pig livers, 77 were left in favourable shape for needle intervention (Figure 2). Since several lesions dropped away through bile ducts and veins, 23 hyperdense, 28 isodense, and 26 hypodense lesions were left for measurement.

The mean width was 20.2 (standard deviation (SD): 9 mm) mm, length and height of these lesions were 18.6 (SD: 6 mm) mm, and 16 (SD: 5 mm) mm, respectively, according to the macroscopic standard of reference. Two isodense lesions were missed based on CT data due to poor visibility on the CT images only, whereas both lesions were clearly seen on combined PET/CT images only. The lesions were confirmed by liver dissection. There were no failed punctures in hypo- and hyperdense lesions. The mean RMS for the CT measurements and the combined

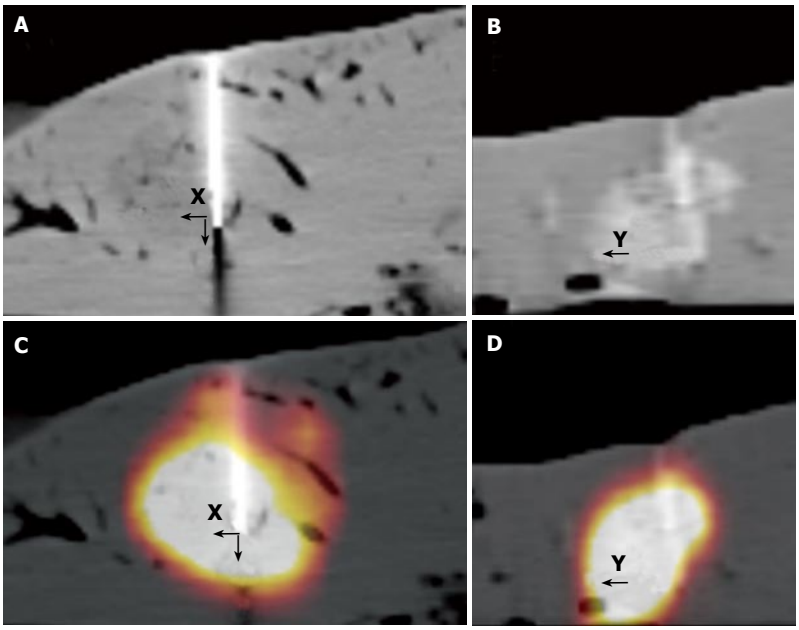

Figure 4 It shows the measurements on the commercially available computer workstation for CT alone (**A**/**B**) and combined PET/CT (**C**/**D**). The lesions´ width and height was measured in coronal direction (Figure 4**A** and **C**, X/Z), the lesions´ length in sagittal direction (black arrow (Y) in Figure 4 **B** and **D**).

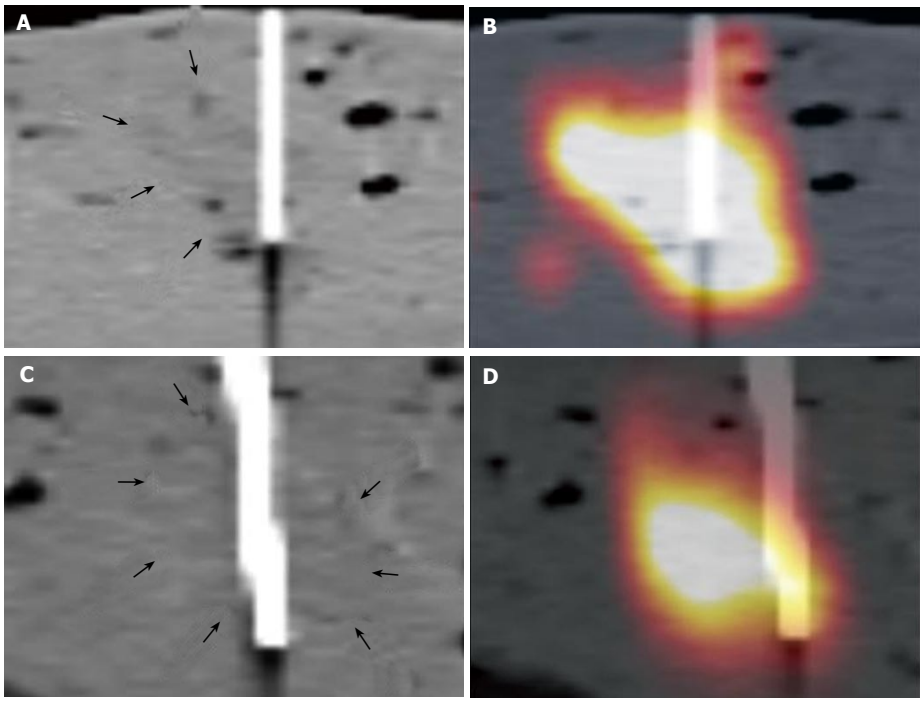

Figure 5 It shows an example of two isodense lesions on CT alone and combined PET/CT with the inserted biopsy needles. The lesions margins are barely seen on CT images alone (fine black arrows, **A** and **C**). In comparison, the true extent and localisation of the lesion are well shown on the combined PET/CT images. Based on poor visibility on CT alone, the lesion in the bottom row was nearly missed (**D**).

PET/CT in hyperdense lesions showed nearly identical values (mean RMS for CT: 10, mean RMS for PET/CT: 10.1) (Figure 3). On the other hand, significant differences were found for the comparison of isodense lesions (mean RMS for CT: 12.1, mean RMS for PET/CT: 6.1). The values for hypodense lesions were less different, however, a significant difference was found here as well (mean RMS for CT: 10.4, mean RMS for PET/CT: 7.4) (Figure 3). Differences between CT and PET/CT were statistically significant in hypodense and isodense lesions ($P < 0.05$ for hypo-, and isodense lesions). Thus, the definition of the needles' position within the lesion on combined PET/CT images was significantly more accurately defined than on CT images alone in hypodense and isodense lesions (Figure 4 and Figure 5). No statistically significant difference between CT-based and PET/CT-based measurements in hyperdense lesions was found. The distribution of mean Hounsfield Units (HU) for hypodense lesions, isodense lesions and hyperdense lesions, as well as for the surrounding liver tissue with corresponding standard deviation was measured and calculated as well (Figure 6).

The difference between mean values for hypodense lesions compared to the liver background was less than the difference of mean values of hyperdense lesions compared to liver background. Mean values of isodense lesions were only slightly different compared to mean values of the liver background, thus the model of artificial lesions met the requirements of the study.

DISCUSSION

PET/CT imaging proved more accurate when assessing

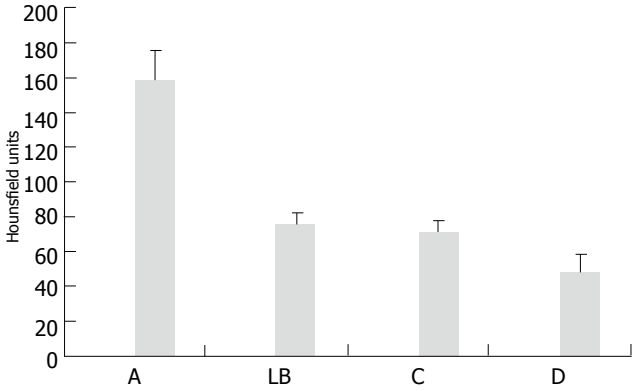

Figure 6 This shows the mean Hounsfield Units (HU) of all measured lesion types, the compared liver background and the corresponding standard deviation. **A**: hyperdense lesions; **LB**: liver background; **C**: isodense lesions; **D**: hypodense lesions.

the position of an interventional device within a liver lesion than imaging analysis based on morphological data alone. Hence, based on the option of more accurate detection of viable tumor tissue, PET/CT promises to add substantial information in selected liver interventions in which equivocal findings on morphologic imaging procedures complicate accurate determination of the interventional needle within the tumour.

Exact positioning of different interventional devices is indispensable for appropriate results in different interventional procedures. In cases of radiofrequency ablation (RFA), complete tumor necrosis is required, because inaccurate position of the ablation device will lead to much higher recurrence rate [9-11]. In biopsy procedures, however, differentiation of viable tumor tissue and necrosis might sometimes be difficult based on only slight differences in density. Furthermore, exact positioning may sometimes be problematic due to unfavourable shape, size, position and visibility of the lesions.

Although CT is a widely used tool for guidance of interventional procedures [12-14], even contrast-enhanced CT images may sometimes not be able to distinguish, both the margin of the lesion and the necrotic region from viable tumor fractions. Lesion attenuation values may differ after i.v. contrast enhancement depending on the tumour entity. Thus, metastases of colorectal cancer appear as hypodense lesions within a contrast enhanced liver, whereas HCC as a contrast-enhancing lesion appears with a higher attenuation than the surrounding liver tissue in the arterial phase. However, some lesions may not show lesion to background contrast after i.v. contrast enhancement, they appear isodense. Therefore a lesion model with different densities, representing several contrast enhancing patterns was chosen.

PET/CT provides additional data by adding functional information to morphology and may be of benefit in cases with impaired visibility and only partly viable lesions [15, 16]. In our study, the needles' positions were significantly better determined on combined PET/CT data sets in hypodense and isodense lesions than with CT alone. Two advantages may arise from these methods from the clinical point of view: first, the placement of the needle may be performed more accurately in cases with impaired visibility. Although PET/CT puncture itself was not performed in this study, these results are suggesting that the use of the additional functional information can possibly lead to the correct position of the ablative device within the lesion. Thus complete tumour ablation in RFA can be achieved. Second, the assessment of the positioning of interventional devices may be more accurate as well.

Needle positioning was significantly more accurate in hypodense and isodense lesion. While this result may be expected for isodense lesions, it seems rather surprising for hypodense lesions. After all, the needle position in hyperdense lesions was assessed equally well with CT alone and combined PET/CT. These results may be explained by attenuation values of the different lesions types. Differences between attenuation values of hypodense lesions and liver background were less than the difference between the mean Hounsfield Units of hyperdense lesions and liver background. Thus, lesion to background contrast was substantially higher for hyperdense lesions than for hypodense lesions (Figure 6). A further decrease in CT attenuation of hypodense lesions may lead to similar results as detected for hyperdense lesions in this study.

Additionally, two isodense lesions were missed by needle placement based on CT images alone. Primary puncture based on PET/CT images might presumably bring an advantage in visibility and might lead to a successful puncture in these cases.

The study has different limitations. Since it was conducted as an *ex-vivo* trial, patient-induced organ movement was excluded and unfavourable tumor sites close to the diaphragm or the liver hilum could be disregarded. Furthermore, this *ex-vivo* model did not include any background activity which may lead to an increased lesion detectability when assessing the needles' position on combined PET/CT. However, the concentration of FDG injected was chosen based on the described ratio calculated on measurements in patients with different liver malignancies. Thus, we tried to come close to a lesion to background ratio similar to those of patients with a broad average of liver malignancies.

Clinical disadvantages are the small size of the evaluated lesions less than 4 cm in diameter. However, small lesions are the ones most difficult to place an ablative device in. Thus, these are the ones in which CT or other morphological imaging procedures may fail. In addition, interventional procedures with a curative intent are limited to smaller lesions. For example, in RF-ablation the maximal diameter that may successfully be treated is 5 cm. Therefore, the lesion size chosen here met the requirements to simulate clinically often difficult procedures.

Additionally, effective treatment (especially in RFA) can particularly be delivered only to lesions with an appropriate, more or less round, shape. However, primary liver tumors for example can grow beside vessels and bile ducts. In these cases, other therapeutic options have to be considered. Since several lesions in our study setting drained away through bile ducts or veins, these unfavourable lesions were excluded from the evaluation as well.

A general consideration when implementing PET/CT-guided interventions in clinical practice will be its com-

plexity and additional cost compared to CT-guided liver interventions. This approach will be more time-consuming, based on patient preparation with FDG administration one hour prior to the intervention and a substantially longer scan time based on the PET component. Hence, this approach should be limited to cases in which CT alone is not able to accurately assess lesion size, shape and location. A possible clinical procedure when evaluating patients for (diagnostic or therapeutic) interventions could be to conduct a contrast enhanced CT scan first. If the target is well visualized and the lesions' margins are clearly defined on non-enhanced or contrast-enhanced images, a conventional CT-guided intervention might be performed. If the lesion is not clearly defined (hypodense or isodense), a separate PET/CT might be performed to identify the exact localisation and margins of the lesions. The combined PET/CT image then can possibly serve as a landmark tool for the CT-guided intervention. Another step further can be the direct, PET/CT-guided intervention. In this case, the interventional procedure has to be performed directly within the PET/CT system. However, the PET component will require, compared to CT alone, significantly more time, which presumably will be one limiting factor in this scenario. To date, there is no software available where the needle's position can be defined online under combined PET/CT-guidance. Hence, further studies with new technologies have to address the feasibility of this approach. In our opinion, this should be furthermore chosen with focus on cases in which a curative approach is aspired, based on the additional complexity, duration and costs of this procedure.

We conclude that combined PET/CT adds substantial additional information to CT alone when assessing the position of an interventional device within a liver lesion. Thus, co-registered PET/CT can be recommended to date for planning selected liver interventions but, based on its additional complexity and cost, should be limited to those procedures where CT alone is not able to accurately delineate the lesion in question.

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RAPID COMMUNICATION

Hepatocytic differentiation of mesenchymal stem cells in cocultures with fetal liver cells

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Abstract

AIM: To investigate the hepatocytic differentiation of mesenchymal stem cells (MSCs) in co-cultures with fetal liver cells (FLC) and the possibility to expand differentiated hepatocytic cells.

METHODS: MSCs were marked with green fluorescent protein (GFP) by retroviral gene transduction. Clonal marked MSCs were either cultured under liver stimulating conditions using fibronectin-coated culture dishes and medium supplemented with stem cell factor (SCF), hepatocyte growth factor (HGF), epidermal growth factor (EGF), and fibroblast growth factor 4 (FGF-4) alone, or in presence of freshly isolated FLC. Cells in co-cultures were harvested, and GFP+ or GFP- cells were separated using fluorescence activated cell sorting. Reverse transcription-polymerase chain reaction (RT-PCR) for the liver specific markers cytokeratin-18 (CK-18), albumin, and alpha-fetoprotein (AFP) was performed in different cell populations.

RESULTS: Under the specified culture conditions, rat MSCs co-cultured with FLC expressed albumin, CK-18, and AFP-RNA over two weeks. At wk 3, MSCs lost hepatocytic gene expression, probably due to overgrowth of the cocultured FLC. FLC also showed a stable liver specific gene expression in the co-cultures and a very high growth potential.

CONCLUSION: The rat MSCs from bone marrow can differentiate hepatocytic cells in the presence of FLC *in vitro* and the presence of MSCs in co-cultures also provides a beneficial environment for expansion and differentiation of FLC.

INTRODUCTION

The existence of putative liver stem cells in the bone marrow was first described by Petersen *et al*[1] who showed that bone marrow cells transplanted into lethally irradiated mice could engraft in the recipient's liver and differentiate into hepatic stem cells (oval cells) or hepatocytes. First *in vitro* data suggest that several types of bone marrow cells / stem cells can differentiate towards hepatocytic cells under the appropriate culture conditions[2-5]. Oh *et al*[6] found that the liver specific proteins alpha-feto protein (AFP) and albumin are expressed in cultures of unsorted rat bone marrow cells after 21 d. Furthermore, several recent *in vitro* studies indicate the possibility of hepatocytic differentiation of MSCs *in vitro*[7-9]. Kang *et al*[7] reported that MSCs from the rat bone marrow can express the liver specific marker AFP, and produce albumin and urea *in vitro* when cultured in the presence of cytokines, fibroblast-growth factor (FGF-4) and hepatocyte growth factor (HGF). Hong *et al*[8] demonstrated that the liver specific genes cytokeratin (CK) 18, AFP, and albumin are expressed in cultures of human umbilical cord-blood derived MSCs. Furthermore, Lee *et al*[9] also showed liver specific functions of cytochrome P450 activity, urea and albumin-production, and glycogen storage in hepatocytic differentiated umbilical cord blood-derived MSC cultures. We had also shown that rat MSCs have a differentiation potential towards hepatocytic cells when co-cultured with adult rat liver cells[10,11]. The fetal milieu, however, might permit a more suitable environment for rapid MSC-maturation into hepatocytic cells.

In this study, we investigated the potential of rat mesenchymal stem cells derived from adult bone marrow to differentiate into hepatic cells *in vitro* under the direct influence of fetal liver cells for the initiation of liver specific gene expression.

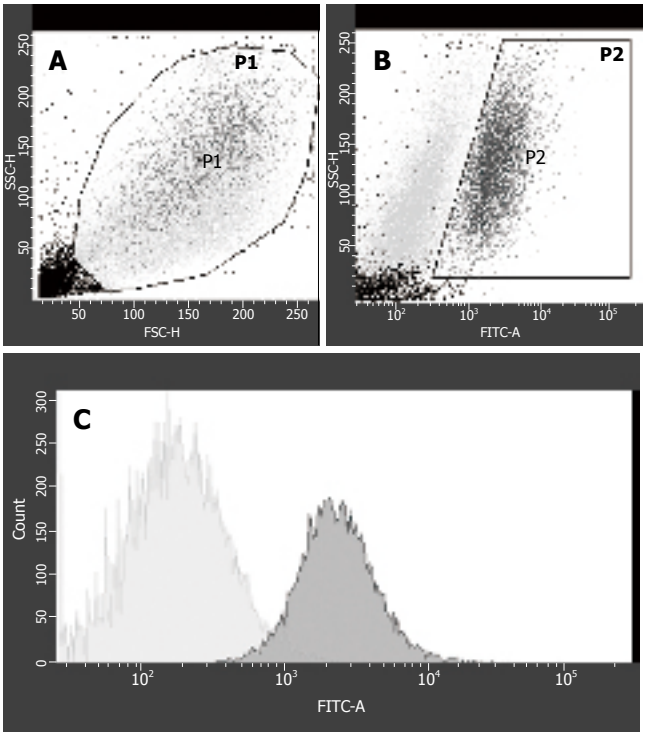

Figure 1 FACS sorting of GFP+ and GFP- cells of co-cultures at wk 1. **A**: Viable cells were gated as P1 cells; **B**: P1 cells were gated in GFP- or GFP+ (P2); **C**: For the highest purification of GFP+ cells, P2 cells were analyzed and only GFP+ cells were sorted for PCR-analysis (light grey peak), 34.5 % of cells from P2 were GFP+ and 65.5 % were GFP-.

MATERIALS AND METHODS

Rat mesenchymal stem cells and fetal liver cells

Isolation, transduction and cloning of rat mesenchymal stem cells were carried out as described previously[10]. Rat embryos of embryonal/ fetal day (ED) 16 were harvested by section after the mother was sacrificed by an overdose of ether-anesthesia. Fetuses were dissected under the dissection microscope. Fetal livers were harvested and liver cells were isolated by a collagenase digestion using collagenase type IV (Sigma, St. Louis, USA) as described previously[12]. A Percoll (P = 1 124 g/mL, Biochrom, Berlin, Germany) gradient centrifugation was used to enrich the viable cell fraction. The Percoll concentration used was 76%. Magnetic cell sorting was used for the removal of fetal OX-43 and OX-44 positive hematopoietic cells from the freshly isolated cells as described elsewhere[13]. In brief, isloated cells were marked with mAb OX-43 and mAb OX-44 (both Serotec, Eching, Germany). Unbound antibodies were removed and secondary marking was done with goat-anti-mouse IgG bound magnetic microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany). Positive cells were absorbed in a magnetic field by Vario-MACS columns, the depleted cell fraction was collected in a tube.

Co-culture of rMSCs with fetal rat liver cells

Cultures were performed according to our protocol published previously[10]. In brief, cells were seeded on culture wells coated with fibronectin (Sigma) on 24-well plates (Greiner). rMSCs (9×10^4) per well were seeded for MSC-controls, or 6×10^4 fetal liver cells per well for controls. For co-cultures of rMSCs with fetal rat liver cells, 9×10^4 rMSCs per well were preceded in 24-well plates for 2 - 3 h. Then, 6×10^4 fetal rat liver cells per well were added to the cultures. Medium was changed twice a week. Stem Span SFEM (Stem Cell Technologies, St. Katherinen, Germany) medium was supplemented with penicillin/streptomycin (Gibco), dexamethasone (Sigma), 100 ng/mL human recombinant stem cell factor (SCF), 20 ng/mL hepatocyte growth factor (HGF) (both Immunotools, Friesoythe, Germany), 50 ng/mL epidermal growth factor (EGF), and 10 ng/mL fibroblast growth factor-4 (FGF-4) (both R&D, Wiesbaden, Germany). Cultures were analyzed on d 7, 14, and 21.

Separation of GFP positive and negative cells in co-cultures by fluorescence activated cell sorting (FACS)

After the culture period, cells from co-cultures were trypsinized, counted with trypan blue, and re-suspended in 3mL PBS. To get single cell suspension, cells were filtered through a 35 μm filter (BD, Heidelberg, Germany). Cells were sorted using the FACS Aria™ Cell Sorter (BD) into GFP-positive (GFP+) or GFP-negative (GFP-) cells, focussing on the highest possible purity of GFP+ cells.

RNA extraction from cells by RT-PCR

RNA was extracted using the Invisorb Spin Cell-RNA™ Mini-kit (Invitek, Berlin, Germany) according to the manufacturer's instructions and stored at -80℃. Reverse transcription (RT) of extracted RNA was performed using the bulk first-strand c-DNA synthesis kit (Amersham, Freiburg, Germany). The cDNA was stored at -20℃. For PCR, 5 μL of cDNA-template was mixed with 2.5 μL of 10× PCR-buffer, 0.5 μL of 10 mmol dNTP´s, 0.5 μL of each primer (50 ng/μL), and 0.5 μL of polymerase (AmpliTaq., Gibco) in a total volume of 25 μL for each probe. PCR was carried out in a programmable Biometra Uno-Thermobloc (Biometra, Göttingen, Germany) using the primers for albumin, AFP, and CK-18 as described previously[10]. Negative controls were performed for each set of primers. Samples were analyzed on 1% agarose gels. The size of the PCR-fragments was estimated using the 100-base-pair ladder (Gibco BRL).

RESULTS

FACS of co-cultured cells

The sorting strategy concentrated on maximal purity of GFP+ cells to exclude contamination with FLC-derived RNA. A typical example for FACS 1 wk after co-culture is shown in Figure 1. The viable cells were gated according to forward and side scatter properties (Figure 1A). Two populations varied in size and granulation. GFP-expression of the gated cells was investigated in two populations, differing in granulation (Figure 1B) and gated as P2 cells. From these P2-cells, the contaminating GFP- cells were sorted out (Figure 1C). All non-P1, non-P2 and non-GFP cells were collected as GFP- cells. When the number of viable cells (gate P1) was set as 100%, the fraction of GFP positive cells decreased from 24.2 % of viable cells at wk 1 to 1.3 % of viable cells at wk 3 (Table 1). This was also con-

Table 1 Fractions of FAC-sorted cells (%)

	Wk 1	Wk 2	Wk 3
Viable Cells	70.2	79.1	77
GFP-positive cells	34.5	5.6	1.7
GFP-negative cells	65.5	94.4	98.3

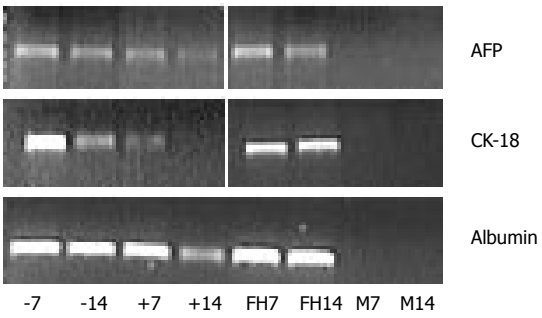

Figure 2 Expressions of the liver specific markers (AFP, CK-18, or albumin) of cultured rMSCs (M), fetal liver cells (FH), and GFP+ (+) or GFP- (-) cells of the co-cultures on d 7 or 14. Cultured rMSCs lacked expression of AFP, CK-18, or albumin on d 7 or 14 (lanes M7 and M14). Cultured fetal liver cells showed expression of AFP, CK-18 and albumin on d 7 or 14 (lanes FH7 and FH14). The GFP+ cells (lanes +7 and +14) showed clear expression of albumin and AFP, and lower expression of CK-18. The GFP- cells (lanes -7 and -14) showed a stable expression of AFP, CK-18, and albumin.

sistent with the morphological findings in the co-cultures, where a massive overgrowth of the adherent MSCs to the fetal liver cells was observed.

Liver specific geneexpression (AFP, CK-18, albumin)

Glyceraldehyde phosphate dehydrogenase (GAPDH) expression in cultured rMSCs, fetal liver cells, or GFP+ and GFP- FACS cells was equal at all time points (data not shown). Cultured rMSCs showed no expression of AFP, CK-18, or albumin on d 7 or 14 (Figure 2, lanes M7 and M14). Cultured fetal liver cells showed expression of AFP, CK-18 and albumin over the two wk culture period (Figure 2, lanes FH7 and FH14). The GFP+ cells (Figure 2, lanes +7 and +14) showed a strong expression of albumin and AFP, and a declining expression of CK-18. The GFP- cells (Figure 2, lanes -7 and -14) showed a stable expression of AFP, CK-18, and albumin. Negative controls without template were negative at all time points and probes (data not shown). On d 21, no liver specific genes were detected in the GFP+ cells (data not shown) probably due to the small cell number.

DISCUSSION

In this study, cloned GFP+ rMSCs from passage ⩾9 were used for the differentiation analysis towards liver cells. For liver specific differentiation, cells were cultured on a fibronectin matrix in serum free medium containing the liver growth factors: HGF, EGF, FGF-4, and the stem cell growth factor: SCF. The impact of FLC on hepatic differentiation was assessed in co-cultures of GFP+ rMSCs with freshly isolated fetal rat liver cells (GFP-negative), and compared to cultures with rMSCs or FLC alone. For gene-expression analysis, cells from co-cultures were separated in GFP+ or GFP- cells by fluorescence-activated cell sorting before RT-PCR analysis.

Albumin is a typical marker of mature hepatocytes, whereas CK18 is expressed in several liver cell types, including biliary epithelial cells and hepatic oval cells[14]. AFP in the liver is a marker of immature (e.g. fetal) liver cells or oval cells in the adult liver[15]. Our data indicate that rMSCs possess a differentiation potential towards hepatocytic cells *in vitro*. Expression of the liver specific genes CK-18, albumin, and AFP was demonstrated in GFP+ cells of the cocultures for two weeks. Additionally, we found that liver specific gene expression in mesenchymal stem cells was induced by the coculture with isolated FLC. Cultured rMSCs alone did not express any of the liver specific genes studied in the presence of fibronectin (FN)-coating and liver-differentiation stimulating growth factors. Avital *et al*[16] have highlighted the effects of co-cultured hepatocytes (separated by a PTFE-membrane) for β2m-negative thy1-positive stem cells from rat bone marrow expressing the liver marker albumin[16]. An important influence of hepatocytes on the differentiation of stem cell-enriched bone marrow has also been highlighted by Okumoto *et al*[17] who found that MSCs-enriched bone marrow stem cells cultured in the presence of HGF and fetal bovine serum (FBS) express the markers: hepatic nuclear factor 1 (HNF1-α) and CK-8 only after 7 d. In co-cultures with hepatocytes separated by a semi-permeable membrane the stem cells additionally express the liver specific markers: AFP and albumin. We found a beneficial effect for differentiation and growth of the co-cultured fetal liver cells (GFP-), which also showed a stable liver specific gene expression and viability over the whole observation period. This is consistent with findings of Hoppo *et al*[18] who showed a positive influence of MSCs on cultured endodermal fetal liver cells of the mouse in culture. However, the FLC tended to overgrow the adherent layer of MSCs at the end of the culture period. Thus, we could not detect any liver specific gene expression in the few remaining GFP+ MSC-derived cells in the co-cultures at wk 3. Our results support the notion of a faster differentiation of MSCs into hepatocytic cells under the influence of fetal liver cells compared to adult hepatocytes. The growth potential of MSCs seems to be impaired by the huge overgrowth of FLC, thus limiting the desired expansion of differentiated MSCs. Based on the declined GFP-positive fraction of cells and the decreased intensity of semi-quantitative PCR-signals over the experimental time of 3 wk, differentiation of rMSCs into hepatocytic cells could be suggested. However, fusion events can not be ruled out by our data.

Our *in vitro* data indicate that mesenchymal stem cells from rat bone marrow possess a differentiation capacity towards hepatocytic cells *in vitro*. Furthermore, we showed a strong influence of co-cultures with fetal liver cells on the induction of liver specific gene expression of cultured stem cells. However, fetal liver cells tended to overgrow the adherent MSCs in the co-cultures.

In conclusion, the co-culture of MSCs and FLC is a feasible culture model which might provide more insights into the relationship between fetal hepatopoietic cells during liver development.

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RAPID COMMUNICATION

Does *Helicobacter pylori* infection eradication modify peptic ulcer prevalence? A 10 years' endoscopical survey

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Abstract

AIM: To compare peptic ulcer prevalence in patients referred for upper gastrointestinal endoscopy in two Italian hospitals in pre-*Helicobacter* era and ten years after the progressive diffusion of eradication therapy.

METHODS: We checked all the endoscopic examinations consecutively performed in the Gastroenterology Unit of Padova during 1986-1987 and 1995-1996, and in the Gastroenterology Unit of Parma during 1992 and 2002. Chi Square test was used for statistic analysis.

RESULTS: Data from both the endoscopic centers showed a statistically significant decrease in the prevalence of ulcers: from 12.7% to 6.3% ($P < 0.001$) in Padova and from 15.6% to 12% ($P < 0.001$) in Parma. The decrease was significant both for duodenal (from 8.8% to 4.8%, $P < 0.001$) and gastric ulcer (3.9% to 1.5%, $P < 0.001$) in Padova, and only for duodenal ulcer in Parma (9.2% to 6.1%, $P < 0.001$; gastric ulcer: 6.3% to 5.8%, NS).

CONCLUSION: Ten years of extensive *Helicobacter pylori* (*H pylori*) eradication in symptomatic patients led to a significant reduction in peptic ulcer prevalence. This reduction was particularly evident in Padova, where a project for the sensibilization of *H pylori* eradication among general practioners was carried out between 1990 and 1992. Should our hypothesis be true, *H pylori* eradication might in the future lead to peptic ulcer as a rare endoscopic finding.

Key words: Ulcer prevalence; *H pylori*

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INTRODUCTION

It is now well established that *Helicobacter pylori* (*H pylori*) eradication can significantly modify the natural history of peptic ulcer disease. Marshall *et al* first demonstrated in 1988 that when *H pylori* was cleared 92% of ulcers healed and only 21% relapsed during a 12 mo follow-up period[1]. Several studies have then confirmed these data over the years. Follow-up studies have in fact shown lower relapse rates of both gastric and duodenal ulcers after successful *H pylori* eradication, in the short term as in the long term[2-4]. So by the end of the eighties *H pylori* was generally accepted as a causal factor in the pathogenesis of chronic gastritis and peptic ulcer. Between the end of the eighties and the beginning of the nineties eradication of *H pylori* has become a widespread approach for acid-related disorders, first among GI specialists, then among general practitioners.

Leerdam *et al* (2003) described a reduction in the incidence of upper gastrointestinal bleeding between 1993/94 and 2000[5]. They hypothesized that the decrease in incidence might partly be explained by the fact that *H pylori* is more often eradicated in patients with dyspeptic complaints and peptic ulcer disease, thus reducing the possibility of development of complications as bleeding. Furthermore, it is often reported as an impression by endoscopists that we see fewer ulcers than we did years ago, but data on the real prevalence are still scanty. Xia *et al* (2001) studied the prevalence of *H pylori* infection, peptic ulcer disease and reflux esophagitis in consecutive patients referred for upper GI endoscopy in an endoscopy unit in Sydney in a three-month period in different years (1990, 1994 and 1998), reporting a decrease in peptic ulcer disease

(22%, 15% and 13%, respectively, $P = 0.003$). They also described a lower prevalence of *H pylori* infection as well as a significant decrease in NSAIDs consumption, leading to the hypothesis that both these two risk factors likely contributed to the reduction of peptic ulcer disease[6].

Therefore, the aim of this study was to compare the prevalence of peptic ulcer disease among patients referred for upper GI endoscopy between the eighties and the nineties and after a period of 10 years, in two Italian GI units of two hospitals.

MATERIALS AND METHODS

Patients

We retrospectively analyzed all the upper GI endoscopies performed in the Gastroenterology Unit of Parma in two different years (1992 and 2002), and in the Gastroenterology Unit of Padova in two periods: from Feb 1, 1986 to Dec 31, 1987 and from Feb 1, 1995 to Dec 31, 1996. We selected the patients with a diagnosis of gastric or duodenal ulcer. Both the endoscopic units of Parma and Padova serve in-patients and out-patients, and are the major endoscopy centers of the area. Out-patients are directly sent by general practitioners or by specialists. Both the units have an informatic database (DB3 engine). We searched for gastric and duodenal ulcers both manually and informatically, with the strings "(gastrica or gastriche or angolare or antrale or del corpo-fondo) and/or (duodenale/i or bulbare or bulbari or del duodeno) and (ulcera or ulcere)".

Between 1990 and 1992 a project was performed in Padova in order to stimulate the aptitude towards the *Helicobacter pylori* (*H pylori*) eradication among physicians. It involved both specialists and general practitioners.

H pylori status was obtained for all patients accessing the hospital, through the dosage of serum antibodies. Data about upper GI symptoms and history of acid-related disorders were also collected. All patients positive for *H pylori* infection were treated with a triple one week therapy ("ulcer-free hospital" project).

Several meetings were held by the Gastroenterology Clinic of Padova between 1990 and 1992, with the participation of groups of 30 general practitioners. Statements discussion and interactive clinical case analysis were conducted, with initial and final testing of *H pylori* . General practitioners were sensibilized to test all patients suffering from upper GI symptoms or with a history of gastritis or peptic ulcer, diagnosed with a structured questionnaire ("ulcer-free ambulatory") and eradicate *H pylori*, when positive.

Statistical analysis

Chi Square test was applied on the changes of prevalence of peptic ulcers (total), gastric and duodenal ulcers after a decade. $P < 0.05$ was considered as significant.

RESULTS

In Parma we analyzed a population of 3779 subjects in 1992 (2185 out-patients and 1594 in-patients), with a mean age of 69.4 years (range 5-94 years) and a sex distribution of 54.4% males and 45.6% females, as well as a population of 3828 subjects in 2002 (1985 out-patients and 1843 in-patients), with a mean age of 62.3 years (range 12-97 years) and a sex distribution of 53.4% males and 46.6% females. We found 588 ulcers in 1992 (239 GU and 349 DU), 459 ulcers in 2002 (223 GU and 236 DU). *H pylori* status was evaluable only for 28.7% of patients with peptic ulcer in 1992 and 47.7% in 2002, so it could not be useful for statistical analysis. Among gastric ulcers, we found neoplastic lesions in 56 subjects in 1992 and 20 subjects in 2002.

In Padova we analyzed a population of 3703 subjects during 1986-1987, with a mean age of 54 years (range 15-91 years) and a sex distribution of 53.5% males and 46.5 females, as well as a population of 5727 subjects during 1995-1996, with a mean age of 51 years (range 14-98 years) and a sex distribution of 39.7% males and 60.3% females. We found 470 ulcers during 1986-87 (144 GU and 326 DU), 361 ulcers during 1995-1996 (86 GU and 275 DU). *H pylori* status was available only for patients with peptic ulcer during 1995-1996 and showed a prevalence of 83.6% (68.6% for GU and 88.4% for DU). It was determined by histology of mucosa, with appropriate staining.

Table 1 and Table 2 summarize the changes in prevalence of peptic ulcer after ten years in Parma and in Padova. Table 3 and Table 4 describe the epidemiological characteristics of the populations we studied.

Both the endoscopic centers showed a statistically significant decrease in the prevalence of ulcers: 12.7% to 6.3% ($P < 0.001$) in Padova, 15.6% to 12% ($P < 0.001$) in Parma. The decrease was greater for duodenal ulcer (8.8% to 4.8%, $P < 0.001$ in Padova, 9.2% to 6.1%, $P < 0.001$ in Parma) than for gastric ulcer (3.9% to 1.5%, $P < 0.001$ in Padova, 6.3% to 5.8%, NS in Parma).

Table 1 Changes in prevalence of peptic ulcer after ten years in Padova

Padova	1986-1987 *n* (%)	1995-1996 *n* (%)	*P*
Population	3703	5727	
Total of ulcers	470 (12.7)	361 (6.3)	< 0.001
Duodenal ulcer	326 (8.8)	275 (4.8)	< 0.001
Gastric ulcer	144 (3.9)	86 (1.5)	< 0.001

Table 2 Changes in prevalence of peptic ulcer after ten years in Parma

Parma	1992 *n* (%)	2002 *n* (%)	*P*
Population	3779	3828	
Total of ulcers	588 (15.6)	459 (12)	< 0.001
Duodenal ulcer	349 (9.2)	236 (6.1)	< 0.001
Gastric ulcer	239 (6.3)	223 (5.8)	NS

Table 3 Characteristics of the population from Parma

	1992	2002
Population (*n*)	3779	3828
Origin	2185 out-patients, 1594 in-patients	1985 out-patients, 1843 in-patients
Age (yr)	69.4 (range 5-94)	62.3 (range 12-97)
Sex (M/F)	54.4%/45.6%	53.4%/46.6%

Table 4 Characteristics of the population from Padova

	1986-1987	1995-1996
Population (*n*)	3703	5727
Age (yr)	54 (range 15-91)	51 (range 14-98)
Sex (M/F)	39.7%/ 60.3%	53.5%/ 46.5%

DISCUSSION

The study suggests that the incidence of peptic ulcer among patients referred for upper GI endoscopy significantly decreased through the years. Our hypothesis is that *H pylori* eradication could have changed the natural history of peptic disease. Eradication of the bacterium by the general practitioners in symptomatic subjects has become a common approach through the nineties. So we identified those patients who have not been eradicated or who were still symptomatic after the therapy.

One limit of the present study is the examination of a selected population, which was referred for upper GI endoscopy and probably had been given antisecretory drugs during the weeks preceding the access to the endoscopy. We have asked ourselves if the reduced prevalence of ulcer after ten years could be related with differences in drug prescription of anti-secretive agents among general practitioners. We did not collect data on drug intake, however, we thought this finding might only play a secondary role in the observed trend. In fact, significant differences in drug prescription could be seen when comparing the early eighties to the nineties, and since the late eighties histamine H2-receptor antagonists (anti-H2s) and proton pump inhibitors (PPIs) have been widely used in both the Italian areas we examined.

Capurso *et al* (1996)[7] retrospectively analysed upper gastrointestinal endoscopies performed in their center in Rome between January 1981 and December 1991. They reported an incidence of 4.1%±0.6% and a mean annual prevalence of 6.9%±0.7%. These data are quite similar to those in our center at the beginning of the observation period.

Data in Parma for peptic ulcer prevalence are quite similar to those reported by Xia *et al* in Sidney during the same decade. The authors concluded that both the decreased use of NSAIDs and the decline of *H pylori* infection have likely contributed to the reduction of peptic ulcer disease. Regretfully we did not have data about the *H pylori* status and the NSAIDs use of the population we examined. A reduced use of NSAIDs during the last decade has, however, not been reported in our areas, so we do not think it could have played a significant role.

The two populations examined in the present study showed different prevalence of both duodenal and gastric ulcer. We are not sure if this reflected a really different prevalence in the general populations of Parma and Padova, since no available data were collected on this subject. On the other hand, it must be underlined that the organization of both endoscopic units was similar; they tested both in-patients and out-patients, directly sent by general practitioners or by specialists.

Padova showed a greater significant decrease in the prevalence of ulcers through the decade. This might be due to the fact that the Gastroenterology Department of the University of Padova performed during those years a diffuse sensibilization of general practitioners about the eradication of *H pylori*, by means of the so called "Ulcer Free Project", as above described.

The decrease in prevalence was greater for duodenal ulcer than for gastric ulcer in both the studied populations. This is probably related to the different role played by *H pylori* in gastric and duodenal ulcer pathogenesis: it is known that more than 90% of duodenal ulcers but only 70% of gastric ulcers are associated with *H pylori* infection. This is in line with the results of the meta-analysis conducted by Ford *et al*[8]. They showed a reduction of relative risk of 54% in the recurrence of duodenal ulcer after *H pylori* eradication, and a still significant but smaller reduction of relative risk of 37% for gastric ulcer.

Additionally, it must be stressed that the role of anti-inflammatory drugs in the pathogenesis of gastric ulcer could be important in trying to correctly explain these data, but as previously mentioned, we lack at present epidemiological data on this subject.

In conclusion, we think *H pylori* eradication may in the future lead to peptic ulcer as a rare endoscopic finding, particularly in areas where a diffuse information program among general practitioners is performed. By now, the absolute number of ulcers we have diagnosed is still high, and there is need for more prevention strategies.

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RAPID COMMUNICATION

Conventional colonoscopy and magnified chromoendoscopy for the endoscopic histological prediction of diminutive colorectal polyps: A single operator study

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Abstract

AIM: To accurately differentiate the adenomatous from the non-adenomatous polyps by colonoscopy.

METHODS: All lesions detected by colonoscopy were first diagnosed using the conventional view followed by chromoendoscopy with magnification. The diagnosis at each step was recorded consecutively. All polyps were completely removed endoscopically for histological evaluation. The accuracy rate of each type of endoscopic diagnosis was evaluated, using histological findings as gold standard.

RESULTS: A total of 240 lesions were identified, of which 158 (65.8%) were non-neoplastic and 82 (34.2%) were adenomatous. The overall diagnostic accuracy of conventional view, and chromoendoscopy with magnification was 76.3% (183/240) and 95.4% (229/240), respectively ($P < 0.001$).

CONCLUSION: The combination of colonoscopy and magnified chromoendoscopy is the most reliable non-biopsy method for distinguishing the non-neoplastic from the neoplastic lesions.

Key words: Colorectal polyps; Colonoscopy; Chromoendoscopy; Magnifying endoscopy

De Palma GD, Rega M, Masone S, Persico M, Siciliano S, Addeo P, Persico G. Conventional colonoscopy and magnified chromoendoscopy for the endoscopic histological prediction of diminutive colorectal polyps: A single operator study. *World J Gastroenterol* 2006; 12(15): 2402-2405

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INTRODUCTION

Recently, colorectal polyps have been identified with increasing frequency as a result of the wide use of colonoscopy for screening. The detection and subsequent removal of neoplastic colorectal lesions, including adenomatous polyps and early cancers can reduce the incidence and mortality of colorectal cancers. Although initial data suggest that 80%-90% of diminutive colorectal polyps (≤5 mm) are histologically hyperplastic, more recent data indicate that 40%-60% of such polyps are neoplastic [1-5]. The ability to accurately differentiate the adenomatous from the non-adenomatous polyps is useful because it obviates the need for biopsies during colonoscopy and for removal of all diminutive polyps[6-8]. Some investigators suggest that the combination of magnified chromoendoscopy and colonoscopy can make the histopathological diagnosis of colorectal polyps. However, how this technique increases the accuracy of differential diagnosis of adenomatous from non-adenomatous polyps over conventional colonoscopy is unclear.

This study was to compare the observations by conventional colonoscopy to these obtained by magnified chromoendoscopy in a group of patients with diminutive colorectal polyps (≤5 mm), using histopathology as a "gold standard".

MATERIALS AND METHODS

Patients and exclusion criteria

Patients were eligible to participate in the study if they were scheduled to undergo flexible colonoscopy in our center. Patients with polyps larger than 5 mm were excluded from this study. Patients with inflammatory bowel disease (IBD), hereditary non-polyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP) were also excluded.

The study protocol was approved by the institutional review board of our hospital. An informed consent was

Table 1 Characteristics of patients and indications for chromoendoscopy

Characteristics of patients	
Patients (*n*)	150
Gender (males/females)	93/57
Age (yr), mean (range)	58.9 (37-85)
Indications for chromoendoscopy	
Screening	58
Family history	50
Lower GI bleeding	26
Altered bowel habit	16

Table 2 Characteristics and distribution of colonic lesions

No. of lesions	240
0-Ip (protruded, pedunculated)	18
0-Is (protruded, sessile)	122
0-IIa (superficial, elevated)	100
Adenomatous polyps	82
Non-adenomatous polyps	158
Distribution of colonic lesions	
Rectum	76
Sigmoid	96
Left colon	22
Transverse	6
Right colon	40

Table 3 Actual and predicted histology of 240 polyps by conventional colonoscopy

	Histology	
	AP[1]	non-AP[1]
Endoscopic prediction: AP	75/82	50/158
Endoscopic prediction: non-AP	7/82	108/158

[1]AP = adenomatous polyps; non-AP = non-adenomatous polyps.

obtained from each patient for the inclusion in the study.

Diagnostic procedures

A zoom-colonoscope (Olympus CF-Q160ZL, Olympus Optical Co., Ltd., Tokyo, Japan) providing both conventional and magnified images was used. The colonoscope was introduced up to the cecum. Subsequently, while the instrument was retracted, the colorectal mucosa was scrutinized in detail. When a polyp was detected, the mucus on the surface of the lesion was washed away and the polyp was first examined with the conventional view. Then 0.5% indigo carmine dye was sprayed over the lesion with a special catheter and observation was carried out. On conventional view, the key endoscopic findings for distinguishing non-neoplastic from neoplastic lesions were based on the gross appearance at visual inspection, namely the size, shape, overlying mucus, and color of the lesions. Macroscopic appearance was reported according to the Paris endoscopic classification of superficial neoplastic lesions[9].

The polyps were categorized as neoplastic or non-neoplastic as previously described[10]. The diagnosis at each step was recorded consecutively and all polyps were completely removed endoscopically. The accuracy rate of each type of endoscopic diagnosis was evaluated, using histological findings as gold standard. Morphology, size, and anatomical location of all lesions were documented. The diameter of each lesion was estimated using standard fully open biopsy forceps (4 mm) with the height estimated by placing the closed forceps tip adjacent to the lesion (2 mm). A single colonoscopist who was well trained in magnified colonoscopy, performed all procedures.

Statistical analysis

The number of patients in each group to demonstrate statistical significance with 5% alpha error and 90% power was estimated to be 47 and calculated on the basis of previous data[11-14], assuming a 25% difference in negative predictive value (NPV) for adenomatous polyps between the two groups. A chi-square analysis was performed for comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

Of the 1982 consecutive patients attending for routine colonoscopy, 150 (93 men and 57 women with a mean age of 58.9 years) with polyps smaller than 5 mm in diameter were recruited between December 2004 and July 2005. Characteristics of the patients enrolled and indications for endoscopy are shown in Table 1. Total colonoscopy and histological evaluation were carried out in all patients without complications. A total of 240 lesions were identified. A single lesion was found in 101 (67.3%) cases, and 35 (23.3%) and 14 (9.3%) patients had two or more than three lesions, respectively.

A total of 158 lesions (65.8%) were non-neoplastic (including hyperplastic polyps, inflammatory polyps, and lymphoid aggregates) and 82 (34.2%) were adenomatous. Of these lesions, 194 (80.8%) were located in the distal colon (below the splenic flexure) and 46 (19.2%) in the proximal colon. The mean size of the non-neoplastic and neoplastic lesions was 3.46 mm and 3.83 mm, respectively. Although the neoplastic lesions might be larger than the non-neoplastic ones, the difference was not significant. The characteristics and distribution of colonic lesions are shown in Table 2.

The surface pattern of the lesion could be obtained and interpreted adequately in all cases. The overall diagnostic accuracy for distinguishing the neoplastic from the non-neoplastic lesions at each step (that is, conventional colonoscopy and magnified chromoendoscopy) was 76.3% (183/240), and 95.4% (229/240), respectively. The method of magnified chromoendoscopy with indigo carmine dye was significantly better for distinguishing the neoplastic from the non-neoplastic lesions, compared with conventional colonoscopy ($P < 0.0001$).

Fifty-seven of 240 lesions were misdiagnosed by conventional colonoscopy, including 38 hyperplastic polyps, 8 inflammatory polyps and 4 lymphoid aggregates which were overdiagnosed and 7 adenomas with mild atypia which were underdiagnosed. For the detection of adenomatous and non-adenomatous polyps, conventional colonoscopy had a sensitivity of 91.4%, specificity of 68.3%, NPV of 93.9% and PPV of 60.0% (Table 3). Eleven of 240 lesions were misdiagnosed by magnified chromo-

Table 4 Actual and predicted hystology of 240 polyps by high-resolution chromoendoscopy

	Histology	
	AP[1]	non-AP[1]
Endoscopic prediction: AP	80/82	9/158
Endoscopic prediction: non-AP	2/82	149/158

[1]AP= adenomatous polyps; non-AP= non-adenomatous polyps.

endoscopy, including 5 hyperplastic polyps, 2 inflammatory polyps and 2 lymphoid aggregates which were overdiagnosed and 2 adenomas with mild atypia which were underdiagnosed. For the detection of adenomatous and non-adenomatous polyps, high-resolution chromoendoscopy had a sensitivity of 97.5%, specificity of 94.3%, NPV of 98.69% and PPV of 89.9% (Table 4).

DISCUSSION

Given the incidence of colorectal cancer in the Western population and the recent thrust to implement broad-based screening methods, the issue of endoscopic resection of potentially cancerous or precancerous lesions assumes immense practical importance. It has been estimated that approximately two thirds of colorectal cancers arise from adenomatous polyps. The endoscopic detection and removal of polypoid tumors, therefore, have been emphasized to prevent the development of advanced colorectal cancers[2,15,16]. Since 10%-30 % of all colorectal polyps are non-neoplastic, it is very important to distinguish non-neoplastic from neoplastic lesions by colonoscopy, because removal or biopsy of non-neoplastic lesions would not only waste time and money but may also increase procedure-associated complications[6-8]. In particular, since reported data suggest that 80%-90% of diminutive colorectal polyps ($\leqslant$5 mm) are histologically hyperplastic, the ability to establish an immediate endoscopic diagnosis that is virtually consistent with the histology would be useful because it would obviate the need for biopsies during colonoscopy and also the need to remove all diminutive polyps, which is time consuming, expensive, and associated with risk.

Various data have been reported on the diagnostic abilities of conventional colonoscopy and magnified chromoendoscopy for such differentiation[11-15]. However, to the best of our knowledge, there is no prospective study on the accuracy for the differential diagnosis of non-neoplastic from neoplastic colorectal lesions using conventional colonoscopy and high-resolution chromoendoscopy consecutively.

In the present study, the diagnostic accuracy by conventional endoscopy and magnified chromoendoscopy using indigo carmine was 72.0% and 95.6%, respectively, with statistically significant difference ($P < 0.0001$). Fifty-seven of 240 lesions were misdiagnosed by conventional endoscopy, including 38 hyperplastic polyps, 8 inflammatory polyps and 4 lymphoid aggregates which were over-diagnosed and 7 adenomas with mild atypia which were underdiagnosed. For the detection of adenomatous and non-adenomatous polyps, conventional colonoscopy could show over 90% of the neoplastic lesions, but less than 70% of the non-neoplastic lesions, suggesting the possibility of excessive treatment, waste of time and resources (Table 3). Eleven of 240 lesions were misdiagnosed by magnified chromoendoscopy, including 5 hyperplastic polyps and 2 inflammatory polyps, 2 lymphoid aggregates which were over-diagnosed and 2 adenomas with mild atypia which were underdiagnosed. Magnified chromoendoscopy displayed 97.5% of the neoplastic lesions and 94.3% of the non-neoplastic lesions. Two (0.8%) false negative cases due to mixed polyps were diagnosed by magnified ehromoendoscopy.

Because the NPV was 98.6% indicating that some polyps were overlooked, it could be argued that high-resolution chromoendoscopy is not completely useful in clinical practice. Since no screening tests currently used have a NPV of 100%, the combination of magnified colonoscopy and dye spraying is the most reliable non-biopsy method for distinguishing the non-neoplastic from the neoplastic lesions at present. Moreover, with improved dyes and higher resolution chromoendoscope, better results might be obtainable. In this study all procedures were performed by a single operator who was well trained in magnified chromoendoscopy. The problem of a single operator study can represent the difficulty in deciding whether such results can be generalized. The time necessary for learning to recognize the mucosal crypt patterns is important, but has not been precisely reported. The endoscopist in this study had 5 years of experience in magnified endoscopy. In our previous experience, a foreign doctor without knowledge of pit patterns took only 3 months to acquire a differential diagnostic accuracy of over 90 %, which was similar to that of the well-trained endoscopists in this study (unpublished data). We believe that the time for learning could be shortened by showing trainees numerous typical endoscopic pictures of pit patterns before they perform the diagnostic procedure. However, this should be confirmed by future prospective studies including a large number of endoscopists.

Further questions include whether magnified endoscopy is as effective as total colonoscopy, how much time this adds to per case, and what is the cost associated with this procedure. In this study, with an instrument that was slightly more difficult to handle, intubation of the cecum was achieved for all patients without complications. Therefore, our results support the conclusion that magnified endoscopy is safe and can be used routinely. In our experience, the procedure usually only takes about 10-20 seconds for one polyp, and is less troublesome and time-wasting than colonoscopy.

In conclusion, the combination of colonoscopy and magnified chromoendoscopy is the most reliable nonbiopsy method for distinguishing non-neoplastic from neoplastic lesions. The use of this technique could decrease the time and resources required during colonoscopy by reducing the need for biopsy and resection of non-neoplastic polyps.

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RAPID COMMUNICATION

Phosphate-activated glutaminase activity is enhanced in brain, intestine and kidneys of rats following portacaval anastomosis

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Abstract

AIM: To assess whether portacaval anastomosis (PCA) in rats affects the protein expression and/or activity of glutaminase in kidneys, intestines and in three brain areas of cortex, basal ganglia and cerebellum and to explain the neurological alterations found in hepatic encephalopathy (HE).

METHODS: Sixteen male Wistar rats weighing 250-350 g were grouped into sham-operation control ($n = 8$) or portacaval shunt ($n = 8$). Twenty-eight days after the procedure, the animals were sacrificed. The duodenum, kidney and brain were removed, homogenised and mitochondria were isolated. Ammonia was measured in brain and blood. Phosphate-activated glutaminase (PAG) activity was determined by measuring ammonia production following incubation for one hour at 37℃ with O-phthalaldehyde (OPA) and specific activity expressed in units per gram of protein (μkat/g of protein). Protein expression was measured by immunoblotting.

RESULTS: Duodenal and kidney PAG activities together with protein content were significantly higher in PCA group than in control or sham-operated rats (duodenum PAG activity was 976.95±268.87 μkat/g of protein in PCA rats *vs* 429.19±126.92 μkat/g of protein in sham-operated rats; kidneys PAG activity was 1259.18 ± 228.79 μkat/g protein in PCA rats *vs* 669.67± 400.8 μkat/g of protein in controls, $P < 0.05$; duodenal protein content: 173% in PCA *vs* sham-operated rats; in kidneys the content of protein was 152% in PCA *vs* sham-operated rats). PAG activity and protein expression in PCA rats were higher in cortex and basal ganglia than those in sham-operated rats (cortex: 6646.6 ± 1870.4 μkat/g of protein *vs* 3573.8 ± 2037.4 μkat/g of protein in control rats, $P < 0.01$; basal ganglia, PAG activity was 3657.3 ± 1469.6 μkat/g of protein in PCA rats *vs* 2271.2 ± 384 μkat/g of protein in sham operated rats, $P < 0.05$; In the cerebellum, the PAG activity was 2471.6 ± 701.4 μkat/g of protein *vs* 1452.9 ± 567.8 μkat/g of protein in the PCA and sham rats, respectively, $P < 0.05$; content of protein: cerebral cortex: 162% ± 40% *vs* 100% ± 26%, $P < 0.009$; and basal ganglia: 140% ± 39% *vs* 100% ± 14%, $P < 0.05$; but not in cerebellum: 100% ± 25% *vs* 100% ± 16%, P = ns).

CONCLUSION: Increased PAG activity in kidney and duodenum could contribute significantly to the hyperammonaemia in PCA rats, animal model of encephalopathy. PAG is increased in non-synaptic mitochondria from the cortex and basal ganglia and could be implicated in the pathogenesis of hepatic encephalopathy. Therefore, PAG could be a possible target for the treatment of HE or liver dysfunction.

Key words: Hyperammonaemia; Minimal hepatic encephalopathy; Glutamine; Protein content; Sham-operated

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INTRODUCTION

Hyperammonaemia plays a major role in the pathogenesis of hepatic encephalopathy (HE). Phosphate-activated glutaminase (PAG) catalyzes the hydrolysis of glutamine (Gln) to yield glutamate (Glu), energy, nucleotide synthesis and ammonia. Two main isoforms of PAG have been described: the kidney-type (K-PAG) and the liver type (L-PAG). The K-PAG has been found in kidney, brain and villi enterocytes, while L-PAG is restricted to the liver. Hyperammonaemia is largely considered to be derived from urea breakdown by intestinal bacteria, thus most treatments have been focussed on reducing ammonia production from colon bacteria[1]. However, some data suggest that small intestine plays an important role in ammonia production during the pathogenesis of hepatic encephalopathy[2]. Indeed, duodenal PAG activity has been found to be nearly four times higher in cirrhotic patients than in healthy controls, and moreover it is closely related to minimal hepatic encephalopathy[3]. In rats, the highest PAG activity along the length of gastrointestinal tract has been measured in the small intestine[4]. Lower but still substantial activity (15% of total PAG activity) has been found in large intestine. PAG activity distribution along the gastrointestinal tract in humans is similar to that in rats[5]. PAG plays another important role at renal level. Glutamine is filtered and reabsorbed in the proximal convoluted tubule where it is deamidated by PAG. Two thirds of this ammonia production is excreted in urine. This process is pH sensitive and helps to maintain acid-base homeostasis and to excrete nitrogen[6]. However, PAG activity in kidney remains largely unexplored in portacaval shunted rats. Distribution and location of PAG in brain as well as its role in the pathogenesis of HE, are widely controversial, while PAG activity in portacaval shunted rats still remains unknown. Portacaval anastomosis (PCA; or portacaval shunt PCS) in rats is widely accepted as a model of type B hepatic encephalopathy that mimics minimal hepatic encephalopathy in humans. The aim of this study was to assess whether portacaval anastomosis in rats affects the expression and/or activity of glutaminase in kidney, intestines and the astrocytes of three brain areas (cortex, basal ganglia and cerebellum) involved in the neurological alterations of hepatic encephalopathy.

MATERIALS AND METHODS

Animal surgery

Sixteen male Wistar rats (n = 16) weighing 250-350 g were randomly allocated into sham operation controls (n = 8) or portacaval shunt (n = 8). All animals were pair fed two weeks before and four weeks after operation and kept under standard laboratory conditions. Both PCA and sham-operated rats were fasted overnight in single wire-net floor cages with free access to tap water. All animal procedures were approved by our institution and met the guidelines of Spain (RD 223 erased 14th March 1998) and European (Directive 86/609/CEE) Union for care and management of experimental animals.

Rats were operated under general anaesthesia with isofluorane to avoid liver metabolism of intravenous agents and end-to-side portacaval shunt was performed as previously described[7]. Briefly, after middle laparotomy for viscera exteriorization, inferior vena cava and portal veins were exposed, dissected and clamped laterally together with a Satinsky clamp. Longitudinal incisions in both veins and lateral anastomosis with running suture as usual were performed. Finally, portal trunk was tied and cut next of liver hilum, turning the portacaval lateral shunt in functionally terminal. Sham-operation was performed following laparotomy, the inferior vena cava was isolated and clamped for 30 s. Sham-operated animals served as controls.

Determination of ammonia in brain and blood

Ammonia was measured in cerebral cortex and blood as previously described[8]. Cerebral cortex was homogenized and deproteinised in 5 volumes of ice-cold 100 g/L trichloroacetic acid, and kept on ice for 15 min. After centrifugation at 12 000 r/min for 10 min at 4 ℃, the supernatants were collected, neutralized with 2 mol/L $KHCO_3$ and centrifuged at 12 000 r/min for 10 min at 4 ℃. The neutralized supernatants were used to measure ammonia in μmole/g tissue. Blood (150 μL) was taken from the tail vein the third week after surgery. Blood samples were deproteinized with one volume of ice-cold 100 g/L trichloroacetic acid and kept on ice for 15 min. After centrifugation at 12 000 r/min for 10 min at 4 ℃, the supernatants were collected, neutralized with 2 mol/L $KHCO_3$ and centrifuged at 12 000r/min for 10 min at 4 ℃. The neutralized supernatants were used to measure ammonia. In a final volume of 100 μL, the reaction mixture contained 50 μL or 60 μL of sample, 30 mmol/L α-ketoglutarate, 0.5 mmol/L nicotinamide adenine dinucleotide (reduced form) in potassium phosphate buffer (pH 8.0). After recording of the initial fluorescence, reactions were started by the addition of 5 μg of glutamate dehydrogenase (Boehringer Mannheim, Germany) and monitored by the fluorimeter (Fluoroskan Ascent; Labsystems; Oy, Helsinki, Finland) for at least 70 min. Standards containing up to 25 nmol of ammonia were included in each assay. Assays were performed in Costar 96-well UV plates (cat. No. 3635; Corning Costar Corporation, Cambridge, MA).

Animal handling and brain tissue preparation

All procedures were carried out in the cold room at 2℃-4℃. The rats were killed by cervical dislocation. The skull of each rat was opened to remove blood from the surface of the tissue and this procedure needed to be performed in <30 s. The tissue was placed in 5 mL of isolation medium in a Petri dish maintained in an ice bath. The brain was chopped with fine scissors and the chopped material was washed frequently with isolation medium to remove blood. Meanwhile, the first portion of small intestine (3 cm of length) was removed and placed in 5 mL of isolation medium in Petri dish maintained in an ice bath. The duodenum was washed and mucosa was removed by glass film and quickly frozen in liquid air. Samples of kidney were similarly obtained. Tissue samples were then homogenized manually in 1 mL of isolation medium per 100-150 mg of tissue using a Dounce

homogenizer fitted with a Teflon pestle having a total clearance of 0.1 mm. Usually, six up- and down-strokes were sufficient to generate a rough homogenate which was then diluted with isolation medium to a final volume of 1.2 mL and homogenized further with four up- and down-strokes.

Preparation of mitochondria

The procedure for isolation of brain mitochondria was based on previously reported methods[9] except for 1 mmol/L ethylene glycol-bis (β-aminoethyl ether) tetra-acetic acid (EGTA) being used in the homogenisation medium instead of EDTA. The homogenate was centrifuged at 2 000 r/min for 3 min, the pellet was washed with 400 μL of homogenisation medium and re-centrifuged at 2 000 r/min for 3 min. Both supernatants were pooled and centrifuged for 8 min at 12 000 r/min to obtain the crude mitochondrial pellet. The pellet was suspended in 300 μL of the 30 g/L Ficoll medium (see below) and layered onto 1.2 mL of 60 g/L Ficoll medium and centrifuged at 12 000 r/min for 30 min. The 60 g/L Ficoll medium contained 6% (w/w) Ficoll, 0.24 mol/L mannitol, 0.06 mol/L sucrose, 0.05 mmol/L K-EDTA and 10 mmol/L Tris-HCl, pH 7.4. The 30 g/L Ficoll medium was the 60 g/L Ficoll medium diluted 1:1 with glass re-distilled water. The loose, fluffy, white upper layer of the pellet was removed, the remaining brown pellet was re-suspended in isolation medium without EGTA and the suspension was centrifuged at 12 000 r/min for 10 min. The pellet was re-suspended in incubation medium (isolation medium with 2.5 mL/L buffer containing protease inhibitor, 7 g/L Triton X-100, 5 mmol/L β-mercapto-ethanol) to obtain a protein concentration in the range of 5-10 g/L. After incubation on a mixing wheel for 30 min at 4 ℃, the samples were frozen at -80 ℃ for batched activity measurement.

PAG activity assay

Mitochondrial protein was measured by the method of Bradford *et al*[10] with bovine serum albumin (BSA) as standard. Briefly, 25 μL of mitochondrial solution was added to 35 μL of reaction medium (150 mmol/L K_2HPO_4, pH 8; 171 mmol/L L- GLn; 1 mmol/L NH_4Cl; pH 8). After incubation for 60 min the reaction was stopped with 10 μL of 100 g/L trichloroacetic acid (TCA). Blanks were prepared separately following the incubation of the reaction medium and samples were mixed before the addition of TCA. When the sample-mixture reaction was stopped, the reaction mixture was placed in ice for 15 min and then centrifuged at 12 000 r/min for 5 min at 4 ℃. The micro-titre plate was loaded with 5 μL of supernatant and 150 μL of OPA reagent (0.2 mol/L K_2HPO_4, pH 7.4; 56 mL/L ethanol; 10 mmol/L *O*-phthaldialdehyde; 0.4 mmol/L *β*-mercapto-ethanol). The plate was incubated in dark at room temperature for 45 min. Standards of NH_4Cl were prepared to concentrations of 50-300 mg/L. Absorbance was measured at 405 nm with a spectrophotometer (R&D System, Palo Alto, USA). Specific activities of enzymes were expressed in international units per gram (μkat/mg) of mitochondrial homogenate protein.

Analysis of glutaminase protein content by immunoblotting

Samples from sham-operated rats or rats with PCS were homogenized in medium containing 66 mmol/L Tris-HCl (pH 7.4), 10 g/L SDS, 1 mmol/L EGTA, 100 ml/L glycerol, 1 mmol/L sodium orthovanadate and 1 mmol/L sodium fluoride and the protein concentration was determined by the bicinchonic acid method (Pierce, Rockford, IL, USA). Samples were subjected to gel electrophoresis and immunoblotting as previously described[11] using isoform-specific polyclonal antibodies raised in rabbits against K- glutaminase proteins diluted at 1:1000. After incubation with anti-rabbit IgG conjugated with alkaline phosphatase (Sigma, Germany) and development with alkaline phosphatase colour developer (Sigma, Germany), the image was captured using the Gel Printer Plus System (TDI, Madrid, Spain) and the densities of the spots were measured using the Intelligent Quantifier ™ software Version 2.5.0 (BioImage®, Madrid, Spain). Results were relativized to the optical density respect to controls.

Statistical analysis

Data were expressed as mean ± SD. Statistical analyses were performed using the SPSS 11.0 software (spss, Chicago, IL). Differences in glutaminase activity or protein content were analysed by Student *t*-test. $P \leqslant 0.05$ was considered statistically significant for all tests applied.

RESULTS

Ammonia measurement

Ammonia was significantly higher in PCA group than in control rats. Plasma ammonia level was 166 ± 51 μmol/L in PCA rats and 83 ± 12 μmol/L in control rats ($P < 0.05$). In the cortex, brain ammonia was 0.9±0.4 μmol/g of tissue in PCA rats and 0.3 ± 0.1 μmol/g of tissue in sham operated rats ($P < 0.05$).

PAG activity in duodenum and kidneys

Duodenal and kidney PAG activities were significantly higher in PCA group than in control rats. In the duodenum, PAG activity was 976.95 ± 268.87 μkat/g of protein in PCA rats and 429.19 ± 126.92 μkat/g of protein in sham-operated rats ($P < 0.05$). In mitochondria from kidneys, PAG activity was 1259.18 ± 228.79 μkat/g protein in PCA rats and 669.67 ± 400.8 μkat/g of protein in controls ($P < 0.05$).

Glutaminase protein content in intestine and kidneys

There was a significant effect of PCA on PAG protein in duodenum. The content of protein was 173% in PCA compared to sham-operated rats and in kidneys the content of protein was 152% in PCA compared to sham-operated rats (Figure 1).

PAG activity in brain

The PAG activity was 6646.6 ± 1870.4 μkat/g of protein in the cortex of PCA rats and 3573.8 ± 2037.4 μkat/g of protein in that of control rats ($P < 0.01$). The PAG activity was 3657.3 ± 1469.6 μkat/g of protein in basal ganglia

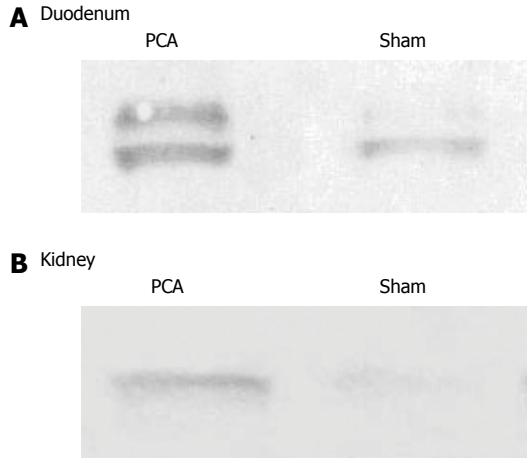

Figure 1 Representative immunoblotting from duodenum (**A**) and kidneys (**B**). Glutaminase content in enterocytes from rats with portacaval shunt (PCA) and 6 control rats (sham) homogenised and subjected to immunoblotting, 10 μg of protein applied in each lane.

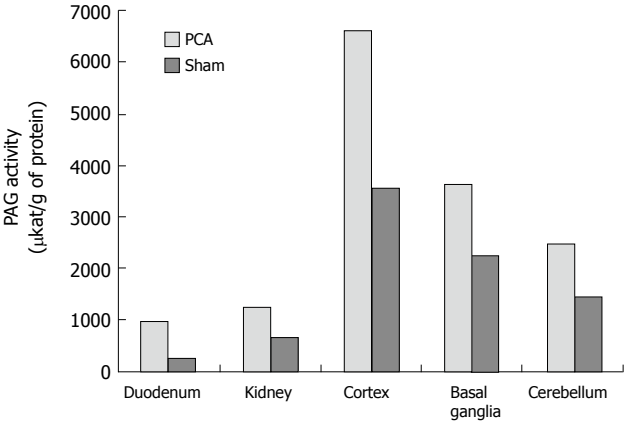

Figure 2 PAG activity (kat/g of protein) in duodenum, kidneys and brain of PCA and control rats (sham).

of PCA rats and 2271.2 ± 384 μkat/g of protein in that of sham-operated rats ($P < 0.05$). In the cerebellum, the PAG activity was 2471.6 ± 701.4 μkat/g of protein and 1452.9 ± 567.8 μkat/g of protein in the PCA and sham-operated rats, respectively ($P < 0.05$). In PCA rats, the PAG activity was increased up to 186% in cortex, 161% in basal ganglia and 170% in cerebellum compared to sham-operated rats. The highest activity was found in the cortex (Figure 2).

Glutaminase protein content in cerebral cortex and basal ganglia

The content of glutaminase was significantly increased in cerebral cortex of PCA rats compared to sham-operated animals (162% ± 40% and 100% ± 26%; $P < 0.009$) and the content of glutaminase was significantly higher in basal ganglia of PCA rats than in that of sham-operated animals (140% ± 39% and 100%±14%; $P < 0.024$), but there was no significant difference between the groups after PCA in cerebellum (100% ± 25% and 100% ± 16%; P = NS) (Figure 3).

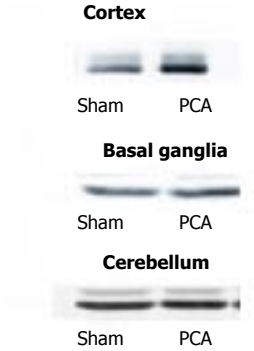

Figure 3 Glutaminase content in cerebral cortex and basal ganglia. Cerebral cortex basal ganglia or cerebellum from 7 rats with portacaval shunt (P) and 6 control rats (C) was homogenised and subjected to immunoblotting, 10 μg of protein was applied in each lane. The codes under the bands of representative blots indicate the different rats with portacaval shunt (P) or control rats (C).

DISCUSSION

The PCA performed in rats is widely accepted as a model of liver dysfunction. In this study, PAG activity was increased in kidney and duodenum, which could contribute significantly to systemic hyperammonaemia. PAG activity was increased in cortex and basal ganglia, which might be responsible for brain hyperammonemia and intra-mitochondrial ammonia levels derived from glutamine hydrolysis that are maintained because successful detoxification by glutamine-synthetase is precluded.

During the 2nd half of the 20th century, hyperammonaemia was considered to be derived from urea breakdown by intestinal bacteria and the majority of treatments are targeted against bacteria-derived ammonia from the colon[1]. However, the hypothesis was not universally accepted[12]. Hyperammonaemia following portacaval shunting in rats has been found to be similar in germ-free as well as in non-germ-free animals [13, 14], providing support to the concept that hyperammonaemia and encephalopathy could develop without participation of bacteria[15]. The highest hyperammonaemia has been found in portal-drained viscera and derived mainly from glutamine deamidation[16]. Hence, increased PAG activity in small intestine could explain these observations at least in part. Moreover, increased PAG in duodenum has been demonstrated in cirrhotic patients suffering from minimal hepatic encephalopathy[3].

Recently, renal ammoniagenesis has been implicated in several forms of hepatic encephalopathy. In patients suffering from hepatic encephalopathy due to variceal bleeding or overdose of diuretics, ammonia production from kidney seems to be the main factor involved in the development of hepatic encephalopathy[17]. Glutamine is filtered in the glomeruli and enters the lumen of the nephron. The filtered glutamine is reabsorbed in the proximal tubule where glutamine is deamidated by PAG. The main factor in the regulation of kidney PAG activity seems to be pH. During metabolic acidosis, PAG activity increases which induces higher excretion of ammonia[18]. In hepatic encephalopathy, metabolic alkalosis is observed more frequently than acidosis and increased PAG activity in kidney could be a protective mechanism. This PAG increase could be even more protective than the event associated with the systemic hyperammonaemia. However, this possibility needs to be explored in greater detail in future studies.

Ammonia reaching the brain can be detoxified to

glutamine only in astrocytes due to the predominance of glutamine synthetase in these cells. Glutamine accumulation as a by-product of ammonia metabolism has been implicated in the pathogenesis of hepatic encephalopathy[19]. Glutamine is an osmotic amino acid and plays a major role in the regulation of cell volume. A raised peak of glutamine-glutamate/creatine ratio is a typical feature of brain spectroscopic magnetic resonance imaging in hepatic encephalopathy. However, glutamine synthetase has not been found to be increased in brain of portacaval shunted rats[20]. Indeed, administration of amino acid mixtures induces hyperammonaemia, raises glutamine peak in the brain and is associated with impairment in neuropsychological function[21]. Also, use of methionine-sulfoxamine blocks the activity of glutamine synthetase and improves abnormalities induced by ammonia, such as seizures or astrocytes swelling[22]. Nevertheless, accumulation of glutamine following treatment with drugs that are able to block N-methyl-D-aspartate (NMDA)-receptors has not been shown to be associated with neurological impairment[23]. Indeed, in astrocyte culture, glutamine metabolism is linked to free radical production and oxidative stress and this may represent a key mechanism in ammonia neurotoxicity [24]. Thus, glutamine accumulation in the brain is neither a pathological event *per se* nor a safe ammonia detoxification pathway. Glutamine accumulating in the astrocytes can be considered as a "Trojan horse" leading circuitously to neurological impairment. In cultured astrocytes, glutaminase inhibitors such as 6-diazo-5-oxo-L-norleucine (DON) induce a complete blockade of glutaminase activity and preempt free radical production and neurotoxicity induced by ammonia[25]. Most of the glutamine in astrocytes is metabolized by mitochondrial PAG[26]. Since ammonia induces free radical production, the PAG can be implicated in free radical production. The inhibition of this enzyme could be a new therapeutic target. However, DON has also been reported to inhibit γ-glutamyl-transpeptidase, increase glutamine release, inhibit transport of glutamine into cells and block the transport of glutamine into mitochondria. All these mechanisms could induce a decrease in the amount of glutamine available for hydrolysis[27]. The distribution of K-type PAG in the brain has been strongly debated. Some studies have reported that PAG is absent in astrocytes of cerebellum[28]. In the current study, PAG activity in non-synaptic mitochondria from the cerebellum showed the lowest activity in the brain and no differences were observed between PCA and sham-operated rats using immunoblotting. Thus, PAG could be detected in cerebellum, but at a low-level in comparison to other areas such as basal ganglia or cortex.

In summary, PAG is enhanced in the intestine and kidney of PCA rats and induces hyperammonaemia and hepatic encephalopathy. Furthermore, PAG activity and glutaminase content are increased in astrocytes from cortex and basal ganglia. Mitochondrial glutaminase activity in astrocytes could be implicated in the production of ammonia. The induction of the mitochondrial permeability transition and free radicals production as the end product of glutamine metabolism could be responsible at least in part for the pathogenic effect observed in hepatic encephalopathy. Hence, PAG might be a new therapeutic target in the management of hepatic encephalopathy. Further studies using PAG inhibitors or PAG knock-out mice help clarify the role of increased PAG expression in the pathophysiology of hepatic encephalopathy.

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RAPID COMMUNICATION

Lamivudine therapy for children with chronic hepatitis B

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Abstract

AIM: To assess the effectiveness and side-effects of lamivudine therapy for children with chronic hepatitis B (CHB) who fail to respond to or have contraindications to interferon-α (IFN-α) therapy.

METHODS: Fifty-nine children with CHB were treated with 100 mg lamivudine tablets given orally once daily for 12 mo. Alanine aminotransferase (ALT) activity was evaluated monthly during the therapy and every 3 months after its discontinuation. HBe antigen, anti-HBe antibodies, HBV DNA level in serum were evaluated at baseline and every six months during and after the lamivudine therapy. Sustained viral response (SVR) to lamivudine therapy was defined as permanent (not shorter than 6 mo after the end of the therapy), namely ALT activity normalization, seroconversion of HBeAg to anti-HBe antibodies, and undetectable viral HBV-DNA in serum (lower than 200 copies per mL). The analysis of the side-effects of the lamivudine treatment was based upon interviews with the patients and their parents using a questionnaire concerning subjective and objective symptoms, clinical examinations, and laboratory tests performed during clinical visits monthly during the therapy, and every 3 mo after the therapy.

RESULTS: ALT normalisation occurred in 47 (79.7%) patients between the first and 11th mo of treatment (mean 4.4 ± 2.95 mo, median 4.0 mo), and in 18 (30.5%) of them after 2 mo of the therapy. There was no correlation between the time of ALT normalization and the children's age, the age of HBV infection, the duration of HBV infection, inflammation activity score (grading), staging, ALT activity before treatment, serum HBV DNA level, and lamivudnie dose per kg of body weight. HBeAg/anti HBe seroconversion was achieved in 27.1% of cases. The higher rate of seroconversion was connected with lower serum HBV DNA level and longer duration of HBV infection. There was no connection between HBeAg/anti HBeAb seroconversion and the children's age, age of HBV infection, grading, staging, ALT activity before treatment, and lamivudnie dose per kg of body weight. No complaints or clinical symptoms were observed during lamivudine therapy. Impairment of renal function or myelotoxic effect was noted in none of the patients.

CONCLUSION: One year lamivudine therapy for children with chronic hepatitis B is effective and well tolerated. Seroconversion of HBeAg/HBeAb and SVR are connected with lower pre-treatment serum HBV DNA level.

Key words: Chronic hepatitis B; Children; Lamivudine

Liberek A, Szaflarska-Popławska A, Korzon M, Łuczak G, Góra-Gębka M, Łoś-Rycharska E, Bako W, Czerwionka-Szaflarska M. Lamivudine therapy for children with chronic hepatitis B. *World J Gastroenterol* 2006; 12(15): 2412-2416

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INTRODUCTION

Hepatitis B virus (HBV) infection is still an important problem due to its high incidence and may lead to chronic hepatitis[1]. Up to 90% of infected children develop chronic hepatitis[2]. Chronic hepatitis B greatly increases the risk of liver cirrhosis or hepatocellular carcinoma[3]. Spontaneous seroconversion of HBeAg/HBeAb during the course of chronic hepatitis B is observed only in less than 10% of children, and total recovery with the elimination of all virus antigens and the presence of anti-HBs occurs in approximately 2% of cases[4]. In recent years lamivudine treatment for chronic hepatitis B has been recommended for patients who fail to respond to IFN-α therapy or have contraindications for this therapy[5]. However clinical data concerning nucleotide analogue treatment for CHB children are lacking.

The aim of the present study was to analyze prospectively the results, tolerance, and side-effects of lamivudine therapy for children with chronic hepatitis B who fail to respond to or have contraindications for IFN-α treatment.

Table 1 Characteristics of the patients

	n (%)	Mean	Median
Sex			
Boys	48 (81.4)		
Girls	11 (18.6)		
Age (yr)	6-18	10.5±3.24	10
Age of HBV infection (yr)	1-14	3.7±3.09	3
Duration of HBV infection (yr)	1-16	6.8±3.09	6
Completed previous IFN-α treatment			
Yes	51 (86.4)		
No	8 (13.6)		
ALT activity before treatment (IU/L)	20-664	101±96.3	76
Inflammation activity score			
Grade 1	24 (40.7)		
Grade 2	33 (55.9)		
Grade 3	2 (3.4)		
Grade 4	0		
Staging			
Stage 0	7 (11.9)		
Stage 1	37 (62.7)		
Stage 2	12 (20.3)		
Stage 3	2 (3.4)		
Stage 4	1 (1.7)		
Serum HBV DNA level (copies/mL)	200-200 000	135 632±81 018	200 000
Lamivudnie dose (mg per kg of body weight)	1.3-4.1	3.0±0.85	2.9

MATERIALS AND METHODS

Fifty-nine children, 48 boys and 11 girls, aged 6 - 18.0 years were included in the study. The age of HBV infection varied from 1 to 14 years and the known duration of infection was 1-16 years (Table 1). The precise route of infection was not determined. Most of the children had a history of multiple hospital admissions. None of these children suffered from onco-hematological disorders. Fifty-three children were previously treated with recombinant (3 million units) IFN-α given subcutaneously, three times a week for 20 wk, recommended by the Polish Working Liver Group[6]. Fifty-one of them completed the therapy, in 2 cases the therapy was discontinued at the 8th and 12th wk due to repeated seizures. Interferon therapy was completed 1 - 7.5 years before the present study (mean 4.0 ± 1.8 years, median 4.0 years). Six patients (10.2%) were not previously treated because of relative contraindications for IFN-α therapy, including high grade fibrosis on liver biopsy in 3 cases and epilepsy in 1 case.

The inclusion criteria for lamivudine treatment were increased aminotransferase activities in serum noted at least three times during the last six months before therapy, the presence of HBsAg and HBeAg in the blood, measurable (above 200 genome copies/mL) HBV-DNA in serum for at least six months prior to the study, negative HCV-RNA and anti-HCV antibodies in serum, and evidence of inflammation on liver biopsy performed within 24 mo before the study.

Patients with coexisting clinically significant illness or other types of liver disease, or having received antiviral agents, immunomodulatory drugs within the previous 6 mo were excluded from the study. The teenage pregnant girls were excluded and advised not to get pregnant during and after 6 mo after the lamivudine therapy.

ALT activity as well as total and direct bilirubin, alkaline phosphatase (ALP) and gammaglutamyl-transpeptidase (GGT) activity in serum were measured before treatment, monthly during and every 3 mo after the therapy by routine laboratory method. Serological markers of HBV infection were analyzed before and every six months during and after the therapy by immunoenzymatical methods: HBeAg, anti-HBe and anti-HBs by Roche's diagnostics tests while HBsAg by Micro Elisa tests. HBV-DNA serum concentration was measured in all patients before and every six months during and after the lamivudine therapy by quantitative PCR method using the Roche Cobas Amplicor HBV Monitor Assay (Roche Diagnostics, Pleasanton, USA). Activity of inflammation (grading) and fibrosis (staging) of liver biopsy was classified according to Scheuer's scale modified by International Working Party in 1995[7].

All patients were treated with 100 mg lamivudine tablets given orally once daily for 12 mo (Zeffix, GlaxoSmithKline Pharmaceuticals, SA). A single dose of lamivudine varied from 1.3 mg/kg to 4.1 mg/kg (Table 1)

The analysis of the side-effects of the lamivudine treatment was based upon interviews with the patients and their parents using a questionnaire concerning subjective and objective symptoms, clinical examinations, and laboratory tests (level of hemoglobin, blood cell count, urea and creatinine level) performed monthly during and every 3 mo after the therapy. The duration of clinical observation was at least 6 mo after the therapy in all cases.

Sustained viral response (SVR) to lamivudine therapy was defined as permanent (not shorter than 6 mo after the end of the therapy): ALT activity normalization, seroconversion of HBeAg to anti-HBe antibodies, and undetectable viral HBV-DNA in serum (lower than 200 copies per mL).

In patients with ALT elevation during the lamivudine therapy mutations in the YMDD (tyrosine, methionine, aspartate, and asparate) motif of the reverse-transcriptase domain in the HBV polymerase gene were assessed by polymerase chain reaction and restriction-fragment-length polymorphism assay. Analysis of HBV genotype was performed only in these patients because of economical reasons.

The results of the study were statistically analyzed using Statistica 5.77 (StatSoft, Inc., Tulsa, OK, USA). The differences in frequency were analyzed using χ^2 test with Yate's correction if necessary. The differences between groups were achieved using U Mann-Whitney's test. $P < 0.05$ was considered statistically significant.

Children over 12 years of age and their parents or legal guardians provided their written informed consent. The study was approved by the Ethics Committee of the Medical University of Gdańsk.

Table 2 Statistical analysis of factors predicting response to lamivudine treatment

Factors that may predict response	ALT normalisation	HBe/anti-HBe seroconversion	Sustained viral response
Sex	$\chi^2_{(Y)}$=0.37, P=0.54	$\chi^2_{(Y)}$=0.13, P=0.72	$\chi^2_{(Y)}$=0.01, P=0.76
Previous IFN-α treatment	$\chi^2_{(Y)}$=0.70, P=0.76.	$\chi^2_{(Y)}$=3.29, P=0.07	$\chi^2_{(Y)}$=4.42, P=0.04
Children's age	Z=0.10, P=0.92	Z=1.83, P=0.07	Z=2.28, P=0.02
Age of HBV infection	Z=0.70, P=0.49	Z=-1.49, P=0.14	Z=-1.40, P=0.15
Duration of HBV infection	Z=0.13, P=0.89	Z=2.99, P=0.002	Z=3.33, P<0.001
Inflammation activity score (grading)	Z=0.23, P=0.82	Z=1.56, P=0.12	Z=1.76, P=0.08
Staging	Z=0.88, P=0.38	Z=1.46, P=0.14	Z=1.69, P=0.09
ALT activity before treatment	Z=-0.48, P=0.63	Z=1.49, P=0.14	Z=1.23, P=0.23
Serum HBV DNA level	Z=-0.80, P=0.07	Z=-3.29, P=0.001	Z=-3.22, P=0.001
Lamivudnie dose per kg of body weight	Z=-0.46, P=0.64	Z=-1.75, P=0.08	Z=-1.59, P=0.11

RESULTS

ALT activity before the lamivudine therapy varied from 20 to 664 IU/L (Table 1). The ALT level was lower than 100 U/L in 42 (71.2%) patients and higher than 100 U/L in 17 (28.8%) patients. All 59 children who completed the 12-mo therapy had normal serum, total and direct bilirubin, and ALP. GGT level in serum was slightly elevated only in 3 (5.1%) patients.

Inflammation activity in liver biopsy specimens was found at either low or medium levels in 96.6% of patients: grade 1 in 24 and grade 2 in 33 children. Only in 2 patients grade 3 inflammation activity was observed (Table 1). No liver fibrosis was found in 7 patients. Fibrosis of stages 1-4 was found in 37, 12, 2, and 1 patient, respectively (Table 1).

Before lamivudine treatment HBV-DNA serum level ranged between 200-200 000 copies/mL. HBV-DNA level was over 200 000 copies/mL in 33 children (55.9%), 10 000-200 000 copies/mL in 16 (27.1%) children, and below 10 000 copies/mL in 10 (16.9%) children (Table 1).

ALT normalization was achieved in 47 (79.7%) patients at the end of therapy. It occurred mostly between the first and the 11th mo of treatment (mean 4.4 ± 2.95 mo, median 4.0 mo). ALT normalization was observed in 37 of 48 boys and 10 of 11 girls, in 43 of 53 children previously treated and 4 of 6 not treated with IFN-α. There was no connection between the rate of ALT normalization and the children's age, age of HBV infection, duration of HBV infection, inflammation activity score (grading), staging, ALT activity before treatment, serum HBV DNA level, and lamivudnie dose per kg of body weight. Statistical results are shown in Table 2.

HBeAg/anti-HBeAb seroconversion was achieved in 16 cases (27.1%) at the end of therapy. It occurred mostly after 12 mo of treatment. This seroconversion was observed in 13 of 48 boys and 3 of 11 girls, in 12 of 53 children previously treated and 4 of 6 not treated with IFN-α. The higher rate of seroconversion was connected with longer duration of HBV infection (median 9 *vs* 5 years) and lower serum HBV DNA level (median 50 000 *vs* 200 000 copies/mL). There was no connection between HBeAg/anti-HBeAb seroconversion and the children's age, age of HBV infection, inflammation activity score (grading), staging, ALT activity before treatment, and lamivudnie dose per kg of body weight. Statistical results are shown in Table 2. HBsAg/anti-HBsAb seroconversion was observed six months after the end of the therapy only in one child (1.7%). In 14 patients (23.7%) with ALT normalization and HBeAg/anti-HBeAb seroconversion, sustained viral response (SVR) was achieved at the end of therapy. In these cases HBV DNA level in serum was lower than 200 copies/mL. In two patients with ALT normalization and HBeAg/anti-HBe seroconversion, the serum HBV DNA level remained high (14 400 and 145 000 copies/mL). SVR was observed in 11 of 48 boys and 3 of 11 girls, and more frequently achieved in children previously treated with IFN-α. The rate of SVR was connected with older children's age (median 12 *vs* 9 years), longer duration of HBV infection (median 9 *vs* 5 years), and lower serum HBV DNA level (median 50 000 *vs* 200 000 copies/mL). There was no connection between SVR and the age of HBV infection, inflammation activity score (grading), staging, ALT activity before treatment, and lamivudnie dose per kg of body weight. Statistical results are also shown in Table 2.

No complaints or clinical symptoms were observed during the lamivudine therapy. Slight and transient increase of ALT activity was observed in 4 children (6.8%) between the 3rd and the 12th mo of treatment. No association with hyperbilirubinemia or other signs of hepatic decompensation was found in all cases. Mutations in the YMDD were detected in 2 of 4 patients with ALT elevation during the lamivudine therapy.

Lamivudine did not show myelotoxic effect in treated children. There were no significant differences between erythrocyte or leukocyte peripheral blood count, platelet count, and hemoglobin level during or after the therapy.

Impairment of renal function was observed in none of the patients.

DISCUSSION

This study presented an analysis of the outcome, tolerance and side-effects of lamivudine therapy for children with chronic hepatitis B, who failed to respond to or had contraindications for IFN-α treatment. Up till now IFN-α is the therapy of first choice for children with chronic hepatitis B in Poland. However the treatment with IFN-α is uncomfortable (especially in children) and has many different side effects[8].

Lamivudine is the first oral antiviral therapy for chronic hepatitis B. Positive results of this treatment in adult patients have made lamivudine therapy possible in children with chronic hepatitis B[9-11].

The results of international research conducted in children with chronic hepatitis B have proved that a 52-wk course of lamivudine therapy results in the significantly higher rate of viral response. Furthermore, SVR with HBs Ag/HBsAb seroconversion and ALT normalization has also been observed[12]. There are also some other data confirming positive biochemical and viral response in children with chronic hepatitis B treated with lamivudine[13,14].

This study demonstrated that 100 mg lamivudine treatment for 12 mo resulted in a 23.7% sustained virologic response. Special stress must be put on the fact that 86% of patients did not respond to IFN-α therapy.

Lamivudine therapy is mainly used for children with chronic hepatitis who fail to respond to IFN-α[11-15].

Other nucleoside analogues (like adefovir) used in the treatment of adult patients with chronic hepatitis, are not widely accessible for pediatric patients[16,17]. Data on the results of combined IFN-α and lamivudine therapy vary[18-21], seem no more effective than monotherapy with either IFN-α or lamivudine.

Lamivudine has been proved to be more effective than IFN-α for chronic hepatitis HBeAg-minus[22,23]. Most trials on the effectiveness of lamivudine therapy in both adults and children with chronic hepatitis B showed that ALT normalization is significantly more frequent even though it is often not associated with the viral response[10-16].

In the present group of children, ALT normalization within the first 11 mo of therapy was observed in almost 80% of patients and in 30% of children in the first 2 mo. No connection was noted between the time of ALT normalization and clinical data, biochemical tests, histopathological changes in the liver tissue, viral load, or lamivudine dose per kg body weight. Jonas *et al*[12] showed that the median time of ALT normalization was 24 wk.

In our group of patients, HBeAg/HBeAb seroconversion was observed in 27.1% of children and after 12 mo of treatment in most cases. Only in one patient, HBsAg/HBsAb seroconversion took place 6 mo after the lamivudine therapy. The low rate of positive response to lamivudine therapy expressed by HBsAg/HBsAb seroconversion is consistent with other reports[12,13,15]. SVR was noted in 23.7% of patients. ALT normalization and HBeAg/HBeAb seroconversion were still accompanied with high viral load. The results of lamivudine therapy are similar to other reports[12,24]. Some authors investigating lamivudine therapy effectiveness demonstrated a higher viral response rate of 36-44%[13,14]. However, Kocak *et al*[11] observed HBeAg/HBeAb seroconversion only in 5% of cases while viral load significantly decreased in 90% of cases[11].

In the analyzed group of patients the positive response to the lamivudine treatment was connected with the older age of patients and lower serum DNA level before therapy. The similar connection between SVR and the pretreatment viral load has also been observed by other authors[12,13]. On the contrary to the published data[9,25], no connection between SVR and pretreatment ALT activity or grading was noted in our study.

Special attention must be paid to the histopathological improvement within the liver tissue and in ALT activity in patients with no viral response after lamivudine therapy[9].

Liver biopsy after lamivudine therapy was performed only in a few patients. No consent of patients and their parents for invasive diagnostic procedure was available at that time. Control liver biopsy has not been performed by other researchers[11].

Due to the limited number of data on representative groups of patients, establishing the optimal dose especially for children under the age of 12 years appears still problematic. It appears that increase in daily dose or in frequency of lamivudine administration does not improve the results[12,13,15,26]. The recommended dose of lamivudine results in the same serum concentration of the drug in children as in adults receiving 100 mg per 24 h[26]. The experiences with treatment of chronic hepatitis B in adult patients suggest that this dose of lamivudine seems to be satisfactory.

In our group of patients, 100 mg lamivudine tablets were administered, and the dose per kilogram of body weight varied from 1.3 to 4.1 mg/kg per d, mean 3.0 mg/kg per d. Lamivudine in suspension is not widely available. The average dose of lamivudine in our group is comparable with that recommended by other authors[12,13,15,26].

Based upon interviews with the patients and their parents using a questionnaire concerning subjective and objective symptoms, clinical examinations, and monthly laboratory tests during and every 3 mo after the therapy, no particular side-effects were observed in our group of children.

Lebensztejn *et al*[27] reported a case of a child with chronic hepatitis B treated with lamivudine who developed thrombocytopenia and found that withdrawal of lamivudine could normalize platelets count, while reintroduction of the drug results in relapse of thrombocytopenia.

Slight and transient increase of ALT activity during lamivudine therapy (between the 3rd and the 12th mo) was noted in 4 children of our group of patients. YMDD mutation was detected in 2 of them. Termination of lamivudine therapy after 12 mo did not result in any increase of ALT activity or any other symptoms of liver impairment in the follow-up. Some authors have reported the risk of liver dysfunction and even acute liver failure after cessation of lamivudine therapy especially in patients with advanced liver fibrosis or cirrhosis[9]. Advanced fibrosis (stages 3 and 4) could be detected only in 5.1% of children with chronic hepatitis B, which may be the reason for no complications in our group of patients.

Because of economical reasons, only patients with ALT increase were evaluated for YMDD mutation and thus no conclusions about the incidence of YMDD mutation in children with chronic hepatitis B treated with lamivudine can be established. Thus, it can not be ruled out that mutation in the YMDD motif is responsible for the development of resistance to lamivudine in non-responders.

Duration of lamivudine therapy increases not only the rate of positive viral response, but also the risk of YMDD mutations[9], which appears to rise up to 60% in patients treated with lamivudine for more than 4 years[16,24,28]. How-

ever, besides the high percentage of viral mutants, lamivudine is still capable of inducing HBeAg/HBeAb seroconversion and improving histopathological changes within liver tissue in the treated patients[29,30].

In conclusion, one year lamivudine therapy for children with chronic hepatitis B is effective and well tolerated. Seroconversion of HBeAg/HBeAb and SVR are connected with lower pre-treatment serum HBV DNA level.

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RAPID COMMUNICATION

Pegylated IFN-α 2b added to ongoing lamivudine therapy in patients with lamivudine-resistant chronic hepatitis B

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Abstract

AIM: To investigate the role of pegylated-interferon (IFN) α-2b in the management of patients with lamivudine-resistant chronic hepatitis B.

METHODS: Twenty consecutive anti-HBe positive patients were treated with pegylated IFN α-2b (100 μg sc once weekly) for 12 mo. There was no interruption in lamivudine therapy. Hematology, liver biochemistry, serum HBV DNA levels were detected by PCR, and vital signs were also assessed. Liver histology was assessed in some patients at entry and at wk 52 for comparison.

RESULTS: Nine patients (45%) had a partial virological end-treatment response; seven patients (35%) showed complete virological end-treatment response. Eight patients (40%) showed biochemical end-treatment response. There was a trend for higher virological response rates in patients who had previously responded to IFN and relapsed compared to IFN non-responders (four out of seven patients *vs* none out of six patients, respectively; $P = 0.1$). Patients without virological end-treatment response showed significant worsening of fibrosis [median score 2 (range, 1 to 3) *vs* median score 3 (range, 1 to 4)], in the first and second biopsy respectively ($P = 0.014$), whereas necroinflammatory activity was not significantly affected. Patients with complete or partial virological end-treatment response did not show any significant changes in histological findings, possibly due to the small number of patients with paired biopsies ($n = 5$). Nevertheless, after 12 mo of follow-up, only one patient (5%) showed sustained virological response and only 2 patients (10%) showed sustained biochemical response. Two patients (10%) discontinued pegylated IFN both after 6 mo of treatment due to flu-like symptoms.

CONCLUSION: Pegylated IFNα-2b, when added to ongoing lamivudine therapy in patients with lamivudine-resistant chronic hepatitis B, induces sustained responses only in a small minority of cases.

Key words: Pegylated interferon; Lamivudine resistance; HBeAg negative chronic hepatitis B; Adefovir

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INTRODUCTION

Lamivudine has been a major breakthrough in the care of patients with chronic hepatitis B (CHB). Nevertheless, almost all patients with HBeAg-negative CHB, which accounts for the majority of patients in Greece, require long-term therapy with lamivudine to maintain a response[1,2]. This strategy is associated with the frequent emergence of viral resistance with reported rates of 10%-27% at 1 year, 40%-56% at 2 years and 67% at 3 years of treatment[3-7]; in a previous study in 77 patients with anti-HBe-positive CHB we have found resistance rates of 1.6% at 9 mo, 3.3% at 12 mo, 12.7% at 15 mo, 15% at 18 mo and 31% at 48 mo[8]. Once lamivudine resistance occurs, patients may experience an attenuation of the initial clinical, virologic and histologic benefits of therapy[9]. The wide use of lamivudine in CHB during the last 5-6 years has resulted in the constant increase of patients with lamivudine-resistant mutants. Their treatment may be the most pressing task for the current anti-HBV strategies in clinical practice[10]. Adefovir dipivoxil is the only approved agent that has been

Table 1 Baseline demographic and clinical features of the study population

No	Sex	Age (yr)	*t* (prior lamivudine therapy) mo	Lamivudine resistance mutation	HBV DNA level (10^3 log_{10} copies/L)	ALT level (times the ULN)
1	M	53	26	L180M + M204V	9.36	1.3
2	M	55	14	L180M + M204I	6.56	6
3	M	63	18	L180M + M204I	7.23	7
4	M	53	25	L180M + M204V	6.68	2.75
5	F	55	19	M204I	6.57	8.5
6	M	63	11	L180M + M204V	6.58	9
7	M	62	10	M204I	8.16	1.3
8	M	47	24	L180M + M204V	7.23	2
9	M	53	18	L180M + M204V	6.86	2.5
10	M	46	16	L180M + M204I	7.98	10
11	M	66	24	L180M + M204V	6.00	1.5
12	F	48	22	L180M + M204I	7.60	3
13	M	54	21	L180M + M204V	9.30	7
14	M	60	41	L180M + M204I	7.51	3.8
15	M	38	29	L180M + M204V	6.26	2.5
16	M	61	22	L180M + M204V	6.57	5
17	M	42	8	M204I	7.43	3.5
18	M	61	25	L180M + M204V	6.98	7
19	M	65	12	L180M + M204I	7.26	5
20	M	54	12	M204I	7.55	2

shown to be effective in this setting, whilst entecavir is also a potential candidate[11-13]. The efficacy of interferon-α (IFN) therapy has not yet been evaluated in any well-designed study in these patients, and therefore no conclusions can be drawn.

IFN-α has a dual mode of action, antiviral via inhibition of viral replication, and immunomodulatory via enhancement of the immunological response of the host against the virus[14]. Pegylation of interferon leads to improved pharmacokinetic and pharmacodynamic profiles, which translated to superior efficacy, compared with conventional, nonpegylated IFN, in the treatment of chronic hepatitis C, and more recently, CHB[15-20]. Two modulations of pegylated IFN (PEG-IFN) are currently being used in clinical practice, namely PEG-IFN-α 2b and PEG-IFN-α 2a. This study was designed to explore the role of PEG-IFN-α 2b in the management of patients with CHB with lamivudine-resistant HBV.

MATERIALS AND METHODS

Subjects

Between November 1999 and February 2003, a total of 20 consecutive anti-HBe positive patients [18 males (90%)], with a median age of 54 (range, 38-66) years, were enrolled in this prospective study. Patients eligible for the study were aged 18 years and older, hepatitis B surface antigen (HBsAg) positive, and receiving ongoing lamivudine therapy for CHB for at least 6 mo at the time of screening; median duration of prior lamivudine therapy was 20 (range, 8-41) mo. All patients were HBeAg negative and antiHBe positive (both at the beginning of prior lamivudine therapy and at the beginning of the present study), genotype D, and had confirmed HBV polymerase gene mutation within the YMDD motif by DNA sequencing (Trugene HBV genotyping, Visible Genetics Inc); lamivudine resistance mutations are shown in Table 1. Patients were required to have a screening HBV DNA level $>10^9$ copies/L (Amplicor HBV-DNA Monitor Test; Roche Diagnostics, Branchburg, NJ, USA, with a sensitivity of 400×10^3 copies/L) as well as elevated serum alanine aminotransferase (ALT) levels >1.2 times the upper limit of normal (ULN) on at least 2 occasions at least 1 mo apart within the preceding 6 mo. The exclusion criteria are as follows: absolute neutrophil count $\leqslant 10^9$ cells/L; hemoglobin $\leqslant 100$ or $\leqslant 90$ g/L (males or females, respectively); platelet count $< 50 \times 10^9$/L; prior treatment with interferon or other immunomodulatory therapies within the 6 mo preceding study screening; serious concurrent medical conditions, including other concurrent liver diseases; coinfection with hepatitis C virus or hepatitis delta virus or human immunodeficiency virus; current alcohol or substance use; and pregnancy and/or lactation. None of the patients had liver cirrhosis.

Amongst the 20 patients enrolled, 7 (35%) were naive to IFN and 13 (65%) had been previously treated with IFN 5 MU sc three times weekly for at least 12 mo (before receiving lamivudine); six of the latter had shown no response and seven had responded to IFN (i.e. had shown both reduction in serum HBV DNA level to $<10^8$ copies/L and normalization of ALT level at the end of IFN administration) but relapsed after discontinuing therapy.

Methods

Patients were treated with pegylated interferon α-2b (100 μg sc once weekly) for 12 mo. There was no interruption in lamivudine therapy, even after the cessation of PEG-IFN-α 2b. Patients were evaluated every month. At each visit, any untoward medical occurrences, regardless of causality, were recorded as adverse events. Hematology, liver biochemistry, serum HBV DNA levels, and vital signs were also assessed. Liver histology was assessed in 13 patients at entry and at wk 52 for histological comparison; the rest of the patients denied a liver biopsy. A single pathologist, who was blinded to the sequence of the biopsies, evaluated all

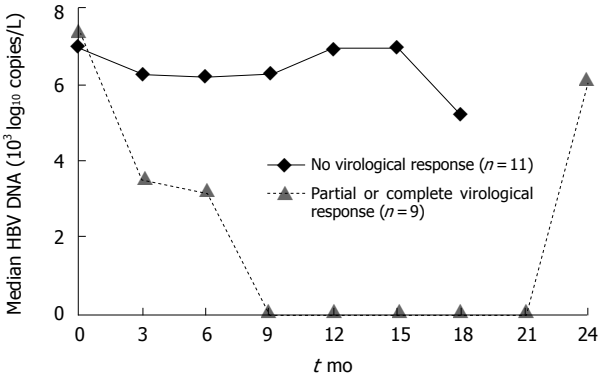

Figure 1 Median HBV DNA titers during PEG-IFN-α 2b therapy and during follow-up.

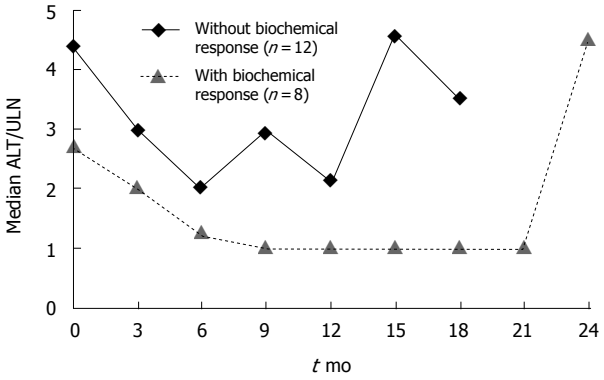

Figure 2 Median ALT values, expressed as multiples of ULN, during PEG-IFN-α 2b therapy and during follow-up.

specimens. Inflammation and fibrosis were each classified into 4 stages according to the Scheuer system.

After the cessation of PEG-IFN-α 2b, adefovir dipivoxil, 10 mg once daily, was given to patients who did not show a reduction in serum HBV DNA level to below 10^8 copies/L at wk 52 and to patients who demonstrated virologic breakthrough during follow-up (defined as reappearance of serum HBV DNA on two consecutive occasions, at least 3 mo apart, after its initial disappearance). The primary end point of the study was a reduction in serum HBV DNA level (partial virological end-treatment response). This was assessed as the proportion of patients with HBV DNA level $<10^8$ copies/L at wk 52. Secondary end points included the proportion of patients with undetectable HBV DNA at wk 52 (complete virological end-treatment response), the percentage of patients with normalization of ALT level at wk 52 (biochemical end-treatment response) and undetectable HBV DNA by PCR and normalization of ALT level after 12 mo of follow-up (virological and biochemical sustained response, respectively).

The study was approved by the ethics committee of our institution, and all patients provided written informed consent before screening. The study was performed in accordance with the principles of the Declaration of Helsinki.

Statistical analysis

All data were analyzed using the statistical package SPSS (version 10.0; SPSS Inc., Chicago, IL). The population analyzed included all patients who received at least one dose of study medication. The Mann-Whitney and Chi-square tests were used for comparisons of quantitative and qualitative variables respectively. The Wilcoxon Signed rank test was used to test the effect of PEG-IFN-α 2b on histological findings in the patients who underwent paired liver biopsies. In all cases, a 2-tailed *P* value less than 0.05 was considered statistically significant.

RESULTS

Baseline demographic and clinical features of the study population are presented in detail in Table 1. At baseline, median HBV DNA level was 7230 log_{10} copies/L (range, 6 000-9 360 log_{10} copies/L) and median ALT level was 3.5 (range, 1.3-10) times the ULN. Changes in median HBV DNA titers during PEG-IFN-α 2b therapy are shown in Figure 1. After 52 wk of treatment, 9 patients (45%) had a partial virological end-treatment response. The median change from baseline in serum HBV DNA levels was -7000 (range, -9 400 to 7 600) log_{10} copies/L. Seven patients (35%) showed complete virological end-treatment response. In these patients HBV DNA became undetectable after a median of 9 (range, 3 to 12) mo. Eight patients (40%) showed biochemical end-treatment response. In these patients normalization of ALT levels occurred after a median time of 9 (range, 3 to 12) mo (Figure 2). Overall, serum ALT level decreased over 52 wk in 16 patients (80%). The median ALT level at baseline was 3.5 times the ULN; by wk 52, this had declined to 2.2 times the ULN.

None of the baseline demographic and clinical features predicted virological (partial or complete) or biochemical end-treatment responses. Nevertheless, it should be noted that none of the six patients who had not responded to prior IFN treatment showed complete virological end-treatment response, while four out of the seven patients who had relapsed after an initial response to IFN showed complete virological end-treatment response ($P = 0.1$). The findings in the 13 patients in whom biopsies were performed are shown in Table 2 and in Figure 3. Patients without virological end-treatment response showed significant worsening of fibrosis ($P = 0.014$) in the second biopsy, whereas necroinflammatory activity was not significantly affected. Patients with complete or partial virological end-treatment response did not show any significant changes in histological findings, possibly due to the small number of patients with paired biopsies ($n = 5$). Likewise, patients without biochemical end-treatment response showed significant worsening of fibrosis ($P = 0.014$) in the second biopsy, whereas necroinflammatory activity was not significantly affected. Also, patients with biochemical end-treatment response did not show any significant changes in histological findings, possibly due to the small number of patients with paired biopsies ($n = 3$).

During follow up, HBV-DNA reappeared in six out of the seven patients who had shown complete virological end-treatment response, giving an overall sustained virological response rate of 5%. The median time to HBV-DNA re-emergence was 5 (range, 1 to 12) mo. Changes in median

Table 2 Histological findings in 13 patients with biopsy performed [median (range)]

	Baseline biopsy		End of treatment biopsy	
Virological end-treatment response	Necroinflammatory activity	Fibrosis	Necroinflammatory activity	Fibrosis
None (n=8)	3 (1-3)	2 (1-3)	2 (1-4)	3 (1-4)
Partial (n=2)	2.5 (2-3)	2.5 (2-3)	2.5 (2-3)	2.5 (2-3)
Complete (n=3)	2 (2-3)	2 (2-2)	1 (1-2)	1 (1-2)

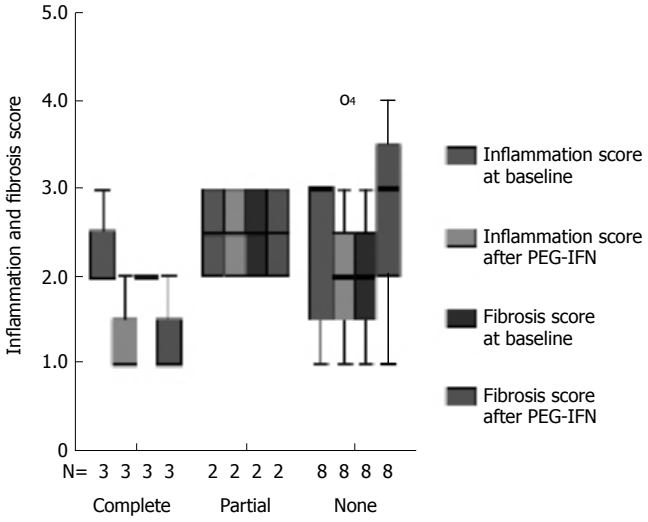

Figure 3 Changes in median inflammation and fibrosis scores according to end of treatment virological response.

HBV DNA titers during follow-up are shown in Figure 1 (patients' data are censored at the time of commencement of treatment with adefovir dipivoxil). Two patients (10%) had a sustained biochemical response while in the remaining 6 patients with biochemical end-treatment response, ALT became abnormal after a median of 4 (range, 1 to 12) mo. Changes in median ALT levels during follow-up are shown in Figure 2 (patients' data are censored at the time of commencement of treatment with adefovir dipivoxil). Two patients (10%) discontinued PEG-IFN both after 6 mo of treatment and both due to flu-like symptoms (fatigue, low-grade fever, arthralgia and headache). Neither of these patients showed virological or biochemical end-treatment response; in fact, one of them showed a rise in HBV-DNA levels and both showed a rise in ALT levels at 52 wk. PEG-IFN was well-tolerated in all other patients and none of them required dose reduction or interruption of therapy. There were instances of hepatic decompensation during the study.

DISCUSSION

Richman[21] has recently defined an antiviral drug as one that selects for resistance. Antiviral drug resistance depends on the viral mutation frequency, intrinsic mutability of the antiviral target site, the selective pressure exerted by the drug, and the magnitude and rate of virus replication. In particular, lamivudine resistance is due to mutations within the YMDD motif in the major catalytic region C of the HBV polymerase gene[22]. Viral resistance is clinically expressed by the virological breakthrough phenomenon, defined as the reappearance of serum HBV DNA after an initial clearance of viraemia despite the continuation of therapy[10]. The emergence of resistance has a negative impact on the efficacy of therapy in CHB patients, since virological breakthroughs are almost invariably followed by increasing viraemia levels, culminating in biochemical breakthroughs, which ultimately have an adverse effect on liver histology[6].

It is clear that the possible adverse effects of YMDD mutants do cast a concern. Rescue therapies for patients with worsening liver disease caused by lamivudine-resistant mutants are being evaluated. Until recently, treatment options for these patients have been limited to continuation or cessation of lamivudine therapy. Continuation of lamivudine aims to further suppress or to prevent the return of wild-type HBV which is more replicative competent than the YMDD mutant[23]. However, this strategy seems ineffective. Lamivudine withdrawal results in re-emergence of wild-type HBV within 3–4 mo[24]. Therefore, acute exacerbations of liver disease might ensue and could, although uncommon, result in hepatic decompensation or acute liver failure[25]. Adefovir dipivoxil effectively inhibits replication of YMDD mutants resistant to lamivudine and hence averts the resultant disease; entecavir has also shown promising results[11-13]. Nevertheless, neither of these novel nucleoside analogues was licensed in Greece during the study period.

There is a paucity of data regarding the role of IFN in the treatment of lamivudine-resistant HBV. Interferon-α has multiple sites of action in the viral life cycle and may be effective against lamivudine-resistant virus[26]. Recently, lamivudine was found to restore cytotoxic T-cell responses in patients with CHB, and, therefore, it may augment the immunomodulatory activity of IFN[27]. Hence, there would be a rationale in treating patients with lamivudine-resistant HBV mutants with IFN. Pegylation is the attachment of a polyethylene glycol (PEG) molecule to the base IFN molecule resulting in effective concentrations of IFN throughout the dosing interval and substantially reduced peak-to-trough ratio, in contrast to conventional IFN, which yields only intermittent drug exposure; pegylation also allows for once weekly frequency of administration[14]. Pegylated IFN has been shown to be highly active against wild-type HBV infection, both HBeAg-positive[15,17-19] and HBeAg-negative[20]. Complete virological end-treatment response was achieved by 35% of our patients. Therefore, this study confirms that PEG-IFN-α 2b is also active against lamivudine-resistant HBV. Of note, complete virological end-treatment response was achieved by 63% of patients with wild-type HBeAg-negative CHB in a recent landmark study[20]. The small number of patients included in the present report might *per se* preclude direct comparisons of efficacy between these two studies; however, different patients' characteristics might have also accounted for the apparently inferior results of PEG-IFN therapy in our study. Even though no pretreatment factor

has been found to be reliably associated with a response to IFN in HBeAg-negative CHB[1], it must be mentioned that our patients were older and had higher ALT and lower HBV-DNA levels at baseline than the patients in the aforementioned study[20]. Of course, it is also possible that PEG-IFN might not be as effective in lamivudine-resistant as in wild-type HBV strains, but this has to be investigated further in large-scale studies.

Patients with HBeAg-negative CHB receiving IFN retreatment respond as well as naive ones, irrespective of the outcome of the initial treatment[28]. In accordance with this, in our study, prior IFN treatment, as well as its outcome, was not associated with the efficacy of PEG-IFN-α 2b. Nevertheless, this could be attributed to the limited number of patients studied, since there was a trend for higher virological response rates in patients who had previously responded to IFN and relapsed compared to IFN non-responders ($P = 0.1$).

In patients with HBeAg-negative CHB, the 12 mo sustained response rates to IFN treatment vary from 10% to 47% (average 24%)[1]; sustained 6 mo biochemical and complete virological response rates with PEG-IFN rise up to 59% and 19%, respectively[20]. The low percentage of sustained biochemical and complete virological response (10% and 5% respectively) in our patients is of concern and renders PEG-IFN-α 2b rather unattractive for patients with lamivudine-resistant CHB. Nevertheless, the already mentioned differences in patients' characteristics between studies and the inherent limitation of the small number of patients included in our report might have contributed to these discrepant findings. Furthermore, it must be pointed out that the time point of evaluation of the sustained response in our study was at 12 mo after treatment completion compared to 6 mo in the wild-type HBV study[20], and this should be taken into account when comparing our results with the latter ones. However, the issue of differing activity of IFN in lamivudine-resistant compared to wild-type HBV strains definitely needs to be addressed.

In conclusion, this study shows that, 52 wk of treatment with PEG-IFN-α 2b, when added to ongoing lamivudine therapy in patients with lamivudine-resistant CHB, induces sustained responses only in a small minority of cases. Therefore, other treatment strategies should be considered for these patients, possibly including more prolonged or earlier (when the viral load is less than 10^6 copies/L) administration of PEG-IFN-α 2b.

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RAPID COMMUNICATION

One-step palliative treatment method for obstructive jaundice caused by unresectable malignancies by percutaneous transhepatic insertion of an expandable metallic stent

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Abstract

AIM: To describe a simple one-step method involving percutaneous transhepatic insertion of an expandable metal stent (EMS) used in the treatment of obstructive jaundice caused by unresectable malignancies.

METHODS: Fourteen patients diagnosed with obstructive jaundice due to unresectable malignancies were included in the study. The malignancies in these patients were a result of very advanced carcinoma or old age. Percutaneous transhepatic cholangiography was performed under ultrasonographic guidance. After a catheter with an inner metallic guide was advanced into the duodenum, an EMS was placed in the common bile duct, between a point 1 cm beyond the papilla of Vater and the entrance to the hepatic hilum. In cases where it was difficult to span the distance using just a single EMS, an additional stent was positioned. A drainage catheter was left in place to act as a hemostat. The catheter was removed after resolution of cholestasis and stent patency was confirmed 2 or 3 d post-procedure.

RESULTS: One-step insertion of the EMS was achieved in all patients with a procedure mean time of 24.4 min. Out of the patients who required 2 EMS, 4 needed a procedure time exceeding 30 min. The mean time for removal of the catheter post-procedure was 2.3 d. All patients died of malignancy with a mean follow-up time of 7.8 mo. No stent-related complication or stent obstruction was encountered.

CONCLUSIONS: One-step percutaneous transhepatic insertion of EMS is a simple procedure for resolving biliary obstruction and can effectively improve the patient's quality of life.

Key words: Expandable metallic stent; Bile duct carcinoma; Gall bladder carcinoma; Pancreatic carcinoma; Gastric carcinoma; Obstructive jaundice

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INTRODUCTION

The incidence of biliary obstruction resulting from malignancies is increasing. As operative techniques and diagnostic imaging have advanced, more and more patients undergo resection. However, in cases where operation is not possible, prognosis remains poor often because of the presence of obstructive jaundice.

Palliative treatment with a biliary stent is carried out in patients with inoperable malignancies in order to relieve symptoms related to obstructive jaundice, prevent cholangitis and prolong survival. In addition, the biliary insertion procedure only requires a short time of hospitalization, which is especially important for patients with unresectable malignancies because of their poor prognosis.

When first introduced, stent insertion is performed using polyethylene endoprosthese. However, the expandable metallic stent (EMS) has also been available for a number of years [1, 2]. EMSs have advantages over plastic stents in that they can be introduced through a smaller delivery catheter, have a larger inner diameter and remain fixed in position after

release [3-6].

It was previously reported that one-step method of percutaneous transhepatic insertion of EMSs can be used for obstructive jaundice due to unresectable common bile duct carcinoma[7], further supporting the one-step method of percutaneous transhepatic insertion of EMS to treat obstructive jaundice caused by unresectable malignancies.

MATERIALS AND METHODS

Patients

This study comprised 14 patients with obstructive jaundice caused by unresectable malignancies, who were admitted into Nippon Medical School or Uchida Hospital from 2002 to 2004. Of the 14 patients (6 men and 8 women), 1 suffered from gastric carcinoma, 3 from recurrence of gastric carcinoma, 4 from pancreas carcinoma, 3 from bile duct carcinoma, 1 from gall bladder carcinoma, and 2 from recurrence of gall bladder carcinoma. The age distribution of the patients ranged between 65-90 years, with a mean age of 77.1 years.

The diagnosis was confirmed by ultrasonography and computed tomography. All patients were diagnosed before cholangiography as having obstructive jaundice caused by unresectable malignancies as a result of very advanced carcinoma or old age.

This study was performed according to the principles of the Declaration of Helsinki, and informed consent was obtained from the patients and/or their families.

Methods

The procedure was performed single-handedly (Hiroshi Yoshida). The portion of the procedure was consistent for all the patients. Following pre-medication with an intravenous injection of pentazocine (15 mg) and hydroxyzine (25 mg), the patient was given a local anesthetic consisting of 1% xylocaine. The appropriate intrahepatic bile duct of the lateral segment or right lobe of the liver was punctured with a sheath needle (19 gauge × 150 mm; Hakko, Tokyo, Japan) under ultrasonographic guidance. Percutaneous transhepatic cholangiography was then performed (Figure 1A).

The bile duct obstruction was cleared using a guide wire (RADIFOCUS GUIDE WIRE M, 0.035 inch × 150 cm; TERUMO, Tokyo, Japan). After a catheter with an inner metallic guide (EV drainage catheter, 17 gauge × 270 mm; Hakko, Tokyo, Japan) was advanced into the duodenum, a contrast material was injected to determine the overall length of stenosis (Figure 1B). The guide wire was reinserted and the EV drainage catheter was substituted with an EMS (SMART stent, 10 mm in diameter × 80 mm long; Cordis Endovascular, Warren, NJ) system. In cases where it was difficult to advance the EMS system into the duodenum, a dilatation catheter (PTCS catheter, 9 Fr × 60 cm; Sumitomo Bakelite, Akita, Japan) was inserted prior to insertion of the EMS system. The EMS was then placed in the common bile duct between a point 1 cm beyond the papilla of Vater and the entrance to the hepatic hilum, irrespective of the size of stenotic lesion present (Figure 2A). Where it was difficult to span the distance between the above points with a single EMS, an additional EMS SMART stent (10 mm in diameter × 60 mm long) was inserted connecting lengthways to the first stent. The EV drainage catheter or PTCS catheter was left in place to act as a hemostat at the site of insertion to the liver (Figure 2B). The patient received antibiotics following the stent insertion procedure. As an additional measure, saline was injected daily into the catheter to flush out the bile duct. The catheter was removed after resolution of cholestasis and stent patency was confirmed 2 or 3 d post-procedure.

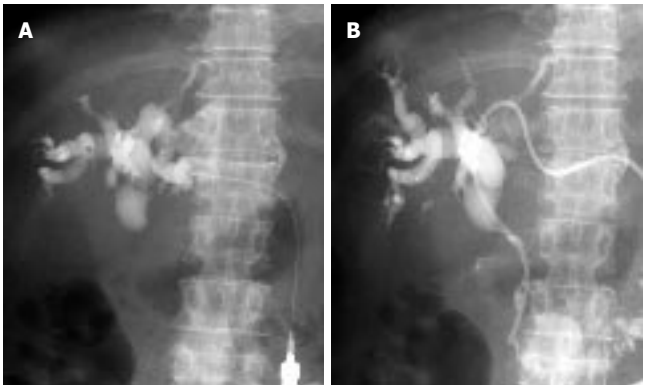

Figure 1 Insertion procedure of EMS (**A**) and the overall length of stenosis (**B**). Appropriate intrahepatic bile duct of the lateral segment of the liver was punctured with a sheath needle under ultrasonographic guidance. Percutaneous transhepatic cholangiography was performed. The bile duct obstruction was cleared using a guide wire. After a catheter with an inner metallic guide was advanced into the duodenum, contrast material was injected to determine the overall length of stenosis.

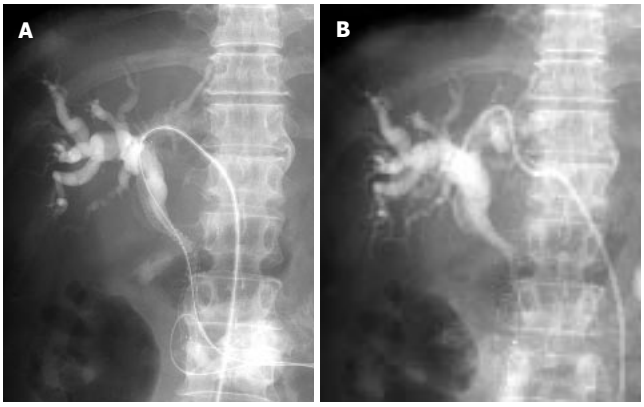

Figure 2 Placement of EMS (**A**) and EV drainage(**B**). The guide wire was reinserted and the EV drainage catheter was substituted with an EMS system. The EMS was placed in the common bile duct between a point 1 cm beyond the papilla of Vater and the hepatic hilum, irrespective of the size of the stenosis lesion present. The EV drainage catheter was left in place to act as a hemostat at the insertion site of the liver.

RESULTS

One-step insertion of the EMS was achieved in all patients. The procedure time ranged between 15-42 min with a mean time of 24.4 min. A single EMS was required in 7 patients, while two EMSs were needed in the other 7.

Table 1 Outcome after insertion EMS

Patient No.	Age	Sex	Disease	Procedure time (min)	No.of EMS	Days[2]	Outcome
1	90	F	Bile duct ca.	32	2[1]	3	death after 8 mo
2	72	F	Bile duct ca.	35	2[1]	2	death after 5 mo
3	87	F	Bile duct ca.	20	2	3	death after 7 mo
4	72	M	Gastric ca.	31	2	2	death after 5 mo
5	67	M	Recurrence of gastric ca.	16	1	2	death after 4 mo
6	66	F	Recurrence of gastric ca.	15	1	2	death after 12 mo
7	90	F	Recurrence of gastric ca.	21	2	3	death after 10 mo
8	72	M	GB ca.	42	2	2	death after 3 mo
9	84	F	Recurrence of GB ca.	20	1	3	death after 17 mo
10	65	F	Recurrence of GB ca.	28	1	2	death after 11 mo
11	82	M	Pancreas ca.	20	1	2	death after 8 mo
12	76	M	Pancreas ca.	18	1	2	death after 5 mo
13	72	M	Pancreas ca.	22	1	2	death after 6 mo
14	84	F	Pancreas ca.	22	2	2	death after 6 mo
Mean	77.1			24.4	1.5	2.3	7.8

EMS:expandable metallic setent ca:carcinoma. GB:gall bladder. 1: Dilation catheter was used before insertion of EMS. 2: Postprocedure d of catheter removed.

In 2 patients, a PTCS catheter was used because of prior difficulty in inserting the EMS system. Out of the patients who required 2 EMSs, 4 of these needed a procedure time exceeding 30 min.

After successful insertion of the stent, cholestasis rapidly resolved itself in all the patients. The catheter was removed after a mean post-procedure time of 2.3 d. All patients died of malignancy with a mean follow-up time of 7.8 mo. No stent-related complication or stent obstruction was encountered (Table 1).

DISCUSSION

Long-term survival is poor in patients with malignant bile duct obstruction and in those who are not candidates for surgical resection. The objective of palliation with a biliary stent is to relieve symptoms related to obstructive jaundice, prevent cholangitis and prolong survival. Stenting has also been shown to improve patient quality of life[8].

Since the development of suitable metallic stents, a debate has arisen regarding when to use a metallic stent in preference to a plastic one. Randomized studies comparing metallic and plastic endoprostheses demonstrated that the metallic stent is associated with a lower incidence of complications, remains patent longer and is more cost-effective, although it is initially more expensive[5,6, 9-11].

Complications arising from metal stent placement for malignant bile duct obstruction, including tumor ingrowth or overgrowth[4,12], viscus perforation [13, 14], fracture[15], and stent migration[16] have been reported. The early occlusion rate ranges between 7%-42% and the late occlusion rate ranges between 12%-38%, with a mean time to stent failure of 6-9 mo[5,6,10,11,17] . In this study, no EMS-related complication or obstruction was encountered. A possible reason is that all the patients suffered from very advanced carcinoma and could not undergo surgery before cholangiography. Therefore, the prognosis of the patients was poor and patients died due to malignancy after just 7.8 mo. A further possible reason is that the EMS was placed in the common bile duct between a point 1 cm beyond the papilla of Vater and the hepatic hilum, irrespective of the size of stenotic lesion, therefore the stenosis was not completely covered to avoid overgrowth. In addition, saline was injected into the catheter on a daily basis to flush out the bile duct and stent.

In this study, percutaneous transhepatic insertion of the EMS was conducted. A different approach is to insert the EMS endoscopically. Recently, it was reported that the EMS can be inserted from the papilla of Vater under endoscope[18-20]. The success rate is below 100% and the time of procedure is relatively long[18-21]. This is because dilation of the stenotic lesion and control of a long EMS system are difficult in this approach. For high-risk patients with very advanced carcinoma or old age, it is imperative to keep operative procedures as straightforward and short as possible. In the endoscopic approach, the lengthy time of procedure is too invasive to be carried out on high-risk patients. With the percutaneous transhepatic insertion in this study, the distribution of the procedure time ranged between 15 - 42 min, with a mean time of 24.4 min. The EMS insertion could be performed single-handedly, easily, and quickly.

A disadvantage of the percutaneous transhepatic approach is that the catheter must be left in place to act as a hemostat at the insertion site of the liver. However, this is also a benefit as the catheter could then be used to confirm the stent patency and to flush the EMS free from coagula or debris for 2 or 3 d post-procedure.

In conclusion, the one-step percutaneous transhepatic insertion of EMS is a simple procedure for resolving biliary obstruction and can effectively improve the patient's quality of life.

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RAPID COMMUNICATION

Effect of oral garlic on arterial oxygen pressure in children with hepatopulmonary syndrome

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Abstract

AIM: To study the effect of oral garlic on arterial oxygen pressure in children with hepatopulmonary syndrome.

METHODS: Garlic powder in a capsule form was given to 15 children with hepatopulmonary syndrome (confirmed by contrast echocardiography) at the dosage of 1 g/1.73 m^2 per day. Patients were evaluated clinically and by arterial blood gas every four weeks.

RESULTS: The garlic capsule was administered to 15 patients with hepatopulmonary syndrome. There were 10 boys and 5 girls with a mean age of 9.4 ± 3.9 years. The underlying problems were biliary tract atresia (4 patients), autoimmune hepatitis (4 patients), cryptogenic cirrhosis (4 patients) and presinusoidal portal hypertension (3 patients). Eight patients (53.3%) showed an increase of 10 mmHg in their mean arterial oxygen pressure. The baseline PaO_2 was 65.6 ± 12.1 mmHg in the responder group and 47.1 ± 11.2 mmHg in non-responder group. At the end of treatment the mean PaO_2 in responders and non-responders was 92.2 ± 7.75 mmHg and 47.5 ± 11.87 mmHg, respectively ($P < 0.01$).

CONCLUSION: Garlic may increase oxygenation and improve dyspnea in children with hepatopulmonary syndrome.

Key Words: Hepatopulmonary syndrome; Garlic; Arterial oxygen pressure; Pediatric

INTRODUCTION

In 1995, Lange *et al* [1] introduced the term "hepatopulmonary syndrome" for those patients with pulmonary vasodilatation associated with chronic liver disease and cyanosis. Hepatopulmonary syndrome is defined by an arterial oxygen pressure < 70 mmHg in room air. In adults, the prevalence is 5%-29% and the overall mortality is 41% in hospitalized patients. In children, the prevalence ranges from 0.5% in those with portal vein obstruction to 20% in children with biliary atresia and polysplenia syndrome. In other causes of childhood cirrhosis, the prevalence is 2% -4%. Hepatopulmonary syndrome has been described in children as young as 6 months. Generally, hepatopulmonary syndrome is seen in chronic liver disease, but it has also been described in acute liver failure and extrahepatic portal venous obstruction [2].

A right to left pulmonary shunt due to intrapulmonary vascular dilation characterizes this syndrome. Blood flowing through the dilated capillaries is less exposed to oxygen contained in the alveoli, resulting in a ventilation-perfusion mismatch [3-6]. In animal models, elevated levels of nitric oxide have been detected in the lung homogenates of animals with the clinical features of hepatopulmonary syndrome. This finding appears to be supported by reports documenting increased exhaled nitric oxide concentration in patients with hepatopulmonary syndrome [7, 8].

The best method to evaluate the shunt in hepatopulmonary syndrome is the contrast-enhanced echocardiogram [1, 2, 9, 10]. Technetium 99m-labeled macroaggregated albumin scanning is a second method of detecting intrapulmonary vascular dilatations. Pulmonary arteriography can suggest two angiographic patterns [1]. Schneck *et al* [13] have reported the beneficial effect of methylene blue on this syndrome [13]. The transjugular intrahepatic portosystemic shunt (TIPS) [14], cavoplasty [15], octreotide [16], indomethacin [17, 18] and embolization [19] in the management of this syndrome have been assessed.

In 1992, clinical improvement in a case report was

Table 1 Baseline characteristics of children with hepatopulmonary syndrome

	Age (yr)	Gender	Type of disease	Duration (yr)	Child score	Dyspnea
Non-responders	14	M	Cryptogenic cirrhosis	10	C	+
	10	M	AIH	5	B	-
	6	M	Biliary atresia	6	C	+
	3	F	Biliary atresia	3	C	+
	13	M	Presinusoidal	10	A	+
	12	F	Presinusoidal	9	C	+
	9	M	Biliary atresia	9	C	+
Responders	7	F	Cryptogenic	4	B	+
	12	M	Biliary atresia	11	C	+
	3	F	AIH	1	A	+
	14	M	Cryptogenic cirrhosis	9	B	+
	13	M	Presinusoidal	8	A	+
	4	M	AIH	1	A	-
	11	M	Cryptogenic cirrhosis	5	B	-
	11	F	AIH	5	A	-

AIH: Autoimmune hepatitis.

achieved following the use of garlic [20]. In a study by Abrams *et al* [21] the use of garlic in adults in treatment of this syndrome was evaluated and garlic was found to be an effective therapy for this syndrome especially in young groups. Ku *et al* [22] revealed that active garlic metabolites are capable of pulmonary vasodilatation in rats. In 1997 Battaglia *et al* [23] reported an improvement in right to left shunt and arterio-alveolar gradient in such patients after liver transplantation. Further studies have confirmed the beneficial effect of liver transplantation on this syndrome [24, 25]. In our country, liver transplantation has a long waiting list. If the oral garlic can improve the arterial oxygenation and dyspnea, it may represent a palliative therapy for patients with hepatopulmonary syndrome awaiting liver transplantation.

MATERIALS AND METHODS

Patients

Fifteen patients (aged 6 months - 14 years) suffering from chronic liver disease, portal hypertension or cirrhosis and intrapulmonary shunt which were confirmed by saline contrast echocardiography were enrolled in this pilot study. Patients with congenital heart diseases, acute and chronic lung diseases and symptoms of severe gastritis, were excluded from the study. This study was performed at Children's Medical Center Hospital of Tehran University from 2002-2003.

Methods

Arterial blood gases were obtained from the radial artery in a sitting position at the same time and in the same room. Echocardiography with saline contrast was performed as previously described [13]. The presence of air bubbles in the left heart between 3 and 6 cardiac contractions indicated intrapulmonary shunt. Garlic was administered to the patients at the dose of 0.5-2 g/1.73 m^2 per day. We obtained a special kind of dried garlic (Hamedan city garlic), which was prepared in the form of 250 mg capsules by the hospital pharmacy.

The parents were asked to add the capsule or its content to their food once or twice a day at the recommended dose. We evaluated the patients every month. Clinical symptoms and signs, probable drug complication, and patient's compliance to the drug were asked in each visit. Arterial blood gas (ABG) was taken in the same condition as mentioned above. During the study we provided some facilities such as free contact if needed.

Response to garlic was defined as a 10 mmHg increment in the PaO_2 or when PaO_2 was more than 70 mmHg. Baseline and monthly data between responders and non-responders were analyzed statistically.

The Ethics Committee of Tehran University approved the protocol. The parents of the children gave their informed consent.

Stastistical analysis

The results were expressed as mean ± SD. Differences between two groups were evaluated by Fisher's exact test, Mann Whitney U and Wilcoxson rank-sum tests. $P < 0.05$ was considered statistically significant.

RESULTS

The baseline data of patients are summarized in Table 1. Fifteen patients with hepatopulmonary syndrome were treated with garlic. One child expired after one month of treatment.

At the end of the first month arterial O_2 pressure was increased significantly ($P = 0.004$). The mean of arterial O_2

Table 2 PaO_2 values of responders and non-responders in relation to dyspnea after treatment

Responders	Baseline	1st mo	2nd mo	3rd mo	4th mo	5th mo	Dypnea after Rx
1	66	85	90	90	90	95	Improved
2	70	86	87	90	99	99	Worsened
3	74	83	85	90	90	95	Improved
4	59	79	85	95	99	99	Improved
5	43	77	79	80	80	80	Improved
6	63	85	95	95	95	95	[a]
7	85	91	90	92	91	95	[a]
8	65	69	76	84	82	80	[a]
Non-responders							
1	34	33	32	35	40	40	No change
2	65	70	71	70	70	69	[a]
3	51	55	54	55	40	40	Worsened
4	57	58	.	.	.	.	Worsened
5	37	42	40	39	45	47	No change
6	45	49	51	54	50	52	No change
7	40	35	37	41	39	37	Worsened

[a]No dyspnea before and after treatment.

Table 3 PaO_2 values after oral garlic administration (mean ± SD, n = 15)

	Baseline	1st mo	2nd mo	3rd mo	4th mo	5th mo
Non-responders	47.1 ± 11.2	48.8 ± 13.2	47.5 ± 14.2	49 ± 13.1	47.3 ± 11.8	47.5 ± 11.8
Responders[b]	65.6 ± 12.1	82 ± 6.8	85.8 ± 6.1	89.5 ± 5.1	90.7 ± 7	92.2 ± 7.7[d]
Total	57 ± 14.7	66.5 ± 19.7	69.4 ± 22	72.1 ± 22.6	72.1 ± 24	73 ± 24.7[d]

[b]P <0.01 *vs* non-responders, [d]P <0.01 *vs* baseline.

pressure increment at the end of the second month was higher than 10 mmHg and over 70 mmHg at the end of the third month. At the end of the fifth month, the mean increment in the arterial O_2 pressure was 15 mmHg (Table 2).

Eight patients (53.3%) were considered as responders. The baseline PaO_2 was 65.6 ± 12.1 mmHg in the responder group and 47.1 ± 11.2 mmHg in non-responder group. There was a statistically significant difference in the initial O_2 pressure between the responders and non-responders (P = 0.009). At the end of treatment the mean PaO_2 in responders and non-responders was 92.2 ± 7.75 mmHg and 47.5 ± 11.87 mmHg, respectively (Table 3).

Dyspnea (including shortness of breath at rest or exercise, orthopnea and platypnea) occurred in 5 responders and 6 non-responders. Four out of these 11 patients had their dyspnea improved after treatment (Table 2).

According to the Child score, 5 patients (33.3%) were in group A, 4 (26.6%) in group B, and 6 (40%) in group C. The mean of arterial oxygen pressure in groups A, B and C, at the beginning of therapy was 56.4 ± 15.6 mmHg, 68.7 ± 11.2 mmHg and 49.6 ± 12.8 mmHg, respectively. At the end of therapy the mean PaO_2 in A, B and C groups was 79.4 ± 19.6 mmHg, 88.2 ± 13.3 mmHg and 53.6 ± 26 mmHg, respectively. No statistical difference in the mean PaO_2 was noted between groups A, B, and C.

DISCUSSION

The role of garlic in the management of hepatopulmonary syndrome is controversial. In 1992, clinical improvement was reported following the use of garlic [20]. In 1998 Abrams *et al* [21] studied the effects of garlic on hepatopulmonary syndrome in adults [21]. Chan *et al* [26] showed that garlic has no effect on the improvement of PaO_2 and hypoxemia as well as clinical status, suggesting that deteriorating oxygenation in patients with chronic liver disease may be an indication for liver transplantation. Abrams *et al* [21] showed that 40% of patients have at least an increase of 10 mmHg in their arterial oxygen pressure after treatment with garlic. They also studied the dyspnea index in these patients and reported that all patients responding to therapy have an improvement in dyspnea [21]. In the present study, 53.3% of patients following the use of garlic had an arterial oxygen pressure > 70 mmHg or an increment > 10 mmHg. Four out of the 8 patients responding to the therapy had an improvement in dyspnea, but non-responders did not show any improvement in their respiratory symptoms.

It was reported that the clinical response is better in younger patients [21]. The results of the present study are consistent with those of Abrams *et al* [21]. We showed an improvement rate of 53.4% in children and Abrames *et al* showed 40% in adults. Nevertheless, no statistically significant differences were noted between responders and non-responders with respect to age.

It is interesting to note that responders had a higher baseline PaO_2, suggesting that non-responders have a severe and profound disease and that medical management can be effective when baseline PaO_2 is higher. Since TC-99 macro-aggregated albumin scan was not performed in the present study, there was a possibility of selection bias, because anatomic shunt failing to respond to medical therapy was not excluded.

The exact mechanism of garlic therapy for hepatopulmonary syndrome is not known. Garlic appears to cause pulmonary vasodilatation [22] and increases the rate of NO synthesis [25, 27,28]. Thus one can conclude that garlic worsens hepatopulmonary syndrome. Garlic causes reduced NO synthesis in macrophages, resulting in reduced concentration in hypoxic tissue [29].

Intrapulmonary vasodilatation occurs primarily in the bases of the lungs, resulting in significant V/Q mismatch in this region. Abrams *et al* [21] speculated that if garlic results in uniform vasodilatation throughout the lung, then a redistribution of pulmonary blood flow to apical and mid lung fields could significantly improve V/Q ratios in these regions. Therefore, garlic may improve V/Q and reduce NO synthesis in the lung bases. Also garlic is known as a hepatoprotective agent and protects liver against tissue and chemical injures. If this is the case, it is probable that garlic might improve liver function and hepatopulmonary syndrome. Garlic might also reduce portal hypertension, indirectly resulting in an improvement in hepatopulmonary syndrome. Additionally, Allicin, Ajoene, and diallyl sulfur may be beneficial for hepatopulmonary syndrome treatment. These ingredients are present in dried garlic in sufficient amount [22]. However, the amounts of these ingredients vary depending on different types of garlic [29].

In children with chronic cholestasis, repeated transcutaneous bedside measurements are a rapid and reliable noninvasive test for characterizing the severity of abnormal oxygenation, and may prove useful also in post liver transplantation monitoring [30]. Although the existence of hypoxemia and hepatopulmonary syndrome has a prognostic value in patients with chronic liver disease [31], controlling the oxygen pressure, is not recommended as a screening tool [9, 10]. Therefore disease in diagnosis and control echocardiography with contrast should be more exact.

In conclusion, garlic can reduce the severity of hepatopulmonary syndrome. Arterial oxygen pressure is increased after treatment with garlic. Further studies are needed to evaluate the garlic therapy for hepatopulmonary syndrome in children.

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RAPID COMMUNICATION

Transfusion-transmitted virus in association with hepatitis A-E viral infections in various forms of liver diseases in India

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Abstract

AIM: To describe the prevalence of transfusion-transmitted virus (TTV) infection in association with hepatitis A-E viral infections in different forms of liver diseases in North India.

METHODS: Sera from a total number of 137 patients, including 37 patients with acute viral hepatitis (AVH), 37 patients with chronic viral hepatitis (CVH), 31 patients with cirrhosis of liver and 32 patients with fulminant hepatic failure (FHF), were analyzed both for TTV-DNA and hepatitis A-E viral markers. Presence of hepatitis B virus (HBV), hepatitis C virus (HCV) and hepatitis E virus (HEV) infections was detected in different proportions in different groups. Moreover, TTV-DNA was simultaneously tested in 100 healthy blood donors also.

RESULTS: None of the patients had hepatitis A virus (HAV) and hepatitis D virus (HDV) infections. Overall prevalence of TTV-DNA was detected in 27.1% cases with AVH, 18.9% cases with CVH, 48.4% cases with cirrhosis and 9.4% cases with FHF. TTV-DNA simultaneously tested in 100 healthy blood donors showed 27% positivity. On establishing a relation between TTV infection with other hepatitis viral infections, TTV demonstrated co-infection with HBV, HCV and HEV in these disease groups. Correlation of TTV with ALT level in sera did not demonstrate high ALT level in TTV-infected patients, suggesting that TTV does not cause severe liver damage.

CONCLUSION: TTV infection is prevalent both in patients and healthy individuals in India. However, it does not have any significant correlation with other hepatitis viral infections, nor does it produce an evidence of severe liver damage in patients with liver diseases.

INTRODUCTION

In 1997, a novel DNA virus was identified in serum of a Japanese patient (TT) having post-transfusion hepatitis[1]. This virus was designated as transfusion-transmitted virus (TTV). Later studies demonstrated TTV to be a non-enveloped single-stranded DNA virus, whose genome consists of 3739 bp and two large overlapping open reading frames (ORF-1 and ORF-2) encoding 770 and 202 amino acids and several small ORFs (22-105 amino acids)[2]. The virus has little sequence similarity with any known virus and so, it is presently not clear to which family it belongs. It is likely that TTV represents a new family. These are dense and 40-nm diameter particles. Individual genome sequences vary by up to 40%. Comparison of the genomic sequence, encoded proteins and the biophysical characteristics of the virus suggests that TTV is closely related to the Circoviridae. The genome exhibits high diversity and is classified into 6 genotypes[3]. As the prevalence of TTV in populations that require frequent transfusion of blood products is higher than that in healthy blood donors, TTV was supposed a parenterally transmissible virus. However, since TTV has been reported to be excreted in feces[4] also, it was felt that virus might also be transmitted non-parenterally. Using PCR with a new set of primers derived from a highly conserved region of the TTV genome, TTV DNA was detected in 92% of the general population in Japan[5], demonstrating that TTV is indeed a common virus.

Since its discovery, it has become clear that TTV infection is present worldwide among blood donors and is common in patients with liver diseases, including cryptogenic cirrhosis and fulminant hepatic failure. In one study, TTV DNA was detected in 46% and 12% of non-A to G hepatitis patients and healthy blood

donors, respectively[4], and it was initially suggested that TTV was a new hepatitis virus. The association of TTV with cryptogenic chronic liver diseases, post-transfusional hepatitis[1] and acute hepatitis of unknown etiology suggested an etiological role of this agent in the development of both acute and chronic hepatitis. Preliminary data from Japan and the United Kingdom indicated that TTV sequences were detectable in 25%-47% of patients with fulminant or chronic hepatitis of unknown origin, 27%-68% of hemophiliacs, and 1.9%-22% of apparently healthy blood donors[6-10]. Moreover, TTV-DNA titres were found to be 10- to 100-fold greater in liver tissue than in serum. All these findings have been interpreted as demonstrating its hepatotropism. Evidence for potential hepatotropism of TTV was supported by the presence of high TTV-DNA concentration in liver[7] and by a correlation between TTV-DNA titres and aminotransferase levels in post-transfusional non-A-G hepatitis patients[11]. In some studies, TTV-DNA has been found more frequently in patients with liver cirrhosis and hepatocellular carcinoma than in those with chronic hepatitis. However, virus DNA is not integrated in tumor cells, which may suggest that the virus is a passenger rather than a cause of the tumor. And therefore, further studies are required to determine the role of TTV. In fact, the significance of TTV infection in liver disease is, at present, analogous to that of HGV.

The aim of this study was to evaluate the prevalence of TTV-DNA in patients with different liver diseases in north India, and at the same time, find out the possible relation between TTV and other hepatitis viral infections in causation or progression of different liver diseases.

MATERIALS AND METHODS

Patients and blood samples

One hundred and thirty seven patients of both sexes and in adult age group were included in the present study. The patients consisted of 37 patients with acute viral hepatitis (AVH, age range: 21-48 years), 37 patients with chronic viral hepatitis (CVH, age range: 19-48 years), 31 patients with liver cirrhosis (age range: 34-57 years) and 32 patients with fulminant hepatic failure (FHF, age range: 28-46). All these patients attended either Outpatient Department or were admitted to the Liver Unit of All India Institute of Medical Sciences, New Delhi, from June 2001 to February 2004. They were evaluated clinically and biochemically and their sera were tested for hepatitis viral markers. The diagnosis of different types of liver diseases was based on accepted clinical, biochemical and histological criteria as outlined elsewhere[12]. AVH was diagnosed when patients exhibited overt jaundice and/or increased alanine aminotransferase levels (at least 3 times above the normal value) documented at least twice at a 1-week interval without any history of pre-existing liver disease. None of the patients had a past history of alcohol intake or using any drug. We also could not find any clinical or serological evidence of autoimmune diseases or biliary infection in these patients. The patients with CVH and cirrhosis of liver were diagnosed by histopathological criteria laid down by International Study Group on Chronic Hepatitis[13]. All these CVH patients had persistent elevation of transaminases level (at least twice the upper limit of normal range) for more than six months and histologic evidence of chronic hepatitis on liver biopsy at the beginning of follow-up. Fulminant hepatic failure was diagnosed if the patients developed hepatic encephalopathy within 4 wk of the onset of acute hepatitis as outlined elsewhere[12]. One hundred age- and sex-matched healthy subjects were used as controls.

From each of the above patients, 6-10 mL of venous blood was drawn and aliquoted in plain tubes without anticoagulant. Serum was separated after centrifugation and then stored at -70℃ until further analysis. Repeated freezing and thawing of serum was avoided as far as possible. These sera samples were used to analyze various hepatitis markers, liver function tests and hepatitis C virus (HCV) core protein.

Hepatitis viral markers

Sera were investigated for hepatitis B surface antigen (HBsAg) and IgM antibodies to hepatitis A virus (IgM anti-HAV), hepatitis B core antigen (IgM anti-HBc), hepatitis D virus (IgM anti-HDV) and hepatitis E virus (IgM anti-HEV). Similarly, all these sera were also tested for total antibodies against hepatitis C virus (anti-HCV). The serological analysis was done using enzyme immunoassay kits of high sensitivity and specificity. Kits for HBsAg, IgM anti-HBc and IgM anti-HAV were purchased from Abbot Laboratories, USA. Anti-HCV was tested using highly sensitive third generation ELISA kit from Ortho Diagnostics. This anti-HCV kit used peptides *versus* core, NS3, NS4 and NS5 regions of HCV genome, as antigen to coat the ELISA plate. IgM antibody to hepatitis D virus (HDV) was tested using an enzyme immunoassay kit from Wellcome, UK. Similarly, IgM anti-HEV was tested using third generation ELISA kit from Genelabs and Diagnostics, Biotechnology, Singapore.

Detection of HCV-RNA by RT-PCR

Total RNA was isolated from 100-μL serum or plasma using High Pure Isolation kit from Roche, Germany according to manufacturer's instructions. Five micrograms of the isolated RNA were applied to reverse transcription and nested PCR with primers located in the highly conserved 5' noncoding region (5' NCR) using BIOHCV kit (B&M Labs., Madrid, Spain). The reverse transcription mixture was incubated for 1 min at 85℃, followed by 30 min at 60℃. First PCR was performed in whole content after adding 40 μL of HCV amplification mixture. Thermal cycler was programmed as follows: 85℃ for 30 s, 94℃ for 2 min, followed by 40 cycles of 94℃ for 30 s, 55℃ for 30 s and 72℃ for 30 s and then further incubation of samples for 5 min at 72℃. Five microliters of first PCR product were subjected to nested PCR using nested PCR mixture containing second round primer and enzymes. The protocol on thermo cycler was the same as mentioned in first PCR. The PCR product was electrophoresed on 20 g/L agarose containing ethidium bromide and visualized under UV. A positive control provided in the kit was used as control. All positive and negative controls were tested in parallel with test samples throughout the entire procedures,

Table 1 Prevalence of TTV-DNA in different liver diseases

Liver disease	*n*	Presence of TTV-DNA *n* (%)
Acute viral hepatitis	37	10 (27.0)
Chronic viral hepatitis	37	7 (18.9)
Cirrhosis	31	15 (48.4)
Fulminant hepatic failure	32	3 (9.4)
Control	100	27 (27.0)

starting with RNA extraction.

Detection of TTV-DNA

Total DNA was extracted from 200 μL of serum using DNA isolation kit from Roche Diagnostics GmbH, Germany. This isolation method utilizes the ability of nucleic acids to absorb to silica (glass) in the presence of a chaotropic salt. Serum sample was treated with buffer containing proteinase K and silica particles where nucleic acids are bound to silica surface of magnetic particles. Since the binding process is specific for nucleic acids, the bound nucleic acids are purified from salts, proteins and other impurities by washing. A low salt buffer is used to elute the DNA. Using 5 μL of the DNA solution as a template, TTV DNA of the open reading frame (ORF-1) sequence, was detected by PCR employing the semi-nested primers reported by Okamoto *et al*[2]. The first-round PCR was carried out for 35 cycles, each cycle consisting of denaturation at 94℃ for 45 s, primer annealing at 60℃ for 45 s and extension at 72℃ for 60 s, followed by an additional extension at 72℃ for 7 min, using the primers NG059 (sense: 5'-ACAGACAGAGGAGAAGGCAAC ATG-3') and NG063 primer (antisense: 5'CTGGCATT TTACCATTTCCAAAGTT-3'). Thereafter, the second-round PCR was carried out using 1 μL of the first-round PCR product, NG061 primer (sense: 5'-GGCAACATG YTRTGGGATAGACTGG-3', where Y=T or C; R=A or G), and NG063 primer for 25 cycles under the same aforementioned conditions. The PCR product (10 μL) was electrophoresed on 20 g/L agarose gel containing ethidium bromide, and observed under ultraviolet light. The product of the first-round PCR was of 286 bp and that of the second-round PCR was of 271 bp.

Diagnosis of viral hepatitis

Liver function tests, including transaminase levels (AST and ALT) in serum, were performed on autoanalyser Hitachi-917 using the established techniques. Similarly, hemogram and coagulation profiles were performed using routine assays established in our laboratory. The diagnosis of different types of viral hepatitis was established as follows: The diagnosis of hepatitis A virus (HAV) infection was confirmed by the presence of IgM anti-HAV in serum. Hepatitis B virus (HBV) infection was established by presence of IgM anti-HBc in sera of AVH and FHF patients and by the persistent HBsAg antigenemia in sera of CVH and cirrhosis cases. Similarly, anti-HCV and IgM anti-HDV in sera samples were used for the diagnosis of HCV and HDV infections, respectively. All anti-HCV sera were also tested for HCV-RNA using nested PCR as described above. However, final diagnosis of HCV was based on anti-HCV antibodies in serum. Active or recent hepatitis E virus (HEV) infection was diagnosed by the presence of IgM anti-HEV in serum. Sera positive for HBsAg but negative for all other viral markers were labeled as HBV-carriers. Absence of all the markers including HBsAg labeled the patients with hepatitis non-ABCDE infection. HCV-RNA in serum was used to confirm active HCV infection.

RESULTS

Analysis of sera for different hepatitis viral markers demonstrated the presence of hepatitis B virus (HBV), hepatitis C virus (HCV), and hepatitis E virus (HEV) infections in these patients. None of the sera analyzed could demonstrate the presence of markers related to hepatitis A virus (HAV) and hepatitis D virus (HDV) infections. HBV infection was detected in 19 of 37 (54.1%) patients with AVH, 30 of 37 (80.1%) with CVH, 18 of 31 (58.1%) with cirrhosis and 13 of 32 (40.6%) with FHF. HCV infection, as indicated by the presence of anti-HCV in serum, was detected in 8 of 37 (21.6%) patients with AVH, 15 of 37 (40.5%) with CVH, 5 of 31 (16.1%) with cirrhosis and 2 of 32 (6.3%) with FHF. All anti-HCV-positive sera were also tested for HCV-RNA which was found positive in 96-98% sera samples. The percentage of HCV infection was based on anti-HCV positivity in these cases. HEV infection was observed in 14 of 37 (37.8%) cases with AVH and 5 of 32 (15.6%) cases with FHF.

Presence of TTV-DNA was detected in 10 of 37 (27.0%) cases with AVH, 7 of 37 (18.9%) cases with CVH, 15 of 31 (48.4%) cases with cirrhosis and 3 of 32 (9.4%) cases with FHF (Table 1). TTV-DNA was also detected in 27 of 100 (27%) healthy blood donors. In order to investigate TTV coinfection with different hepatitis viral infections, we observed TTV-DNA with IgM anti-HBc in 16.2% cases with AVH and none with FHF. TTV-DNA with HBsAg, indicating TTV-HBV co-infection in CVH and cirrhosis, was observed in 13.5% and 32.3% cases, respectively. TTV-HCV coinfection, indicated by simultaneous presence of TTV-DNA and anti HCV, was present in 10.8% case with CVH, 6.5% with cirrhosis and 3.2% cases with FHF. None of AVH patients had TTV-HCV coinfection. TTV-HEV coinfection could be demonstrated in 2.7% cases with AVH only. No other disease group had HEV-TTV coinfection (Table 2).

When TTV-DNA positive cases were analyzed for ALT level in sera, we found that 60% AVH patients had ALT level up to 3 334 nkat/L, 30% up to 6 668 nkat/L and only 2.7% more than 10 002 nkat/L. In CVH, all TTV-positive cases had ALT level less than 3 334 nkat/L. Similarly, in cirrhosis patients, ALT level was found up to 3 334 nkat/L in 93.3% cases and up to 6 668 nkat/L in 6.7% cases. In FHF, all three cases had ALT level more than 10 002 nkat/L. ALT level in serum was used as an index of liver damage in different disease groups (Table 3).

DISCUSSION

To investigate the status of TTV coinfection in relation

Table 2 Presence of TTV-DNA in relation to other viral hepatitis markers in liver diseases

Liver disease	*n*	*n* (%) TTV-DNA with HBsAg/IgM anti-HBc	TTV-DNA with anti-HCV/HCV-RNA	TTV-DNA with IgM anti-HEV
Acute viral hepatits	37	6 (16.2)	0 (NIL)	1 (2.7%)
Chronic viral hepatitis	37	5 (13.5)	4 (10.8)	-
Cirrhosis	31	10 (32.3)	2 (6.5)	-
Fulminant hepatic failure	32	0 (NIL)	1 (3.2)	0 (NIL)

NIL: 0%. - : Not done, as HEV is not present in chronic and cirrhosis cases.

Table 3 Relation between TTV-DNA and ALT levels in different liver disease groups

Liver disease	No. of TTV-DNA + patients	*n* (%)[ALT (nkat/L)] 833.5 - 3334	3335 - 6668	6669 - 10002	>10002
Acute viral hepatitis	10	6 (60)	3 (30)	0 (NIL)	1 (10)
Chronic viral hepatits	7	7 (100)	0 (NIL)	0 (NIL)	0 (NIL)
Cirrhosis	15	14 (93.3)	1 (6.7)	0 (NIL)	0 (NIL)
Fulminant hepatic failure	3	0 (NIL)	0 (NIL)	0 (NIL)	3 (100)

Present value was computed in comparison to total number positive for TTV-DNA. Nil : 0%.

to other hepatitis infections in different liver diseases, we tested TTV-DNA simultaneously with markers related to hepatitis A-E infections. Results are shown in Table 2. Analysis of data showed that TTV-HBV coinfection was detected in all the groups except FHF. Similarly, TTV-HCV coinfection was also recorded in all except AVH group. TTV-HEV coinfection was found in a minor proportion of AVH group. Attempts were also made to study the impact of TTV infection on liver damage both with or without other hepatitis viral infections. For this, serum level of ALT was tested in all the cases. The results showed that TTV infection did not cause severe liver necrosis in these groups of patients. This was indicated by a very moderate elevation in ALT level in the disease groups except FHF, which otherwise always showed a high ALT level.

While reviewing our findings in reference to various other studies, we found that our data are in agreement with several other reports. Earlier reports indicate that a majority of individuals who become TTV-DNA-positive after blood transfusion usually have normal ALT level and do not develop chronic hepatitis, although TTV viremia persists for years[9,19-20]. We found TTV-DNA in 27% normal population with normal ALT level and at the same time no significant increase in ALT in hepatitis A-E, TTV-positive patients, thereby suggesting that TTV alone does not cause much change in ALT level. This raises the possibility that TTV is merely an innocent bystander, both with and without non A-E hepatitis viral infections, and does not add to the damage caused by other hepatotropic viruses. Also, as such it does not appear to be the primary cause of hepatitis, though many more studies are still needed to prove this theory. The results by Vimolket *et al*[21] demonstrated that in non A-E hepatitis cases, mean ALT level was comparable among TTV-positive and negative cases. There was no consistent relationship between ALT and TTV-DNA level among these patients. These results also support our findings.

In conclusion, TTV infection is prevalent both in normal as well as patients populations in India. It shows its presence in all types of liver diseases, though comparable to the normal population and presenting no evidence of increasing liver damage both with or without other hepatitis viral infections. TTV infection has been found to be coinfected with HBV, HCV and HEV infections, but does not significantly increase ALT level in these patients. It appears as if TTV is a benign virus acting as a bystander in the body without causing any damage of the liver.

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RAPID COMMUNICATION

Identification of a new target region on the long arm of chromosome 7 in gastric carcinoma by loss of heterozygosity

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Abstract

AIM: To define the common deleted region on the long arm of haman chromosome 7q linked to primary gastric carcinomas in Chinese by loss of heterozygosity (LOH) and its clinical significance.

METHODS: Nine microsatellite markers distributed over chromosome 7q with an average marker density of 10cM were used to examine 70 primary gastric carcinomas for LOH by PCR amplification. The PCR products were separated by electrophoresis on polyacrylamide gel. Genescan and Genotyper softwares were used to analyze LOH.

RESULTS: LOH with at least one marker on 7q occurred in 34.3% (12/50) of the tumors. Among them, LOH at D7S486 and D7S798 was higher in 24.0% (24/70) and 19.2% (5/26), respectively. By statistical analysis we also observed an obvious genotype-phenotype correlation on 7q ($P < 0.05$). The frequency of LOH at D7S486 in patients with lymph node metastasis was significantly higher than that in those without lymph node metastasis ($P = 0.015$).

CONCLUSION: The high incidence of LOH at D7S486 and its correlation with poorer prognosis suggest that there might be putative tumor suppressor genes in this region involved in the tumorigenesis and progression of gastric carcinoma.

Key words: LOH; Chromosome 7; Gastric cancer

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INTRODUCTION

Gastric carcinoma is one of the most common malignancies in the digestive system. Generally, the incidence and mortality vary greatly depending on the region. The highest incidence is found in Japan and China in comparison with other regions of the world. It is one of the leading causes of cancer-related death[1]. Multi-gene and multi-step changes are involved in the occurrence and progression of tumors, including activation of oncogenes and inactivation of tumor suppressor genes (TSG). However, the vital genetic alterations remain uncertain. Previous genetic researches have demonstrated highly frequent amplifications of 1q, 2q, 6q, 7q, 8q, 17q, 20q and deletions of 1p, 1q, 2p, 3p, 4q, 5q, 6p, 7q, 9p, 11p, 11q, 12q, 15q, 17p, 17q, 18q, 19p, 21q in primary gastric cancers[2-5], indicating that these genetic alterations might relate to the occurrence and progression of gastric carcinoma. We used direct G-banding analysis and FISH on de-colored G-banding to study chromosome aberrations in primary gastric carcinoma and detected the deletion of 7q, 3p, 1p and 17p. The frequency of 7q deletion was particularly high[6], suggesting that there might be a potential TSG on the long arm of chromosome 7 involved in the progression of gastric carcinoma. Given the high rate of morbidity and mortality associated with GC, any means of reducing the occurrence of the disease or increasing its early detection is most desirable. In this study we examined the loss of heterozygosity (LOH) in microsatellite locus on human chromosome 7q of primary gastric carcinoma and corresponding non-tumor gastric mucosa tissues in order to narrow the oriented region of related TSG on 7q.

Table 1 Distribution and frequency of LOH at 9 microsatellite loci in primary gastric carcinomas

Microsatellite loci	Location on chromosome	LOH cases	Heterozygous cases	Positive rate of LOH (%)
D7S515	7q22.1	6	53	11.3
D7S486	7q31.1	12	50	24
D7S530	7q32.1	4	50	8
D7S640	7q33	3	63	4.8
D7S684	7q34	4	58	6.9
D7S661	7q35	3	27	11.1
D7S636	7q36.1	3	60	5
D7S798	7q36.2	5	26	19.2
D7S2465	7q36.3	5	52	9.6

MATERIALS AND METHODS

Materials

Primary gastric cancer and its corresponding normal tissue were obtained from 70 patients resected at the Affiliated Cancer Center of Zhongshan University during December 2000 to January 2002. All the patients did not receive any radiotherapy or chemotherapy before operation. The tissues were taken immediately after excision. One part of the samples was preserved at -80℃ for DNA extraction, the others were treated with routine protocol, embedded with paraffin and stained with HE.

Seventy patients (52 men and 18 women) aged 19 - 76 years (mean age 52.9 years) were enrolled in this study. The histological types included 1 papillary adenocarcinoma, 15 tubular adenocarcinomas, 25 poorly-differentiated adenocarcinomas, 2 mucinous adenocarcinomas, 20 signet-ring cell adenocarcinomas, 6 undifferentiated adenocarcinomas and 1 adenosquamous carcinoma, which were diagnosed according to the WHO classification of gastric carcinoma (1990). Based on the UICC TNM classification of gastric carcinoma, the patients were divided into 7 cases of stage Ⅰ (3 of ⅠA and 4 of ⅠB), 13 cases of stage Ⅱ, 24 cases of stage Ⅲ (14 of ⅢA and 10 of ⅢB) and 26 cases of stage Ⅳ.

DNA extraction

Genomic DNA samples of cryo-preserved gastric carcinomas and matched normal gastric mucosa were isolated by phenol chloroform extraction using standard protocols. DNA purification and concentration were detected by gel electrophoresis.

Selection of microsatellite markers and PCR amplification

A total of 9 microsatellite markers distributed over the whole chromosome 7q were selected from Genethon human genetic linkage map: D7S515, D7S486, D7S530, D7S640, D7S684, D7S661, D7S636, D7S798 and D7S2465. The frequencies of heterozygosity of the markers were over 75% and the average marker density was about 10cM. Synthesized microsatellite primers were respectively labeled with three different color fluorescent dyes: FAM, NED and HEX (purchased from PE Company). DNA samples were divided into 2 groups according to molecular size of the products and color of the fluorescent dyes. Primers (including 4 to 5 pairs) of each group were mixed in a tube for amplification. Each multiplex PCR reaction volume (5 μL in total) contained 0.5 μL 10×buffer, 0.2 μL 10 mmol/L dNTP, 0.3 μL 25 mmol/L $MgCl_2$, 0.05 μL 10 pmol/L primers, 0.2 U hot star Taq™ DNA polymerase (Gene Company, USA) and 45 ng genomic DNA. PCR amplifications were carried out in Gene Amp PCR system 9700 thermal cycler (Perkin-Elmer Co, Norwalk, USA). The reactive conditions were as follows: initial denaturation at 95℃ for 12 min followed by 10 cycles at 94℃ for 30 s, at 63℃ for 60 s (decreasing by 0.5℃ per cycle), at 72℃ for 90 s, and 26 cycles at 94℃ for 30 s, at 58℃ for 60 s, at 72℃ for 90 s and a final extension at 72℃ for 15 min.

Genomic scanning and analysis of microsatellite instability

PCR products (0.5μL) were diluted with 1.0 μL electrophoresis loading buffer containing 70% formamide, 3.75 mmol/L EDTA, 7.5 mg/ml blue dextran, 1.2 nmol/L Genescan 350. Mixture (1.0 μL) was loaded in urea-containing denaturing Sequagel Tm polyacrylamide gel (Gene Company, USA), and subjected to electrophoresis on a 377 DNA sequencer (ABI PRISM, PE Applied Biosystems). The automatically collected data were analyzed with Genescan Version 3.0 and Genotyper Version 2.0 software. Genotype for a given locus with two peaks of PCR fragments was defined as heterozygosity, and genotype with a single peak was regarded to be homozygosity. Only patients heterozygous for a given locus were regarded to be informative. Comparison of the ratios between tumors (T) and their controls (N) was made using the following formulas for calculations:

$(T_1/T_2)/(N_1/N_2)$ (1)

$(T_2/T_1)/(N_2/N_1)$ (2)

where T_1 and N_1 are the peak height of the smaller allele, while T_2 and N_2 are the peak height of the larger allele. Formula (1) was used to calculate the ratio of the smaller allele while formula (2) was used to calculate the ratio of the larger allele. For ratios greater than 1, the reciprocal of the ratio was calculated to give a value between 0.00 and 1.00. A value <0.67 was assigned as indicative of LOH[10-12].

Statistical analysis

The data of two-sample ratio were evaluated by chi-square test and Fisher's exact test. $P<0.05$ was considered statistically significant.

RESULTS

Distribution and frequency of LOH in primary gastric carcinoma

Genomic DNA was extracted from 70 primary gastric carcinomas and paired normal gastric mucosa tissues, 9 microsatellite fragments were amplified. Subsequent genotype analysis of the reliable amplification fragments was performed. LOH at all 9 loci was found in 70 primary gastric carcinoma cases (Table 1). The highest frequency of LOH was 24.0% at D7S486 and 19.2% at D7S798, respectively. LOH was detected in 24/70 (34.3%) of

Table 2 Correlation between frequencies and clinicopathology of LOH in primary gastric carcinomas

Clinicopathological variables		LOH frequency of chromosome 7 (%)	χ^2	*P* value	LOH frequency of D7S486 (%)	χ^2	*P* value
Gender	Male	17/52 (32.7)	0.22	0.636	7/35 (20)	1	0.317
	Female	7/18 (38.9)			5/15 (33.3)		
Age (yr)	>53	14/37 (37.8)	0.43	0.51	7/26 (26.9)	0.25	0.618
	≤53	10/33 (30.3)			5/24 (20.8)		
Clinical stage	I-II	3/20 (15)	6.16	0.046	1/13 (7.7)	2.63	0.268
	III	8/24 (33.3)			5/18 (27.8)		
	IV	13/26 (50)			6/19 (31.6)		
T stage	T1-2	3/13 (23.1)	0.88	0.349	1/8 (12.5)	0.68	0.411
	T3-4	21/57 (36.8)			11/42 (26.2)		
Lymph node metastasis	No	4/19 (21.1)	2	0.158	0/13 (0)		0.015
	Yes	20/51 (39.2)			12/37 (32.4)		
Distance metastasis	No	20/60 (33.3)	0.17	0.683	9/42 (21.4)		0.379
	Yes	4/10 (40)			3/8 (37.5)		
Histopathological type	Tubular adenocarcinoma	5/15 (33.3)	2.79	0.425	2/9 (22.2)	0.12	0.989
	Poorly- differentiated carcinoma	9/25 (36)			6/22 (27.3)		
	Signet-cell adenocarcinoma	6/19 (31.6)			3/12 (25)		
	Undifferentiated adenocarcinoma	4/6 (66.7)			1/4 (25)		

primary gastric carcinomas, 15/24 cases (62.5%) showed LOH at only one locus, 9/24 (37.5%) cases had LOH at two loci, and LOH occurred at more than three loci in 5/24 cases (25.0%).

Correlations between LOH and clinicopathology

Relations between LOH frequency and clinicopathology in 70 primary gastric carcinomas are shown in Table 2. There were statistical correlations between LOH frequencies at 7q and clinical stages. The frequency increased with poorer clinical stages ($P=0.046$). LOH at all 9 loci was detected in 13/26 (50.0%) cases with clinical stage Ⅳ, of which 6 cases (46.1%) were found to have LOH at D7S486. Five cases with LOH at more than 3 loci were all in stage Ⅳ. The frequency of LOH at D7S486 was related with lymph node metastasis. It was significantly higher in cases with lymph node metastasis than in those without metastasis ($P=0.015$).

However, frequencies of LOH showed no statistically differences in tubular adenocarcinomas, poorly-differentiated adenocarcinomas, signet-cell carcinomas and undifferentiated adenocarcinomas. Four cases showed LOH in 6 undifferentiated adenocarcinomas.

DISCUSSION

In our previous study, chromosome aberrations and their roles in the genesis and development of primary gastric cancer were investigated using direct G-banding analysis and FISH[7]. The deletion of chromosome 7q is the most consistent aberration, and 7q31-qter is the commonly lost segment[7-9]. LOH of this region is a very common occurrence in many kinds of human malignancies including cancers of breast[10], prostate[11], colon[12] and ovary[13], as well as primary squamous cell carcinoma of the head and neck[12]. Taken together, a critical TSG probably exists in this region with activation in a broad range of tissues. Some putative TSGs in this region such as ST7, Caveolin-1, ING3, and PPP1R3 have been reported[7,9,14,15]. However, no further researches provide reliable evidence for the correlation between these candidate genes and primary gastric carcinomas.

Tumor occurrence and progression involve multi-genes and multi-steps. Different genetic alterations participate in tumor occurrence and progression, and genetic alteration plays a vital role in different tumors. Several chromosomal amplifications and deletions have been reported in primary gastric carcinomas[2-5]. Kuniyasu *et al*[16] have reported LOH at 5 microsatellite markers on 7q in 32% (26/82) of 98 gastric carcinomas. D7S95 on 7q31-35 is the most frequent change locus. Similar results were also reported by Nishizuka *et al*[17]. Our findings are consistent with these previous studies. In our study, the total LOH at 7q was 34.3% in gastric carcinomas, and the frequency of LOH at D7S486 reached 24.0%. The higher frequencies of LOH at D7S486 and D7S798 than at the other 7 loci indicate the presence of tumor suppressor genes in these regions, particularly near D7S486.

Different results about correlations between LOH and clinical factors of chromosome 7q have been reported in various studies. Kuniyasu *et al*[16] found that LOH at D7S95 on 7q31-35 is much higher in stage Ⅳ gastric carcinomas and that patients with LOH at D7S95 show celiac metastasis compared with those without LOH ($P<0.05$). Moreover, patients of stage Ⅲ-Ⅳ with LOH at D7S95 survive shorter than those without LOH ($P<0.05$). Thus, LOH at D7S95 is likely involved in gastric carcinoma progression and prognosis. In our 70 gastric carcinomas, the frequency of LOH at any locus on 7q increased obviously with the rising of clinical stage ($P=0.046$), and reached 50.0% (13/26) in patients with clinical stage Ⅳ. Moreover, 5 cases with LOH at more than 3 loci were all in stage Ⅳ. The frequency of LOH at D7S486 in patients with lymph node metastasis was obviously higher than that in those without lymph node metastasis ($P=0.015$). There was no significant correlation between LOH and histological types. This lack of correlation may be related to the small number of undifferentiated tumors. Our results suggest

that one or more tumor suppressor genes associated with gastric carcinomas might situate on chromosome 7q and D7S486. Loss of restraining effects on tumor proliferation, infiltration and metastasis of these candidate genes might promote gastric carcinoma progression.

The region around the marker D7S486 may contain a fragile site. In fact, a 7q31.2 fragile site (FRA7G) of 300 kb is located between markers D7S486 and D7S522[18]. FRA7G is a common aphidicolin-inducible fragile site at 7q31.2, showing LOH in human malignancies. Common fragile sites are specific regions in mammalian chromosomes that are prone to breakage and rearrangements. This genetic instability can lead to disease manifestations and may play a role in oncogenesis[19]. The present study delineated a breakpoint of putative TSG near the marker D7S486. Tatareli *et al*[20] investigated the structure of FRA7G spanning the region between marker D7S486 and Met H and have identified a gene encoding a 421-amino-acid protein with three LIM domains with 89% identity to murine Testin. These findings suggest that TESTIN may represent a candidate tumor suppressor gene at 7q31.2.

The genetic intervals of microsatellite markers in our study were relatively wide (10cM). Additional studies are needed to narrow these regions on D7S486 and identify potential tumor suppressor genes.

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RAPID COMMUNICATION

Perioperative artificial nutrition in malnourished gastrointestinal cancer patients

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Abstract

AIM: To investigate the potential role of perioperative nutrition in reducing complications and mortality in malnourished gastrointestinal cancer patients.

METHODS: Four hundred and sixty-eight elective moderately or severely malnourished surgical patients with gastric or colorectal cancers defined by the subjective global assessment (SGA) were randomly assigned to 7 d preoperative and 7 d postoperative parenteral or enteral nutrition *vs* a simple control group. The nutrition regimen included 24.6±5.2 kcal /kg per d non-protein and 0.23±0.04 g nitrogen /kg per d. Control patients did not receive preoperative nutrition but received 600±100 kcal non-protein plus or not plus 62 ± 16 g crystalline amino acids postoperatively.

RESULTS: Complications occurred in 18.3% of the patients receiving nutrition and in 33.5% of the control patients ($P = 0.012$). Fourteen patients died in the control group and 5 in those receiving nutrition. There were significant differences in the mortality between the two groups (2.1% *vs* 6.0%, $P = 0.003$). The total length of hospitalization and postoperative stay of control patients were significantly longer (29 *vs* 22 d, $P = 0.014$) than those of the studied patients (23 *vs* 12 d, $P = 0.000$).

CONCLUSION: Perioperative nutrition support is beneficial for moderately or severely malnourished gastrointestinal cancer patients and can reduce surgical complications and mortality.

Key words: Malnutrition; Perioperative nutrition; Parenteral nutrition; Enteral nutrition; Mortality; Complications

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INTRODUCTION

Malnutrition is common in gastrointestinal cancer patients and the causes are often complex and multi-factorial. Although it is widely accepted that malnutrition adversely affects the postoperative outcome of patients, there is little evidence that perioperative nutrition support can reduce surgical risk in malnourished cancer patients. Early retrospective studies suggested that perioperative nutrition support may effectively reduce postoperative complications[1,2]. Subsequent prospective, randomized clinical trials (RCTs) demonstrated that the benefits of perioperative nutrition support are limited to severely malnourished patients undergoing major surgery[3,4]. Most RCTs of perioperative nutritional support in patients with gastrointestinal cancer have shown that the degree of malnutrition varies considerably, ranging from no weight loss to weight loss exceeding 10 % and the results obtained by these studies are different in malnourished and non-malnourished patients[1-4]. Unfortunately, studies in really malnourished gastrointestinal cancer patients are not available. Meanwhile, parenteral and enteral nutrition is till controversial in perioperative malnourished gastrointestinal cancer patients[5].

The aim of this study was to evaluate the efficacy of optimal perioperative nutrition support in reducing complications and mortality in malnourished gastrointestinal cancer patients.

MATERIALS AND METHODS

This prospective study was carried out from May 2002 to July 2004 at the General Surgical Department of Zhongshan Hospital. All patients who underwent surgery for gastrointestinal (stomach, colon and rectum) malignancies were eligible for inclusion. Patients were excluded if they were admitted for emergency surgery. Within 48 h of admission, patients underwent nutritional assessment by the subjective global assessment. (SGA) performed with a standardized questionnaire including the patient's history (weight loss, changes in dietary intake, gastrointestinal symptoms, and functional capacity), physical examination (muscle, subcutaneous fat, sacral

Table 1 Preoperative characteristics of the patients

	Study group ($n=235$)	Control group ($n=233$)
Mean age (yr)	57.3	56.5
Male/ female	162/95	166/89
Body weight (kg)	59.4±15.2	61.1±14.9
SGA score		
B	147	153
C	88	80
Gastric carcinoma	124	129
Colon carcinoma	65	61
Rectum carcinoma	46	43

SGA: subjective global assessment; B: moderately- malnourished; C: severely-malnourished.

and ankle edema, ascites) and the clinician's overall judgment of the patient's status (normal, moderately or severely malnourished). On the basis of these data, the patients were classified as well-nourished, moderately- or severely-malnourished. A total of 512 moderately- and severely-malnourished gastrointestinal cancer patients were included in this study. The Ethical Committee of the Institution approved this clinical study.

After stratified for age, sex and tumor localization (gastric, colorectal), patients were randomly divided into study group ($n=257$, 95 women, 162 men) and control group ($n=255$, 89 women, 166 men). The mean ages were 57.3 years (range 21 - 84 years) and 56.5 years (range 24 - 86 years) in the study group and control group, respectively.

Perioperative nutrition was administered in the study group by parenteral or enteral route or a combination of the two based on a clinical assessment of intestinal function. Patients due to permanent or temporary intestinal failure were given parenteral nutrition (PN). If the clinician felt that the patients had a functioning gastrointestinal tract, they received enteral nutrition (EN).

Most patients (68%) received PN support during the preoperative and postoperative periods. The PN regimen consisted of 25 kcal/kg per d non-protein and 0.25 g nitrogen /kg per d. The non-protein calorie source included glucose and fat, accounting for 60% and 40% respectively of the energy intake. The protein source was supplied by crystalline amino acid solutions. Electrolytes, vitamins, and trace elements were administered according to the current recommendations. PN mixture was delivered through a central venous catheter or peripheral veins using an "all in one" bag. In addition, the patients receiving preoperative PN had free access to food they preferred.

EN was given to 75 patients (32%) in whom GI function was adequate through a fine bore silicone feeding tube. Where appropriate, EN was alternatively administered via a nasogastric tube or a feeding jejunostomy catheter. Similar target intake of non-protein (25 kcal/kg per d) and protein (0.25 g nitrogen/kg per d) was provided using commercially available enteral formulas. The initial rate of delivery was 40-60 mL/h, increasing stepwise to full intake for 48 h according to patient tolerance. Foods were usually given as a continuous infusion using a volumetric pump for 24 h. Nutritional support was started 8-10 d before surgery and continued for more than a week after surgery.

Patients in the control group were given a standard hospital oral diet before surgery and a hypocaloric parenteral solution (600 kcal non-protein and 60 g amino acid) in the postoperative period until gastrointestinal function recovered completely.

After surgery, patients were monitored daily for postoperative complications including septicemia, intra-abdominal abscess, wound infection, wound dehiscence, fistula formation, urinary tract infection, pneumonia, respiratory insufficiency and phlebitis. Rigid objective criteria were established defining each complication to avoid subjective bias. A diagnosis of septicemia was based on a positive blood culture, hypotension and hypoperfusion. An intra-abdominal abscess was defined as an intra-abdominal purulent collection requiring operative drainage. Fistulae were radiographically documented. A diagnosis of urinary tract infection required a quantitative culture of greater than 100 000 organisms. Pneumonia was documented by an abnormal chest x-ray, positive sputum culture, and treatment with antibiotics. The presence of a wound infection was defined by culture and operative or spontaneous drainage of purulent materials. A wound dehiscence required operative re-closure of the wound. The occurrence and cause of death during hospitalization and the length of hospitalization were recorded.

Statistical analysis

Data were analyzed using standard statistical software (SPSS 10.0). For normally distributed data, a paired Student's t test was used for statistical analysis. $P \leqslant 0.05$ was considered statistically significant. Data were expressed as mean $\pm$ SE.

RESULTS

A total of 512 malnourished patients gave their consent to participate in the study and 16 declined. Of the 512 patients, 28 were not randomized after surgery because of un-resectability. Four hundred and sixty-eight patients were assigned at random to study group ($n=235$, 87 women, 148 men) and control group ($n=233$, 90 women, 143 men). Patient demographics and preoperative parameters of the two groups are presented in Table 1. There were no significant differences in mean age, sex distribution and nutrition status between the two groups. Operative data are listed in Table 2. The mean length of the surgical procedure and the volume of intraoperative blood transfusions were similar in two groups. The volume of postoperative blood transfusions was larger in control group. However, none of these differences was statistically significant. The number of patients requiring albumin infusions and the volume of infused albumin were comparable between the groups.

The number of postoperative complications per study group is shown in Table 3. Forty-three complications occurred in 31 patients in the study group, and five patients died due to major complications. Seventy-eight complications occurred in 64 patients of the control group, and 14 patients died. There were significant

Table 2 Operative data of the patients (mean ± SE)

	Study group (*n* = 235)	Control group (*n* = 233)
Mean operating time (min)	210±84	196±102
Operative blood loss (mL)	540±150	525±120
Mean blood transfused (mL)		
Intraoperative	420±80	400±100
Postoperative	200±60	280±120
Patients with albumin infusion (*n*)	156	173
Mean albumin infusion (g)	45±22	55±30
Kind of operation		
Partial gastrectomy	82	90
Total gastrectomy	42	39
Colon resection	65	61
Rectum resection	46	43

Table 3 Postoperative complications in two groups

	Study group (*n* = 235)	Control group (*n* = 233)
Pneumonia	12	23
Urinary tract infection	6	10
Wound infection	11	20
Septicemia	2	5
Intra-abdominal abscess	4	7
Wound dehiscence	3	4
Fistula	2	4
Respiratory insufficiency	2	3
Phlebitis	1	2
Total	43 in 31 patients	78 in 64 patients

differences in the mortality and complications between the two groups (2.1% *vs* 6.0%, $P = 0.003$ for mortality; 18.3% *vs* 33.5%, $P = 0.012$ for complications). In both groups, the most frequent complication was infection related to muscle weakness and/or prolonged immobilization (respiratory insufficiency, phlebitis).

Table 4 summarizes the incidence of septic complications between PN and EN groups. No significant differences were found in the incidence of septic complications between the two groups of nonrandomized patients. In addition, there was no significant difference in the number of septic complications between the two groups.

The total perioperative and postoperative median length of hospitalization was 22 *vs* 29 d in the study group and 12 *vs* 23 d in the control group, respectively. The total length of hospitalization and postoperative stay of the control patients were significantly longer than those of the study patients ($P = 0.014$, $P = 0.000$).

Table 4 Incidence of septic complications in PN or EN group

	PN group (*n* = 160)	EN group (*n* = 75)	*P*
Incidence of septic complications	25 -15.60%	10 -13.30%	0.36
Mean No. of complications per patient (±SE)	0.22±0.03	0.20±0.04	0.19
Mean No. of complications per infected patient (±SE)	1.33±0.05	1.26±0.06	0.22

PN: parenteral nutrition; EN: enteral nutrition.

DISCUSSION

Malnutrition in hospitalized patients is a critical issue associated with a significant increase in morbidity and mortality. Recent surveys have demonstrated that 30 - 50% of hospitalized patients have a certain degree of malnutrition[6,7]. Malnutrition is closely associated with increased morbidity and mortality after major gastrointestinal surgery. Perioperative nutrition support can restore many of biochemical and immunologic abnormalities in malnourished or normal state. However, it is difficult to demonstrate that perioperative nutrition support can significantly reduce surgical complications except in the most severely malnourished patients[8]. Early retrospective studies from 1970s to 1980s suggested that perioperative nutrition support can reduce surgical complications. Subsequent prospective, randomized trials demonstrated that the benefits of perioperative nutrition support are limited to severely malnourished patients undergoing major surgery[3,4]. Most RCTs of perioperative nutritional support in patients with gastrointestinal cancer have shown that the degree of malnutrition varies considerably, ranging from no weight loss to weight loss exceeding 10%, and that the complication rate decreases from 56% in the control arm to 34% in the TPN arm, with no deaths in the latter group[4,9]. Because nutrition support can only ameliorate but not reverse the catabolic response to trauma, it may effectively improve nutritional state and reduce postoperative complications when started preoperatively[10].

This study represented a large randomized clinical trial to explore the role of perioperative nutrition support in moderately- and severely-malnourished gastrointestinal cancer patients. In these patients nutritional support was started 8 ± 10 d before surgery and continued for more than a week after surgery. Nutrition was given by parenteral or enteral routes or a combination of these two. Postoperative complications were defined by rigid objective criteria to avoid subjective bias. This prospective study demonstrated that adequate perioperative nutritional support could effectively reduce the incidence of postoperative complications in moderately- and severely-malnourished gastrointestinal cancer patients. In perioperative nutrition support patients, there was a two-fold reduction in complications ($P = 0.012$) and a three-fold reduction in death ($P = 0.003$). The most dramatic decrease was noted in major septic complications (14.9% *vs* 27.9%, $P = 0.011$) such as pneumonia and wound infection. In addition, the mortality was statistically lower in artificial nutrition support group than in the control group (2.1% *vs* 6.0%, $P = 0.003$). As a consequence of the lower infection rate, the length of hospital stay of the study group was shorter. These results indicate that malnutrition has a negative impact on postoperative outcomes, which may be efficiently controlled by a perioperative nutrition support

that is adequate in quality, quantity, and duration.

Perioperative nutritional support can be administered by PN or EN or their combination. PN has the advantage of easy administration and essentially immediate provision of optimal nitrogen and caloric requirements once the central venous access is established. A major concern with PN in hospitalized patients is the increased risk of septic complications related to immune dysfunction after PN. Unlike PN, EN is not associated with increased infectious complications. In fact, enteral feeding can maintain structural and functional integrity of the gastrointestinal tract and reduce septic complications in critically ill patients. The major disadvantages of EN support are the time delay when attempting to provide complete nutrition by the enteral route and the inability of patients with postoperative abdominal complications to tolerate enteral feeding. Early postoperative EN has fewer septic complications compared to early postoperative PN [11, 12]. Unfortunately, studies in really malnourished cancer patients are not available. The present prospective study demonstrated that there was no statistically significant difference in the incidence of septic complications between PN and EN (15.6% *vs* 13.3%, $P = 0.36$). In addition, there was no significant difference in the number of septic complications per patient or complications per infected patient between PN and EN between the two groups. The results of our study are different from other prospective randomized trials[13-15]. There are several possible explanations for the discrepancies. First, patients are quite different in terms of age, physiologic and nutritional status. Second, the relative protein and energy intake in previous studies are often not comparable, usually being much higher in patients receiving PN than in those receiving EN[16,17]. Excessive energy intake may result in hyperglycaemia and lead to increased septic complications and mortality[18,19]. In the present study, the prescribed target intake for both PN and EN patients was the same (25 kcal /kg per d and 0.25 g N/kg per d). Third, in previous studies, all patients were randomized to receive either PN or EN, with no consideration given to the issue of gut function. Therefore some patients able to tolerate EN were given PN. In the present study, the route of feeding was dictated by an assessment of gut function, ensuring EN was administered only to patients with adequate intestinal function. The results of this study are in accord with the recent studies[20, 21].

In conclusion, perioperative nutrition support can decrease the incidence of postoperative complications in moderately- and severely-malnourished gastrointestinal cancer patients. In addition, it is effective in reducing mortality. Both parenteral support and enteral nutrition support, or their combination can be used in the management of malnourished patients undergoing gastrointestinal surgery.

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RAPID COMMUNICATION

Influence of acid and bile acid on ERK activity, PPARγ expression and cell proliferation in normal human esophageal epithelial cells

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Abstract

AIM: To observe the effects of acid and bile acid exposure on cell proliferation and the expression of extracellular signal-regulated protein kinase (ERK) and peroxisome proliferator-activated receptor γ (PPARγ) in normal human esophageal epithelial cells *in vitro*.

METHODS: *In vitro* cultured normal human esophageal epithelial cells were exposed to acidic media (pH 4.0-6.5), media containing different bile acid (250 μmol/L), media containing acid and bile acid, respectively. Cell proliferation was assessed using MTT and flow cytometry. The expressions of phosphorylated $ERK_{1/2}$ and PPARγ protein were determined by the immunoblotting technique.

RESULTS: Acid-exposed (3 min) esophageal cells exhibited a significant increase in proliferation ratio, S phase of the cell cycle ($P < 0.05$) and the level of phosphorylated $ERK_{1/2}$ protein. When the acid-exposure period exceeded 6 min, we observed a decrease in proliferation ratio and S phase of the cell cycle, with an increased apoptosis ratio ($P < 0.05$). Bile acid exposure (3-12 min) also produced an increase in proliferation ratio, S phase of the cell cycle ($P < 0.05$) and phosphorylated $ERK_{1/2}$ expression. On the contrary, deoxycholic acid (DCA) exposure (> 20 min) decreased proliferation ratio. Compared with bile acid exposure (pH 7.4), bile acid exposure (pH 6.5, 4) significantly decreased proliferation ratio ($P < 0.05$). There was no expression of PPARγ in normal human esophageal epithelial cells.

CONCLUSION: The rapid stimuli of acid or bile acid increase proliferation in normal human esophageal epithelial cells by activating the ERK pathway.

Key words: Acid; Bile acid; Esophageal epithelial cells; Cell proliferation; Extracellular signal-regulated protein kinase

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INTRODUCTION

The incidence of esophageal adenocarcinoma has been rapidly increasing in western countries. With the improvement of investigation devices, reflux esophagitis and Barrett's esophagus (BE) have also been reported in more and more cases in China. In these diseases, duodenogastroesophageal reflux has been established as a strong risk factor[1]. The main components of refluxates are acid and bile acid, whose potential contributions to the esophageal diseases have widely been discussed. However, the effects of acid and bile acid on esophageal cell proliferation and the related signal transduction mechanisms remain unknown. The mitogen-activated protein kinase (MAPK) family, also known as extracellular signal-regulated kinase (ERK), c-Jun-N-terminal kinase (JNK), and p38 pathways, transmit extracellular growth-regulating signals to effector genes in the nucleus, the activation of the ERK pathway is usually associated with pro-proliferative and antiapoptotic effect[2]. Peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear hormone receptor superfamily, has recently been shown to be implicated in the development of many digestive tumors[3], with the potential role in the downstream of ERK pathway[4].

In the present study, we examined the effects of acid and bile acid on cell proliferation in normal human esophageal epithelial cells *in vitro*, in association with ERK signaling pathway and PPARγ expression.

MATERIALS AND METHODS

Antibodies and reagents

Monoclonal antibody raised against human cytokeratin 14 (CK14) was purchased from Changdao Biotech Company, Shanghai. Polyclonal antibodies raised against human ERK$_{1/2}$ and phospho-ERK$_{1/2}$ were purchased from Cell Signaling Technology, USA. Monoclonal antibody raised against human PPARγ was purchased from Santa Cruz, USA. Keratinocyte serum-free media (K-SFM) supplemented with bovine pituitary extract (BPE) and recombinant epithelial growth factor (rEGF) were from Gibco, USA. Hydrochloric acid (1 mol/L) was from Sanpu Pure Chemical Industries, Xi'an. Sodium glycocholate (GC), sodium glycodeoxycholate (GDC), sodium glycochenodeoxycholate (GCDC), sodium taurocholate (TC), sodium taurodeoxycholate (TDC), sodium taurochenodeoxycholate (TCDC), and cholic acid (CA), deoxycholic acid (DCA) were purchased from Sigma, USA, and dissolved in K-SFM to 250 μmol/L. Histostain kit was purchased from Zhongshan Golden Bridge Biotech Company, Beijing. RIPA cell lysis kit was purchased from Shenergy Biocolor BioScience Technology Company, Shanghai. BCA protein assay kit and ECL kit were purchased from PIERCE, USA.

Cell culture

Normal esophageal mucosa samples were obtained from surgically resected esophagus with esophageal carcinoma, then confirmed by an experienced pathologist to contain neither macroscopic tumor tissue nor histologically detectable metaplastic cells or cancer cells. Samples were acquired with the signed informed consent from the patient. Samples were collected aseptically, stored in sterile K-SFM at 4℃, and processed within 4 h. Primary culture of normal esophageal epithelial cells was undertaken according to the method described elsewhere[5]. The collected cells were suspended in K-SFM supplemented with BPE and rEGF, and seeded into 75-cm uncoated plastic culture flask in humidified air containing 50 mL/L CO_2 at 37℃. After the initial subculture, an aliquot of cells was grown on coverslips for immunocytochemical stain with CK14 antibody.

Experimental groups

Equally seeded epithelial cells were cultured in K-SFM without BPE and rEGF for 48 h, then divided into 3 groups, acid exposure group: cells were exposed to the acidified medium (pH 4.0-6.5) for 3-60 min; bile acid exposure group: cells were exposed respectively to the medium containing different bile acid (250 μmol/L) for 3-60 min; and mixed exposure group: cells were exposed to the medium containing different bile acid (250 μmol/L) with different pH (4.0 and 6.5) for 3 min. Cells of control group were cultivated in normal media (pH 7.3).

Cell proliferation and cell cycle determination

For cell proliferation assay, 5×10^3 cells/well were plated in 96-well plates. Using MTT assay, changes in cell number were measured 24 h after different exposures. Then 10 μL MTT solution (5 mg/mL) was directly added to the cell cultures. After 4 h, media were removed and cells were lysed with 100 μL of dimethyl sulfoxide. Absorbance at 490 nm was read and data were presented as ratio to control (proliferation ratio).

For cell cycle determination, cells were seeded at 5×10^5/well in 6-well dishes. After 24 h of different exposures, cells were trypsinized and washed in PBS. A pellet of 1×10^6 cells was fixed in 750 mL/L ice-cold ethanol and stored at 4℃ until analyzed. Before flow cytometric analysis, cells were stained with 50 μg/mL RNase and 50 μg/mL propidium iodide. Cells in the G1, S, and G2 phase of the cell cycle and cell apoptotic percentage were determined using flow cytometer (FACSCalibur, Becton Dickinson, USA).

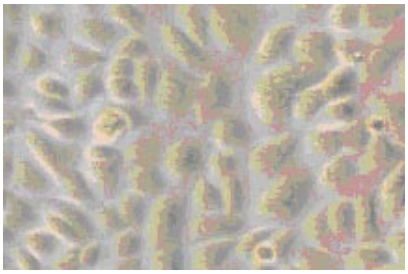

Figure 1 A phase-contrast micrograph of normal human esophageal epithelial cells (× 250).

Immunoblot analysis

After exposure to acid or bile acid for 3 min, cells were immediately treated with cold RIPA cell lysis buffer, followed by centrifugation at 10 000 *g* for 20 min at 4℃ to remove the cell debris. Total protein of the supernatant was measured using a BCA protein assay kit. Protein electrophoresis was performed on a 100 g/L SDS- polyacrylamide gel with 50 μg protein added to each lane. The proteins were then transferred onto a PVDF membrane. The membrane was incubated overnight at 4℃ with the various primary PPARγ (1:100), ERK$_{1/2}$ and anti-phospho ERK$_{1/2}$ (1:1000) antibodies, and then incubated with corresponding secondary antibodies conjugated to horseradish peroxidase for 2 h at room temperature. The protein bands were visualized by ECL and exposed to X-ray film. The relative density of the protein bands was quantified by densitometry using Electrophoresis Documentation and Analysis System.

Statistical analysis

All experiments were performed in triplicate. The data were expressed as mean ± SD. Statistical analysis between two groups was carried out using Student's *t*-test, for comparison of three or more groups using ANOVA test. $P < 0.05$ was considered statistically significant.

RESULTS

Growth characteristics of normal human esophageal epithelial cells

Epithelial monolayer cultures were successfully established with the appearance of cobblestones (Figure 1). As a cytoskeleton protein of esophageal epithelial cells, CK14 was expressed in all secondary cultures using immunocytochemical stain.

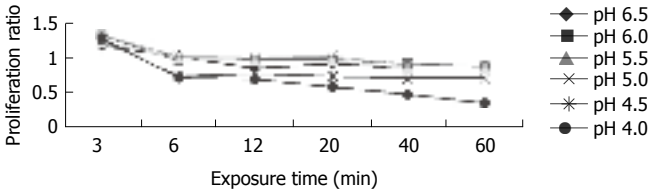

Figure 2 Effect of acid exposure on cell proliferation.

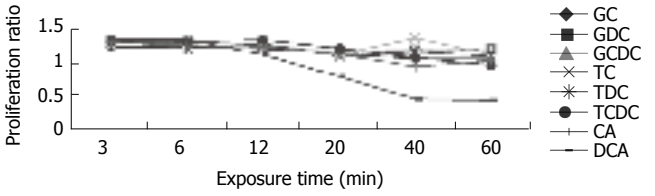

Figure 3 Effect of bile acid exposure on cell proliferation.

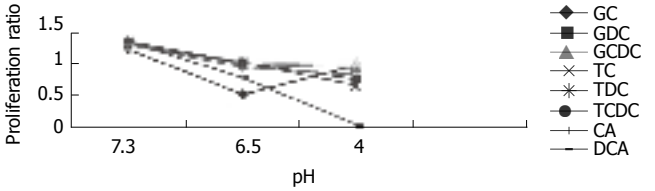

Figure 4 Changes in proliferation of cells exposed to bile acid with different pH for 3 min.

Measurement of cell proliferation and cell cycle

For cells exposed to acid (Figure 2), proliferation ratios were above 1 at 3 min, then decreased gradually to below 1 with prolongation of exposure time, especially when pH < 5.0. For cells exposed to bile acid (Figure 3), proliferation ratios of GC, GDC, GCDC, TC, TDC, TCDC, and CA groups were all above 1 or near 1 with the exposure time of 3-60 min. For DCA group, proliferation ratios were above 1 at 3-12 min, then decreased to below 1 with prolongation of exposure time. For mixed exposure group (Figure 4), proliferation ratios of cells exposed to bile acid with pH 6.5 or 4 for 3 min were markedly less than that of bile acid group (pH 7.4) (P <0.05), for most groups the proliferation ratios dropped to the lowest at pH 4, yet for GC the lowest proliferation ratio was seen at pH 6.5.

The results of cell cycle determination are summarized in Table 1. Compared with the control (pH 7.3), a short-period exposure to acid (3 min) produced an obvious increase of cells in S phase (P < 0.05), and a similar apoptosis percentage. In contrast, longer exposure to acid (6, 12 min) significantly decreased the cell proportion in S phase accompanied by an increased apoptosis percentage (P <0.05). Similarly, a brief exposure to bile acid (3-12 min) also produced an increase of cells in S phase (P<0.05), and a similar apoptosis percentage.

Expression of ERK1/2 and PPARγ

The expression of phospho-ERK1/2 was significantly increased in cells exposed to pH 4.0, 5.0 and 6.0 for 3 min (Figure 5) compared to control (pH 7.3) (P < 0.05). ERK

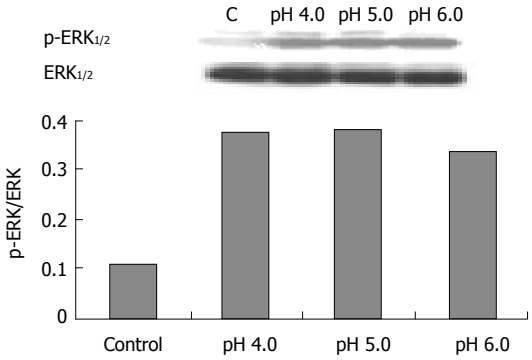

Figure 5 Effect of acid exposure (3 min) on ERK.

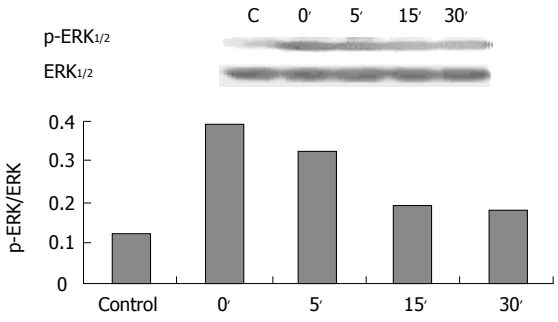

Figure 6 Expression of ERK in 30 min after acid exposure (pH 4.0, 3 min).

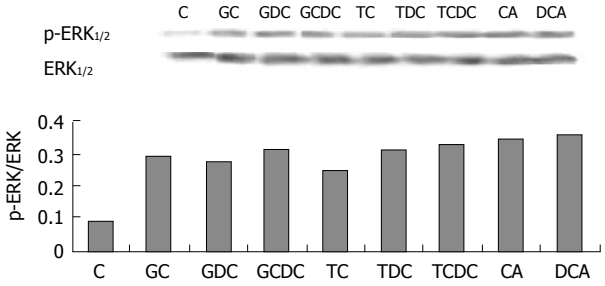

Figure 7 Effect of bile acid exposure (3 min) on ERK.

activity increased immediately after acid exposure (pH 4, 3 min), approximate to the level of control after 15 min (Figure 6). There were also increased expressions of phospho-ERK1/2 in cells exposed to different bile acid for 3 min (P <0.05) (Figure 7). No expression of PPARγ in normal human esophageal epithelial cells, either exposed to acid/bile acid or not, was observed.

DISCUSSION

Increased cell proliferation has been shown in reflux esophagitis and BE[6]. Episodic exposure to acid has been found to increase *in vitro* cell proliferation in organ culture of BE[7] and in Barrett's-associated adenocarcinoma cell line (SEG-1)[8]. Moreover, it was reported the exposure to GCDC for 20 min also increased cell proliferation in SEG-1 cell line[9]. These results suggest that the refluxates probably result in the development of esophageal inflammation, intestinal metaplasia and malignant

Table 1 Effect of acid and bile acid on cell cycle

	3 min		6 min		12 min	
	S (%)	A (%)	S (%)	A (%)	S (%)	A (%)
pH 4.0	32.94±1.87[a]	0.89±0.08	24.12±2.08[a]	2.89±0.11[a]	21.77±1.53[a]	3.50±0.16[a]
pH 5.0	35.51±1.93[a]	0.73±0.05	26.68±1.91[a]	1.70±0.09[a]	25.53±1.72[a]	1.98±0.09[a]
pH 6.0	33.28±2.05[a]	0.85±0.07	27.83±1.06[a]	1.56±0.15[a]	25.49±1.95[a]	1.63±0.10[a]
GC	33.12±1.51[a]	0.92±0.05	35.03±2.06[a]	0.82±0.10	36.83±1.95[a]	0.71±0.09
TC	35.44±2.11[a]	0.76±0.09	37.05±2.14[a]	0.90±0.10	36.96±2.04[a]	0.80±0.11
CA	34.89±2.56[a]	0.88±0.09	34.59±1.97[a]	0.73±0.07	37.04±1.81[a]	0.93±0.08
DCA	33.37±1.64[a]	0.81±0.07	34.21±1.98[a]	0.85±0.16	36.57±1.82[a]	0.79±0.14
Control	29.61±1.82	0.64±0.10	30.56±1.12	0.71±0.09	32.58±1.89	0.58±0.13

S: S phase; A: Apoptosis; [a]$P < 0.05$.

progression by inducing abnormal proliferation. Since there have been some problems unsettled in the primary culture of human normal esophageal epithelial cells, the previous studies were mainly limited to the cells in pathologic status. Based on the newly-established primary culture technique of human normal esophageal cells[5], we observed, probably for the first time, the effects of acid and bile acid on cell proliferation in human normal esophageal cells.

We found an acid-pulse (3 min) enhanced proliferation in normal esophageal epithelial cells, whereas continuous acid exposure blocked cell proliferation. A brief acid exposure (3 min) increased proliferation ratios at 24 h, yet longer exposure (>6 min) led to decreased cell number, which was more significant at pH<5.0. This was further confirmed by the results of cell cycle determination, a brief exposure (3 min) to acid produced an increase of cells in S phase, while longer exposure (6, 12 min) decreased the cell proportion in S phase accompanied by an increased apoptosis percentage.

The above results show that the effects of acid on esophageal cells are time-dependent, the brief exposure has pro-proliferation effect and longer exposure can induce apoptosis. Previous reports have showed the effect of acid on apoptosis, exposure to pH 3.5 for 2 d led to a decreased cell number in mouse esophageal keratinocytes[10], and normal human esophageal epithelial cells could not survive in media below pH 5.5 for 12 h[11]. Esophageal acid reflux is judged clinically by pH < 4. Since cells are cultured *in vitro* with a neutral pH of 7.2-7.4, their tolerance to acid is limited; this is mainly due to the defect of tissue organization, blood transportation and mucosal barrier *in vivo*.

To identify whether bile acid has the similar pro-proliferation effect, we exposed the normal esophageal cells to 6 conjugated bile acids and 2 unconjugated bile acids of 250 μmol/L for 3-60 min. The concentration choice was based on median bile acid concentration found in esophageal aspiration in patients with BE[12]. We found a brief bile acid exposure (3-12 min) also increased proliferation ratios at 24 h and the cell proportion in S phase, while with prolongation of exposure time to the acid and bile acids, especially DCA, the cell number decreased, suggesting that DCA is more toxic than other bile acids. Furthermore, we found that toxic effect of acid was higher compared to bile acid with the same exposure time.

In most cases, duodenogastroesophageal reflux is the mixed reflux of acid and bile. The toxic effects of bile acids are known to vary with pH. Unconjugated bile acids (pKa>4) and glycine conjugates (pKa>6) are soluble in nonionic pattern when pH > 4, whereas taurine conjugates are freely soluble even at pH 2[12]. Bile acids in nonionic pattern are easy to enter cells because of their affinity to lipid. Esophageal perfusion studies in animal models showed that unconjugated bile acids caused mucosal damage selectively in alkaline solutions, whereas taurine conjugates were toxic in acidic conditions[13]. We investigated the influence of pH on the effect of bile acid in cells, and found that compared with bile acid exposure group, the mixed exposure group had a decreased proliferation ratio, which dropped to the lowest at pH 4, yet for GC the lowest proliferation ratio was seen at pH 6.5. So, we could conclude that acid aggravates the toxic effect of bile acid, which may be due to the influence of pH on the ionization degree of taurine conjugates and glycine conjugates. In addition, it may be also related to the synthetic toxic effects of acid and bile acid, since we found that with the same exposure time (3 min), acid or bile acid exposure enhanced cell proliferation, but the mixed exposure decreased the proliferation.

Since acid and bile reflux in esophagus have been clinically shown to be intermittent and repeated, the abnormal proliferation induced by acid and bile acid may play an important role in the development of reflux-related diseases. Though there have been various points of view about the damage mechanisms of refluxates, their pro-proliferative mechanism has been seldom reported. So, we investigated the relation between ERK signaling pathway and the proliferation effects of acid and bile acid. ERK is an important signal transduction factor, unreactive in cytoplasm in normal condition, can be activated by physicochemical stimulus to enter nucleus and is implicated in cell physiological and pathological processes, such as growth, division, proliferation, apoptosis and malignant progression[14]. We found the proliferation induced by acid and bile acid in normal esophageal epithelial cells was related to the up-regulation of ERK expression, which suggest that acid and bile acid may result in esophageal inflammation, intestinal metaplasia and malignant progression by inducing ERK-mediated proliferation.

Recently some reports have suggested the implication

of PPARγ in control of tumor cell growth and its possible role in the downstream of ERK signaling pathway[3,4]. PPARγ is over-expressed in many malignant tissues, but its expression in esophagus remains controversial. Some authors reported PPARγ mRNA level was lower in esophageal squamous cell carcinoma tissues than that of normal esophageal mucosa[3]. Others found PPARγ mRNA was expressed in biopsies of both normal esophageal mucosa and BE, but expression of this protein was detected only in BE[15]. In our study, no expression of this protein was observed either in normal esophageal epithelial cells or the cells exposed to acid or bile acid.

In conclusion, we have shown, probably for the first time, that a brief exposure to acid or bile acid induces *in vitro* proliferation in normal human esophageal epithelial cells, accompanied with the up-regulation of ERK, a potential molecular pathway whereby duodenogastroesophageal reflux may contribute to the development of reflux esophagitis, BE and esophageal adenocarcinoma. Moreover, acid aggravates the toxic effect of bile acid, which supports the view that the mixed reflux facilitates damage to the esophageal mucosa.

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RAPID COMMUNICATION

Antisense angiopoietin-1 inhibits tumorigenesis and angiogenesis of gastric cancer

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Abstract

AIM: To investigate the effect of angiopoietin-1 (Ang-1) on biological behaviors *in vitro* and tumorigenesis and angiogenesis *in vitro* of human gastric cancer cells.

METHODS: Human full-length Ang-1 gene was cloned from human placental tissues by RT-PCR method. Recombinant human Ang-1 antisense eukaryotic expression vector was constructed by directional cloning, and transfected by lipofectin method into human gastric cancer line SGC7901 with high Ang-1 expression level. Inhibition efficiency was confirmed by semi- quantitive PCR and Western blot method. Cell growth curve and cell cycle were observed with MTT assays and flow cytometry, respectively. Nude mice tumorigenicity test was employed to compare *in vitro* tumorigenesis of cells with Ang-1 suppression. Microvessel density (MVD) of implanted tumor tissues was analyzed by immunohistochemistry for factor VIII staining.

RESULTS: Full-length Ang-1 gene was successfully cloned and stable transfectants were established, namely 7Ang1- for antisense, and 7901P for empty vector transfected. 7Ang1- cells showed down-regulated Ang-1 expression, while its *in vitro* proliferation and cell cycle distribution were not significantly changed. In contrast, xenograft of 7Ang1- cells in nude mice had lower volume and weight than those of 7901P after 30 days' implantation ($P < 0.01$, 293.00 ± 95.54 mg *vs*. 624.00 ± 77.78 mg) accompanied with less vessel formation with MVD 6.00±1.73 compared to 7901P group 8.44±1.33 ($P < 0.01$).

CONCLUSION: Ang-1 may play an important role in tumorigenesis and angiogenesis of gastric cancer, and targeting its expression may be beneficial for the therapy of gastric cancer.

INTRODUCTION

Angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2), two main members of angiopoietins family, are involved in both physiological and pathological angiogenesis processes. Ang-1 has been identified as a major activator of the tyrosine kinase receptor Tie2, leading to receptor autophosphorylation on binding. Ang-1 also stimulates endothelial cell migration *in vitro*[1,2]. Ang-2 is the naturally occurring antagonist to Ang-1 and inhibits Ang-1 mediated Tie2 phosphorylation; this effect leads to vessel destabilization, a necessary step in the initiation of angiogenesis[3,4].

Ang-1 and Ang-2 have been reported to be involved in several kinds of cancers, while their roles in gastric cancer progression and angiogenesis are still not fully understood. There have been several reports declaimed the effect of Ang-1 on tumorigenesis of human astrocytomas[5,6], breast cancer[7] and colorectal adenocarcinoma[8]. Previous report has identified that Ang-2 plays an important role in gastric cancer angiogenesis and progression[9,10], while little is known about the roles of Ang-1 in gastric cancer.

In the present study, we aimed to investigate the effect of Ang-1 on the biological behaviors and tumorigenesis and angiogenesis of human gastric cancer cells by modifying Ang-1 expression in SGC7901 gastric cancer cells by *in vivo* and *in vitro* examinations. Our results revealed that inhibition of Ang-1 expression would retard gastric cancer angiogenesis and progression.

MATERIALS AND METHODS

Tissue specimens and cell lines

Fresh placental tissue was obtained from the Department of Gynaecology and Obstetrics, Xijing Hospital, Xi'

an, China, with informed consent from the patients. Human gastric cancer cell line SGC7901 with high Ang-1 expression was preserved in our institute and cultured in RPMI1640 supplemented with 100 mL/L bovine serum.

Ang-1 antisense eukaryotic expression vector was conducted by RT-PCR method and directional cloning. Total RNA of fresh human placental tissue was extracted with Trizol (Life Technologies, Carlsbad, USA) according to the manufacturer's protocol. About 1 μg of total RNA was used for first strand cDNA synthesis according to the manufacturer's instructions. The full-length human Ang-1 cDNA was cloned using primer pairs: 5'-gaggggggaaagagtcaaacaaac-3' and 5'-cttgaccgtgaatctggagcc-3'. PCR parameters were 94 ℃ for 1 min, annealing at 60 ℃ for 1 min, and 72 ℃ for 2 min for 30 cycles, and the product was verified by 8 g/L gel electrophoresis. Sequence of PCR product was verified by the ABI PRISM 377 DNA Sequencer (Sangon, Shanghai, China). After PCR, the 1.9-kb fragment was cloned into the pMD18-T vector (Takara, Dalian, China) followed by *Hind*Ⅲ and *BamH* Ⅰ digestion and ligated into pcDNA3.1-V_5-His C expression vector (Invitrogen, Carlsbad, CA) named as pcDNA3.1-Ang1-. The sequence was confirmed by sequencing analysis.

Cell transfection

SGC7901 cells were plated and grown to 80% confluency without antibiotics. Stable transfections of pcDNA3.1-Ang1- and empty vector, pcDNA3.1-V_5-His C, were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as directed by the manufacturer. Transfected cells were selected with 300 mg/L G418 after 48 h. Clones were picked and expanded for an additional 2 months. Efficiency of Ang-1 suppression was confirmed by RT-PCR and Western blot methods.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was employed to compare the gene expression in different transfectants. The number of optimal replications was determined based on the linear correlations between cycle numbers and PCR products. Gene expression was presented by the relative yield of the PCR product from target sequences to that from GAPDH gene as control. PCR primers and reaction parameters were chosen according to the literature [11] and listed as follows: Ang-1, 5'-ACTGTGCAGATGTATATCAAG C-3' and 5'-GTGGAATCTGTCATACTGTGA A-3'; GAPDH, 5' -TGGGTGTGAACCATGAGAAGTA-3' and 5'-CGCTGTTGAAGTCAG AGGAGA-3'. The PCR parameters were 94℃ for 1 min, annealing at 60℃ for 1 min, and 72℃ for 1 min for 32 cycles for Ang-1 and 94℃ for 30 s, annealing at 52℃ for 30 s, and 72℃ for 45 s for 32 cycles for GAPDH.

Western blot analysis

Equal amounts of the extracted protein of cells were separated by 100 g/L SDS–PAGE; The protein bands were electro-transferred to nitrocellulose membrane. Expression of Ang-1 was analyzed using primary antibody (kindly provided by Micheal Hanner from Renegeron Pharmaceuticals, USA), followed by corresponding second antibody (Zhongshan, Beijing, China), then visualized by enhanced chemiluminescence (ECL, Amersham–Pharmacia Biotech, Beijing, China). Relative protein levels were calculated to β-actin as standard.

MTT assay

Exponentially growing cells were harvested and suspended in 96-well flat-bottomed plates (200 μL /well) (Costar). After incubation for 1, 3, 5 and 7 d, respectively, 20 μL of MTT solution (5 g/L) (Sigma, St louis, MO) was added to each well and further incubated for 4 h. One hundred and fifty μL of dimethylsulfoxide was added before absorbance at 490 nm was measured with a microplate reader BP800 (BIOHIT).

Cell cycle analysis

To evaluate whether the inhibition of Ang-1 would exert any effect on the cell cycle distribution of SGC7901 cells, we performed cytofluorimetric analysis. Briefly, cells were fixed with 70% ice-cold ethanol and stored at 4°C overnight, washed with PBS, and stained with propidium iodide (PI) (50 mg/L) for 30 min. Cell cycle histograms were generated after analysis of PI-stained cells by fluorescence activated cell sorting (FACS) with a Becton-Dickinson FACScan.

In vivo tumorigenicity

To investigate whether or not Ang-1 suppression would alter the tumorigenicity of SGC7901 cells, nude mice bred in our animal facilities were used with body weight range from 18 to 22 kilograms. Four $\times 10^6$ cells were injected subcutaneously into the right flank of nude mice. The experiment was performed on five mice in each group. The mean tumor volume was weekly measured and calculated according to the formula: $a \times (b)^2 \times 0.5$ (a = largest diameter, b = perpendicular diameter). All mice were sacrificed after 30 d and the subcutaneous tumor graft were surgically excised from the mice, fixed with formalin and paraffin-embedded 4-μm sections were prepared for immunohistochemical analysis.

Immunohistochemistry

Factor VIII staining was used to identify the microvessels in the tumor tissues by immunohistochemical method as usual. Briefly, after paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated in alcohol, sections were incubated in 3 mL/L H_2O_2 to block endogeneous peroxidase activity. Each slide was incubated with normal goat serum for 20 min at room temperature, then rabbit anti-factor VIII related antigen antibody (Dako, MedBio Ltd, New Zealand) diluted at 1∶100 was applied on sections and incubated overnight at 4℃. After incubation with biotinylated goat anti-rabbit IgG (dilution 1:200) for 30 min at 37℃, each slide was rinsed in phosphate-buffered saline and was incubated in the avidin-biotin peroxidase complex for 30 min at 37℃. The peroxidase was visualized with 3-3'-diamino- benzidinetetrahydro chloride (DAB) solution and then counterstained with hematoxylin.

MVD evaluation

MVD was assessed according to the international

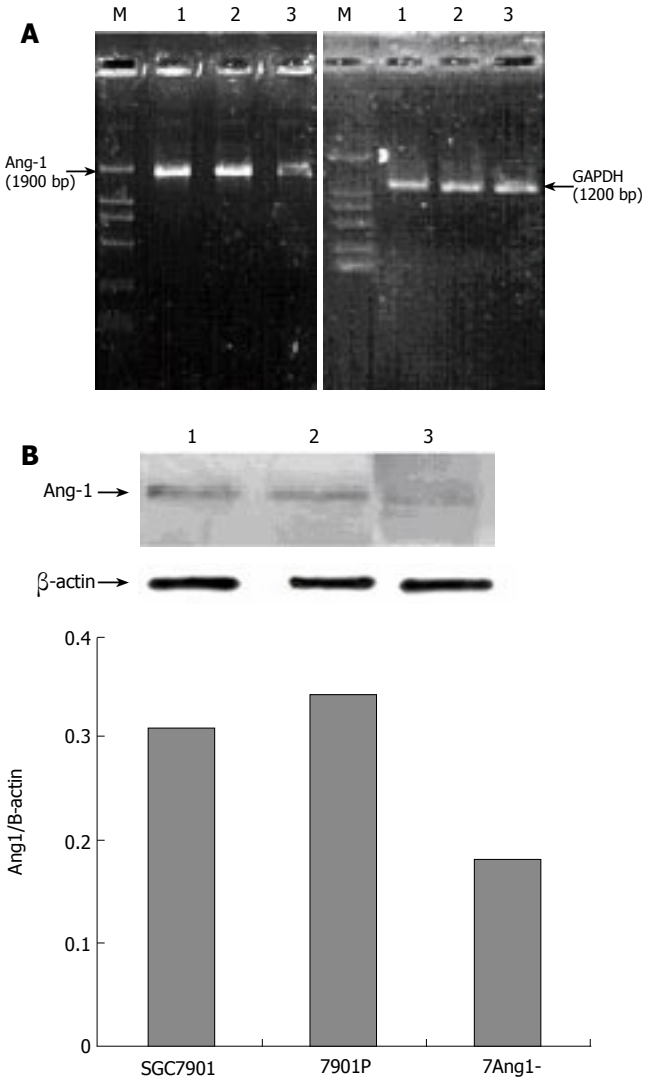

Figure 1 Verification of suppressive efficiency in parental and transfected cells. **A**: semi-quantitive RT-PCR. **B**: western blot. M: DL2000 marker; 1: SGC7901; 2: 7901P; 3: 7Ang1-.

consensus report[12]. Immuno stained slides were scanned at ×100 magnification to identify the areas with the highest number of vessels (so called "hot spot"). Counts were performed on five fields in the hot spot by two independent pathologists at ×200 magnification and the mean was taken and analyzed.

Statistical analysis

Statistical analysis was performed using SPSS software (version 10.0, SPSS Inc, Chicago). The ANOVA test was used to compare the differences between groups for MVD, tumor weight and volume. Differences were considered statistically significant at $P<0.05$.

RESULTS

Construction of Ang-1 antisense eukaryotic expression vector

Full length Ang-1 gene was amplified from placental tissues and cloned into pMD 18-T vector, then cut by by *Hind*Ⅲ and *BamH* Ⅰ digestion which would ensure inverse directional ligation into pcDNA3.1-V5-His C expression vector. The combined antisense expression vector named as pcDNA3.1-Ang1- was verified as expected by sequencing.

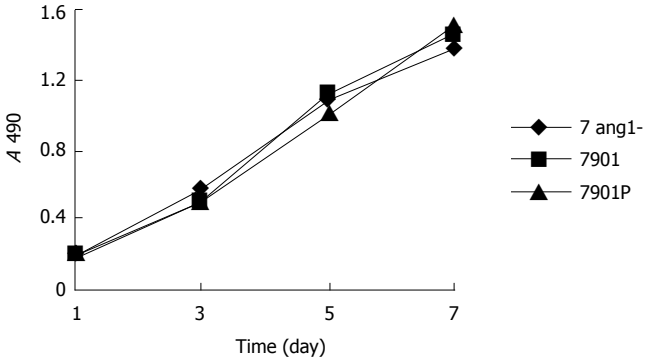

Figure 2 Proliferation of SGC7901-derived cells.

Transfectants of SGC7901-derived cells

Stable transfectants of 7901-derived cells were obtained by Lipofectamine 2000 transfection, named 7Ang1- for pcDNA3.1-Ang1- and 7901P for empty vector-transfected cells. We could clearly find that moderate targeted-inhibition effect was detected in 7Ang1- cells at both mRNA (Figure 1A) and protein (Figure 1B) levels, in contrast, control experiment with the empty vector alone showed a negligible effect on Ang-1 expression (Figure 1).

Proliferation and cell cycle of SGC7901-derived cells

To determine if interference of Ang-1 expression has an effect on the proliferation and cell cycle distribution of SGC7901 cells, MTT assay and FACS method were utilized, respectively. According to Figure 2, the proliferation of 7Ang1- cells was not significantly changed compared to that of 7901P cells. In addition, cell cycle distribution of Ang1- and 7901P were 71.9% and 59.2% for G_1 phase, 15.3% and 12.5% for G_2 phase, 25.5% and 15.6% for S phase, 0.328 and 0.281 for the proliferation index, respectively. Taken together, it suggests that interfering Ang-1 has no direct effect on *in vitro* proliferation and cell cycle of gastric cancer cells.

In vivo tumorigenicity of SGC7901-derived cells

Xenograft model was employed to compare the tumorigenicity of SGC7901 cells before and after Ang-1 inhibition. Subcutaneous tumor node of different groups became palpable almost simultaneously after 7d transplantation. Tumor from 7Ang1- cells appeared to grow slower than those from 7901P cells after 16 d (Figure 3). Finally, tumor grafts were collected and weighed after 30 d, and tumor tissues derived from 7Ang1- cells showed significantly decreased weight compared to those from 7901P cells with mean tumor graft weight (mg) being 293.0±95.5 for 7Ang1- and 624.0±77.8 for 7901P cells ($P<0.01$).

To further elucidate whether reduced angiogenesis account for the suppressed *in vivo* growth of 7Ang1- cells, MVD was assessed by immunohistochemistry. As shown in Figure 4, microvessels could easily be observed by factor

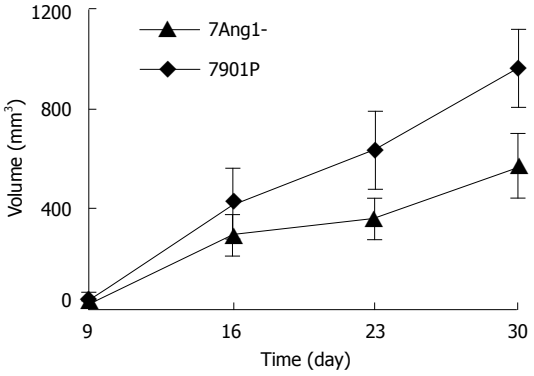

Figure 3 *In vivo* growth of tumor xenograft.

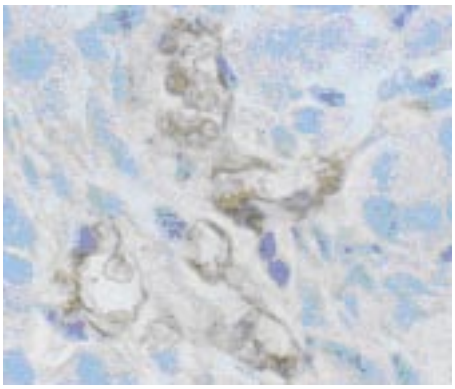

Figure 4 Factor VIII positive tumor microvessels (SABC x 200).

VIII staining. Statistics analysis showed a significantly less MVD was present in 7Ang1- group 6.0±1.7 compared to 8.4±1.3 in 7901P group ($P<0.05$). This result indicated that suppressed *in vivo* tumorigenicity of 7Ang1- cells might be mediated through reduced angiogenesis.

DISCUSSION

Gastric cancer remains a common malignancy in many countries of the world, especially in Asia, and is still among the most important causes of cancer-related death worldwide[13]. Its conventional treatment includes surgery, radiation and chemotherapy. Nowadays, increasing evidence has shown that angiogenesis is essential for the growth of solid tumor and tumor angiogenesis research has become one of the most active fields for anticancer therapies.

Several studies suggested that VEGF receptor pathway and Tie2 pathway are independent and essential mediators of angiogenesis and both play important roles in the tumor angiogenesis[14,15]. Tie2 is a novel endothelial cell-specific molecule involved in both physiological and pathological processes. Tie2 is required for normal vascular development perhaps via the regulation of vascular remodeling and endothelial cell interactions with supporting pericytes and smooth muscle cells[3]. Previous report found that inhibition of Tie2 using a kinase-deficient Tie2 construct or an adenoviral vector delivering a recombinant single-chain antibody fragment into body would inhibit the growth of human tumor xenografts[16,17].

Four ligands for the Tie2 receptor have been identified so far, named Ang-1 to -4. Among them, Ang-1 and -2 were mainly associated with tumor angiogenesis[18]. The findings from functional study of Ang-2 in several types of tumor showed that Ang-2 could stimulate tumor angiogenesis, thus promoting tumor progress[9,19,20], while those from Ang-1 showed great heterogeneity. For example, Zadeh *et al*[6] found that Ang-1 increased the vascular growth of both subcutaneous and intracranial xenografts of astrocytoma; also, Ang-1 promoted tumor angiogenesis and tumor vessel plasticity of human cervical cancer in mice[21]. In contrast, others have reported that Ang-1 could inhibit angiogenesis and growth of hepatic and colon cancer[22] and suppress breast cancer xenograft angiogenesis by blocking tumor neovasculature[7]. Taken together, it suggests that the role of Ang-1 in tumor progression may be context-dependent.

Our previous study found that Ang-1 was highly expressed in SGC7901 cells[23], while its expression in 7Ang1- cells was effectively reduced using antisense method in the present study. Further study revealed that the biological behaviors including proliferation and cell cycle of 7Ang1- cells were not significantly changed, so it seemed that interfering Ang-1 could not alter *in vitro* growth of SGC7901 cells directly; this is similar to other studies[9] in which growth of the gastric cancer cells was not affected by transfection with Ang-2.

Tumorigenicity assay showed that tumor xenograft of 7Ang1- group grew slower as their volume and weight were smaller than those from control group. MVD assessment showed significantly decreased angiogenesis in tumor from 7Ang1- group, indicating inadequate blood supply might account for suppressed *in vivo* growth. To our best knowledge, this is the first direct evidence for the role of Ang-1 in gastric cancer angiogenesis. However, in initial stage of tumor progression, two groups did not show significant difference, and until 16 d after transplantation the suppressive effect appeared dramatic when tumor grew larger. This result is consistent with our previous study in which suppressing the expression of VEGF in SGC7901 cells did not exert any significant effect on its proliferation but restricted its *in vivo* growth in nude mice[24]. Collectively, it infers that suppressing either VEGF or Ang-1 cannot prohibit the onset of tumor, but may slow *in vivo* growth of tumor cells by inhibiting angiogenesis. It was reported that Ang-2 was implicated in angiogenesis and progression of gastric carcinoma via induction of proteases such as MMP-1 and -9[9]. Combining result from our present and previous studies that significant expression of Ang-1 and Ang-2 was found in human gastric cancer tissues and cell lines[23], it infers that both Ang-1 and -2 participate in the angiogenesis and progression of gastric cancer. Previous studies indicated that Ang-1 induced the secretion of MMP-2 and small amounts of proMMP-3 and proMMP-9 in endothelial cells[25], whether or not this effect is involved in the mechanism of Ang-1 contribution to gastric cancer and progression is still unclear, however it still could be concluded from our present study that stimulating angiogenesis by Ang-1, in part at least, could promote tumor progress.

In summary, Ang-1 may contribute to the progression of gastric cancer and inhibiting its expression in gastric

cancer cells could suppress *in vivo* tumorigenicicy. It could also be a useful therapeutic target to prevent gastric cancer or inhibit its malignant progression. Tumor angiogenesis is a multi-factor associated process, and recent study showed that simultaneous phenotypic knockout of VEGF-R2 and Tie2 with an intradiabody could effectively reduce tumor growth and angiogenesis *in vivo*[26]. It suggests that combination of targeting main angiogenesis modulators including VEGF, Ang-1 and Ang-2 and their corresponding receptors can be beneficial for tumor therapeutics.

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RAPID COMMUNICATION

Increase of $CD4^+CD25^+$ T cells in $Smad3^{-/-}$ mice

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Abstract

AIM: To investigate the changes of lymphocyte subpopulations, especially $CD4^+CD25^+$ T regulatory cells in $Smad3^{-/-}$ mice.

METHODS: Hematological changes and changes of lymphocyte subpopulations were detected in $Smad3^{-/-}$ mice using cell counter and flow cytometry, respectively, and compared to their littermate controls.

RESULTS: The numbers of neutrophils and lymphocytes in peripheral blood were significantly increased in $Smad3^{-/-}$ mice compared to littermate controls. $CD19^+$ expressing cells in blood and spleen, and $CD8^+$ T cells in thymus were all markedly decreased in $Smad3^{-/-}$ mice. More important, $Smad3^{-/-}$ mice had an increased population of $CD4^+CD25^+$ T cells in peripheral lymphoid tissues, including thymus, spleen, and lymph nodes.

CONCLUSION: These observations suggest that the changes of lymphocyte subpopulations might play a role in susceptibility to inflammation of $Smad3^{-/-}$ mice.

Key words: $CD4^+CD25^+$ T cells; Lymphocyte subpopulation; SMAD3; TGF-β signaling

Wang ZB, Cui YF, Liu YQ, Jin W, Xu H, Jiang ZJ, Lu YX, Zhang Y, Liu XL, Dong B. Increase of $CD4^+CD25^+$ T cells in $Smad3^{-/-}$ Mice. *World J Gastroenterol* 2006; 12(15):2455-2458

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INTRODUCTION

TGF-β plays an important role in maintaining immune homeostasis. It signals through a set of transmembrane receptor serine/threonine kinases unique to the large superfamily of TGF-β related proteins [1]. As a downstream cytoplasm signaling element of TGF-β receptors, Smad3 mediates a positive signal pathway from the receptor serine/threonine kinases to the nuclei [2]. Previous reports revealed Smad3 plays an important role in mediating TGF-β signal in T lymphocytes and neutrophils, and demonstrated that Smad3 deficiency results in immune dysregulation and susceptibility to opportunistic infection [3].

The immune system discriminates between self and non-self, establishing and maintaining unresponsiveness to self. There is clear evidence that clonal deletion of self-reactive T and B cells is a major mechanism of self-tolerance [4]. However, the fact that potentially hazardous self-reactive lymphocytes are present in the periphery of normal adult individuals [5] reveals that the mechanisms that can prevent pathological autoimmunity exist. In recent years, a burst of papers are focused on a population of $CD4^+$ T cells that constitutively express the IL-2Rα (CD25) T cells and reveal them as key "actors" to self-tolerance[6,7]. A direct experiment to assess the regulatory role of $CD4^+CD25^+$ T cells in self-tolerance reported that the adoptive transfer of $CD4^+CD25^+$-depleted T cells could induce several organ-specific autoimmune diseases in immunodeficient animals [8]. $CD4^+CD25^+$ T cells also regulate antibody responses against self- and non-self-antigens by direct inhibitory effects on B cells or via inhibition of Th cell differentiation [9, 10]. In addition to self-tolerance and autoimmunity, there is evidence that $CD4^+CD25^+$ T cells are actively engaged in negative control of a broad spectrum of immune responses induced by microbial infection [11-13]. They can also mediate transplantation tolerance [14] and maternal tolerance to the foetus [15].

Although great progress in $CD4^+CD25^+$ T cells study has been made in recent years, many issues remain to be solved. For example, the involvement of TGF-β in $CD4^+CD25^+$ T cell immunoregulatory function is still controversial [16-19]. In the present study, we examined the changes of lymphocyte subpopulations in peripheral lymphoid tissues of $Smad3^{-/-}$ mice as well as their controls. Our results showed that $Smad3^{-/-}$ mice were associated with an increased population of $CD4^+CD25^+$ T cells, suggesting that $CD4^+CD25^+$ T cells might play a role in susceptibility to inflammation of $Smad3^{-/-}$ mice.

MATERIALS AND METHODS

Mice

$Smad3^{-/-}$ mice were generated by targeted gene disruption

in murine embryonic stem cells by homologous recombination [3]. Both Smad3$^{-/-}$ mice and their littermate controls (wild-type, Smad3$^{+/+}$) were provided by Xiao Yang (Institute of Biotechnology, Beijing, China). The mice used in these experiments were 6-8 wk of age.

Antibodies and reagents

PE-anti-CD4, FITC-anti-CD8, FITC-anti-CD3, and PE-anti-CD19 were purchased from Southern Biotechnology Associates (Birmingham, USA). FITC-anti-CD25 was purchased from Biolegend (San Diego, CA).

Analysis of leukocytes in peripheral blood

Before mice were sacrificed, approximately 20 μL blood samples were collected through tail vein, diluted, and then analyzed on Sysmex F-820 semi-automatic analyzer (Japan).

Flow cytometry of lymphocytes

Peripheral blood, thymus, spleen and lymph nodes were harvested from mice. Single-cell suspensions were subjected to hypotonic lysis of red blood cells (Becton Dickinson), washed in phosphate-buffered saline, stained with fluorescein-conjugated antibodies according to standard protocols, and then analyzed on an FACScan (Beckman Dickinson). For isolation of peripheral blood mononuclear cells (PBMC), 2 mL of heparinised peripheral blood diluted 1:1 with PBS was layered onto an equal volume of Ficoll-Hypaque density gradient solution and centrifuged at 300 r/min at room temperature. The mononuclear cells were collected, washed twice with PBS.

Statistical analysis

Difference was defined as being statistically significant when P <0.05 was obtained using Student's t test.

RESULTS

Increased numbers of neutrophils and lymphocytes in Smad3$^{-/-}$ mice

We first compared the total numbers of white blood cells and differential distributions of leukocytes in peripheral blood samples from Smad3$^{-/-}$ mice and littermate control mice. A marked increase in absolute white blood cell counts was observed in Smad3$^{-/-}$ mice (P < 0.01). Accordingly, the numbers of neutrophils and lymphocytes were also elevated in Smad3$^{-/-}$ mice compared to their controls (Figure 1). These results are consistent with a previous report that Smad3$^{-/-}$ mice exhibited invasive mucosal infection involving multiple immune organs [3].

Changes of lymphocyte subpopulations

Susceptibility of Smad3$^{-/-}$ mice to infection and tissue inflammation [3] made us wonder whether quantitative changes of lymphocytes were present in these mice. The results showed that numbers of CD19^{+}-expressing cells (most B cells) in the peripheral blood and spleen were significanty decreased in Smad3$^{-/-}$ mice compared to their controls (Figures 2A and 2C). In addition, the number of CD8^{+} T cells was also reduced in thymus in Smad3$^{-/-}$ mice (Figure 2B). Analysis of lymph nodes did not reveal any significant difference between the mutant mice and littermate controls (Figure 2D).

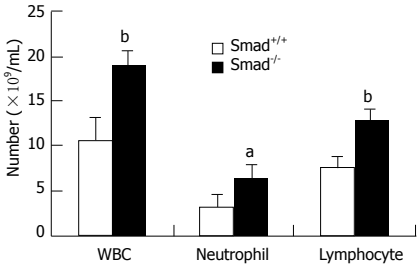

Figure 1 Total numbers and differential distributions of blood leukocytes in Smad3$^{+/+}$ and Smad3$^{-/-}$ mice. Peripheral blood samples were collected from tail veins of mice, and then analyzed on Sysmex F-820. Shown here are the means and standard deviations of total numbers and distributions of blood leukocytes from 4 wild-type and 4 mutant mice (^{a}P < 0.05, ^{b}P < 0.01).

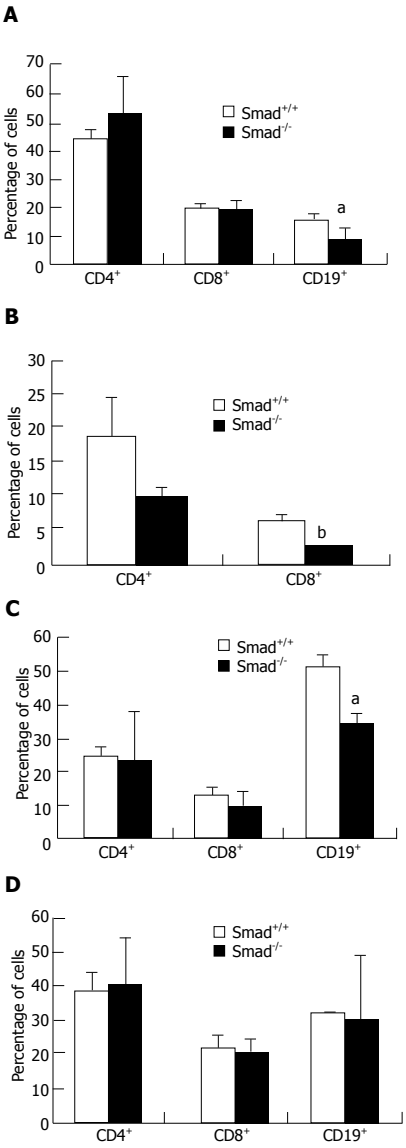

Figure 2 Percentage of lymphocyte subpopulations in peripheral blood (A), thymus (B), spleen (C) and lymph nodes (D) of wild-type and mutant mice. The cells were stained with PE-anti-CD4 and FITC-anti-CD8, or with FITC-anti-CD3 and PE-anti-CD19, and then subjected to cytometric analyses. Shown here are the means and standard deviations of percentage of lymphocyte subpopulations from 4 wild-type and 4 mutant mice (^{a}P < 0.05, ^{b}P < 0.01).

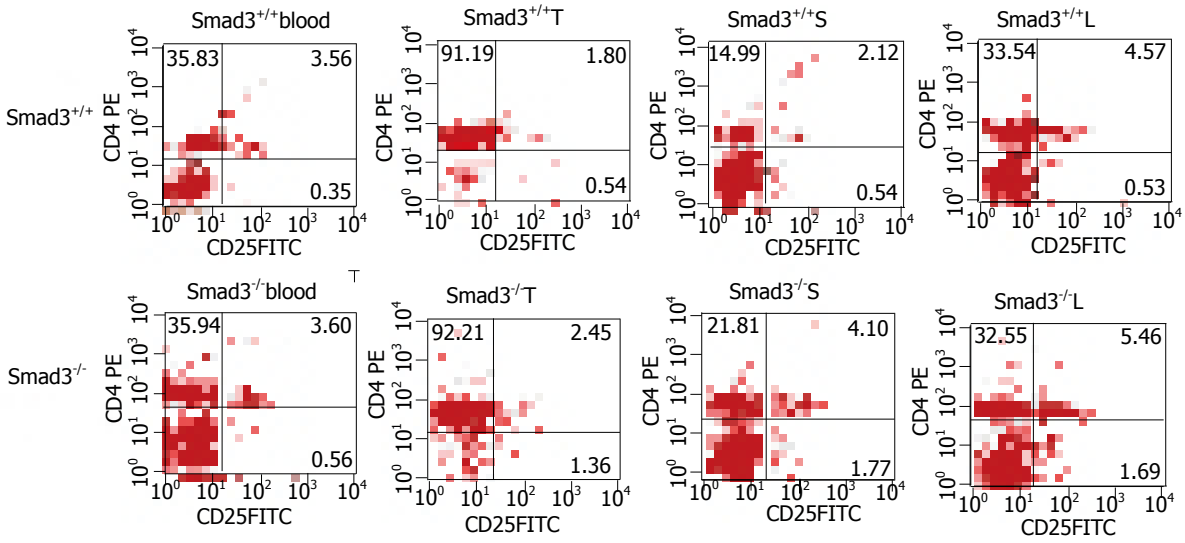

Figure 3 Percentage of CD4$^+$CD25$^+$ T cells in peripheral lymphoid tissues of Smad3$^{+/+}$ and Smad3$^{-/-}$ mice. PBMC (blood) or single-cell suspensions from thymus (T), spleen (S), and lymph nodes (L) were prepared according to "MATERIALS AND METHODS", co-labeled with PE-anti-CD4 and FITC-anti-CD25, and then analyzed by FACScan.

Increased CD4$^+$CD25$^+$ T cells in Smad3$^{-/-}$ mice

CD4$^+$CD25$^+$ T cells play an important role in maintaining the equilibrium between immunity and tolerance[20,21]. Many papers have reported that this population of cells is able to suppress proliferation and effector function of CD4$^+$ and CD8$^+$ T cells [22-24]. To explore whether the decreased lymphocytes in Smad3$^{-/-}$ mice were related to the CD4$^+$CD25$^+$ T cells, we examined this population of cells in peripheral lymphoid tissues of Smad3$^{-/-}$ mice and littermate controls. Smad3$^{-/-}$ mice exhibited a greater percentage of CD4$^+$CD25$^+$ T cells in thymus, spleen and lymph nodes, compared to controls. In peripheral blood, however, no difference was observed between mutant mice and wild type in regarding to CD4$^+$CD25$^+$ T cell proportion (Figure 3).

DISCUSSION

TGF-β is an essential endogenous regulator of T-cell function [25]. It has been recently reported that TGF-β$^{-/-}$ mice have normal numbers of CD4$^+$CD25$^+$ T cells after birth, indicating that CD4$^+$CD25$^+$ T cells are able to develop in complete absence of endogenous TGF-β expression [16, 17]. This made us think whether quantitative or functional changes of CD4$^+$CD25$^+$ T cells occurred in Smad3$^{-/-}$ mice. Our main finding in this study is that Smad3$^{-/-}$ mice had increased CD4$^+$CD25$^+$ T cells compared to their littermate controls (Figure 3). Our results showed that neutrophil and lymphocyte numbers increased (Figure 1) and that lymphocyte subpopulation decreased in the peripheral lymphoid tissues of Smad3$^{-/-}$ mice (Figure 2), which are consistent with the previous reports [3,26].

During infection, the balance between self-reactive effector T cells and regulatory T cells could determine the time of onset, the intensity and duration of autoimmune response [27]. Recent studies have focused on a population of CD4$^+$ T cells that constitutively express CD25. CD4$^+$CD25$^+$ T cells comprise 5%-10% of the peripheral CD4$^+$ T cell pool of normal mice and humans and exhibit immunosuppressive abilities both *in vitro* and *in vivo* [28, 29]. Studies of human diseases indicate that the functional CD4$^+$CD25$^+$ T cells are enriched in inflamed joints of patients with rheumatoid arthritis [30] or with the juvenile idiopathic arthritis [31]. In our study, we showed that an increased population of CD4$^+$CD25$^+$ T cells was present in Smad3$^{-/-}$ mice, which could partially account for the susceptibility to inflammation of these mutant mice. However, our results and those of a previous study [3] did not reveal any significant difference of CD4$^+$ T cells of spleen and lymph nodes between the asymptomatic mice and littermate controls. A possible explanation for the increase of CD4$^+$CD25$^+$ T cells is that they are derived from the CD4$^+$CD25$^-$ cells under the condition of Smad3 gene mutation. It is well accepted that CD4$^+$CD25$^+$ T cells can be generated by the activation of mature, peripheral CD4$^+$CD25$^-$ T cells under different stimulatory conditions [28]. Further studies should concentrate on defining the functional characteristics of the CD4$^+$CD25$^+$ T cells in Smad3$^{-/-}$ mice to gain a better insight into the mechanisms of susceptibility to inflammation.

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RAPID COMMUNICATION

Early removing gastrointestinal decompression and early oral feeding improve patients' rehabilitation after colorectostomy

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Abstract

AIM: To evaluate the feasibility, safety, and tolerance of early removing gastrointestinal decompression and early oral feeding in the patients undergoing surgery for colorectal carcinoma.

METHODS: Three hundred and sixteen patients submitted to operations associated with colorectostomy from January 2004 to September 2005 were randomized to two groups: In experimental group ($n = 161$), the nasogastric tube was removed after the operation from 12 to 24 h and was promised immediately oral feeding; In control group ($n = 155$), the nasogastric tube was maintained until the passage of flatus per rectum. Variables assessed included the time to first passage of flatus, the time to first passage of stool, the time elapsed postoperative stay, and postoperative complications such as anastomotic leakage, acute dilation of stomach, wound infection and dehiscense, fever, pulmonary infection and pharyngolaryngitis.

RESULTS: The median and average days to the first passage of flatus (3.0 ± 0.9 *vs* 3.6 ± 1.2, $P < 0.001$), the first passage of stool (4.1 ± 1.1 *vs* 4.8 ± 1.4 $P < 0.001$) and the length of postoperative stay (8.4 ± 3.4 *vs* 9.6 ± 5.0, $P < 0.05$) were shorter in the experimental group than in the control group. The postoperative complications such as anastomotic leakage (1.24% *vs* 2.58%), acute dilation of stomach (1.86% *vs* 0.06%) and wound complications (2.48% *vs* 1.94%) were similar in the groups, but fever (3.73% *vs* 9.68%, $P < 0.05$), pulmonary infection (0.62% *vs* 4.52%, $P < 0.05$) and pharyngolaryngitis (3.11% *vs* 23.23%, $P < 0.001$) were much more in the control group than in the experimental group.

CONCLUSION: The present study shows that application of gastrointestinal decompression after colorectostomy can not effectively reduce postoperative complications. On the contrary, it may increase the incidence rate of fever, pharyngolaryngitis and pulmonary infection. These strategies of early removing gastrointestinal decompression and early oral feeding in the patients undergoing colorectostomy are feasible and safe and associated with reduced postoperative discomfort and can accelerate the return of bowel function and improve rehabilitation.

Key words: Gastrointestinal decompression; Feeding; Colorectostomy

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INTRODUCTION

At present, gastrointestinal decompression after abdominal operations is still widely practiced in clinic. Routine use of nasogastric tubes after excision and anastomosis of digestive tract is aimed to hasten the return of bowel function, prevent pulmonary complications, diminish the risk of anastomotic leakage, and increase patients' comfort and shorten hospital stay. But nasal insertion of a gastric tube for prolonged gastrointestinal decompression causes extreme discomfort, and can be easily dislodged. Several complications of nasogastric intubation have been described, including sinusitis, injury to the vocal cords, and iatrogenic gastric perforation, nasal trauma, nasal hemorrhage, laryngeal injury, esophageal ulceration, gastroesophageal reflux, fluid and electrolyte imbalances, aspiration pneumonia, feeding dysfunction, and psychological problems[1-4]. Indeed, a unique randomized study by Hoffman *et al* reaffirms that patients find nasogastric tube more inconvenient and uncomfortable than gastrostomy tubes, even though gastrostomy tubes are left *in situ* for up to 4 wk[2]. Some researches have shown that the postoperative nasogastric tube could increase postoperative complications and furthermore, affect the

nutrient intake. Recent evidence seems to indicate that immediate postoperative feeding is feasible and safe after either laparoscopic surgery or laparotomy, including gastrointestinal tract surgery[5,6].

In the light of these evidence, we conducted a randomized study to evaluate the feasibility, and safety as well as the tolerance of early removing gastrointestinal decompression and early oral feeding in the patients undergoing colorectostomy for colorectal tumor in West China Hospital of Sichuan University from January 2004 to September 2005. It is hoped that this study will help to clarify strategies to accelerate postoperative recovery and to reduce patients' complications.

MATERIALS AND METHODS

Cases selection

Between January 2004 and September 2005, three hundred and sixteen consecutive patients receiving excision and anastomosis for colorectal tumor were entered the study. All patients were randomly chosen to undergo early and or late nasogastric tube removal. Nasogastric tubes were routinely placed following anesthetic induction. Patients were divided into two groups. In the experimental group, nasogastric tubes were removed within 12-24 h after the operation. The patients were provided immediately water and gradually to a liquid fiberless diet after one day, and a semi-liquid fiber diet after three days. In the control group, nasogastric tubes were removed upon report of passage of flatus by the patient, usually within three-five days after surgery.

Methods

A nasogastric tube was placed in all patients during operation. The tube was removed in control group with gastrointestinal decompression after passage of gas by intestines with continuous vacuum aspiration. The nasogastric tubes in the experimental group without gastrointestinal decompression were removed from 12 to 24 h after operation. Then, the patients were monitored prospectively, for time to first passage of flatus, time to first passage of stool, postoperative stay, and complications. Febrile morbidity was defined as two armpit temperatures greater than 38.5℃, taken at least 4 h apart, starting 24 h after operation. For the control group patients, we also recorded the time to removal of the nasogastric tube and the postoperative complications. Postoperative stay was calculated from the first postoperative day to the day of hospital discharge. Criteria for hospital discharge included: absence of nausea, vomiting, and abdominal distention; ability to tolerate oral feeding; spontaneous micturition; adequate healing, and absence of fever. Those suffering from anastomotic leaks were subjected to treatments such as anti-infective treatment, nutritional support or colostomy. Correspondingly, acute dilatation of stomach was subjected to placement of nasogastric tubes.

Statistical analysis

The χ^2 test was used for analysis of qualitative variables and Student's *t* test for continuous variables. Results of the two groups were compared using Student's *t* test. $P<0.05$ indicated significant difference. Analyses were performed using SPSS statistical software (SPSS for Windows Ver.11.5).

Table 1 Patient characteristics

General data	Experimental group (n = 161)	Control group (n = 155)
Gender(M/F)	92/69	83/72
Mean age (yr)	55.3±16.7	57.1±19.8
Range	21-78	24-85
Type of tumor		
Colon cancer	29	28
Rectal cancer	132	127
Type of operation		
Right hemicolectomy	17	15
Left hemicolectomy	12	13
Anterior rectal resection	132	127

RESULTS

General data

It was shown that there were no significant differences between two groups in terms of sex ($P>0.05$), and age ($P>0.05$) (Table 1). No significant difference was found between two groups in case distributions ($P=1.000$).

The time to first passage of flatus, stool and the length of postoperative stay and the incidence of complications after operation are shown in Table 2. The time to first passage of flatus was seen, on average, on postoperative day 3.0 in the early removing gastrointestinal decompression and early oral feeding group and on day 3.6 in the control group ($P<0.001$). The first defecation was 0.7 d sooner in the experimental group (postoperative day 4.1) than in the control group (4.8 d; $P<0.001$). The postoperative hospital stays for the experimental and control groups were 8.4±3.4 d and 9.6±5.0 d, respectively. The postoperative complications for the experimental group and control group were seen in 23 cases and 70 cases, respectively. Compared with the control group, the total incidence of complications in the experimental group was evidently higher ($P<0.001$). But the anastomotic leakage (1.24% *vs* 2.58%), acute dilation of stomach (1.86% *vs* 0.06%) and wound complications (2.48% *vs* 1.94%) were similar in the two groups. Symptoms as fever and leakage of intestinal contents were diagnosed as anastomotic leakage. Six cases suffered from the lesion in the two groups. All the leakages occurred during excision and anastomosis of lower or ultra-lower rectal tumor and healed after clinical therapy. Those who suffered from abdominal distension, emesis and succussion splash of stomach were diagnosed as acute dilatation of stomach and then subjected to gastrointestinal decompression. Two cases suffered from the wound infection and 2 cases suffered from the wound dehiscense in the experimental groups, and 3 cases suffered from wound infection but no wound dehiscense in the control group. The fever (3.73% *vs* 9.68%, $P<0.05$), pulmonary infection (0.62% *vs* 4.52%, $P<0.05$) and pharyngolaryngitis (3.11% *vs* 23.23%, $P<0.001$) were much more in the control group

Table 2 Clinical features and complications of experimental and control groups

Parameters	Experimental group	Control group (n = 161)	P value (n = 155)
Time to first passage of flatus (d)	3.0±0.9	3.6±1.2[b]	0.000
Time to first passage of stool (d)	4.1±1.1	4.8±1.4[b]	0.000
Postoperative stay (d)	8.4±3.4	9.6±5.0[a]	0.016
Total complication			
Anastomotic leakage (n%)	2(1.24)	4(2.58)	0.441
Acute dilation of stomach (n%)	3(1.86)	1(0.06)	0.623
Wound complication (n%)	4(2.48)	3(1.94)	1.000
Fever (n%)	6(3.73)	15(9.68)[a]	0.042
Pulmonary infection (n%)	1(0.62)	7(4.52)[a]	0.034
Pharyngolaryngitis (n%)	5(3.11)	36(23.2)[b]	0.000

[a]P < 0.05 *vs* experimental group; [b]P < 0.001 *vs* experimental group.

than in the experimental group. Eight cases of pulmonary infection were found in the two groups by chest X-ray and cured with anti-inflammatory therapy. Any symptom associated with throat discomfort or pain was diagnosed as pharyngolaryngitis; 23.23% of patients suffered from pharyngolaryngitis in the control group and only 3.11% in the experimental group.

DISCUSSION

There are several traditional dicta that are transmitted from generation to generation of surgeons but lack any clear scientific foundation. These include performing gastric decompression after abdominal surgery and delaying oral feeding until the resolution of ileus, as commonly observed in daily surgical practice. In China at present, 97.5% of surgeons routinely place and keep the nasogastric tube until the passage of gas through anus after excision and anastomosis of lower digestive tract, while 2.5% of surgeons discard gastrointestinal decompression 2-3 d after operation before the passage of gas through anus[7]. Indeed, the nasogastric tube can cause moderate to severe discomfort in 88%, severe discomfort in 70% of the patients and significantly delay the return of normal gastrointestinal function[8,9].

Recently, a meta-analysis shows that routine nasogastric decompression does not accomplish any of its intended goals. The analysis included 28 studies fulfilling the eligibility criteria and 4194 patients. It was found that those not having a nasogastric tube routinely inserted experienced an earlier return of bowel function, a marginal decrease in pulmonary complications, and a marginal increase in wound infection and ventral hernia. Anastomotic leakage was similar in the two groups. For this reason, the authors suggest that the routine nasogastric decompression should be abandoned in favor of selective use of the nasogastric tube[10].

We believe that the omission of routine postoperative gastrointestinal decompression may be an important first step in improving the rate of gastrointestinal recovery and shortening hospital stay. Anastomotic disruption after surgical intervention is an infrequent complication, and may lead to severe morbidity and mortality when it occurs. Of the various gastric procedures, the Roux-en-Y gastric bypass (RYGB) has one of the highest risks for anastomotic leakage. Consequently, a nasogastric tube is frequently placed when these operations are performed. But in Italy, a prospective multicenter randomized trial showed that routine placement of a nasogastric tube after Roux-en-Y oesophagojejunostomy is unnecessary in elective total gastrectomy for gastric cancer[11]. Huerta *et al* also suggest that routine placement of a nasogastric tube after RYGB is unnecessary; on the contrary, elimination of postoperative nasogastric decompression decreased postoperative fever and pulmonary problems, and improved patient comfort by decreasing sore throat and nausea[6,12]. In colorectal surgery, the average volume of gastric juice in the group with gastrointestinal decompression was 200 mL daily after operation. Because the total volume of digestive juice is 6000-10000 mL, the gastrointestinal decompression following excision and anastomosis of lower digestive tract can not reduce the pressure of gastrointestinal tract and has no obvious preventing effects on postoperative complications[7,13].

Compared with the control group, the removal of routine gastrointestinal decompression did not increase the postoperative probability of anastomotic leakage (P = 0.441), acute dilation of stomach (P = 0.623) and wound complication (P = 1.000). The data from the present study not only confirmed that placement of a nasogastric tube can be safely omitted in colorectostomy but also demonstrated that routine gastrointestinal decompression may increase the postoperative complications, such as the fever, pulmonary infection, pharyngolaryngitis.

Surgery is a traumatic procedure. The metabolic response of the body is a physiological mechanism that, according to the magnitude and duration of the event, can impact on the patient's morbidity and survival. Early enteral feeding can help to improve energy and protein intake, decrease the negative impact of the metabolic response to injury, stimulate motor, resorption, synthetic, and barrier functions of the small intestine, improve the return of gastrointestinal function and reduce the duration of postoperative ileus, and reduce the risk of serious complications[5,14,15]. Traditionally, tolerance of oral feeding is based on the passage of flatus. However, an in-depth review of the physiology of postoperative ileus suggested that such an approach is excessively conservative. It has been shown that paralysis of the small bowel is transient; the gastric paralysis lasts 24 h, and paralysis of the colon lasts 48–72 h[16]. The gastrointestinal tract motility of the patients undergoing major abdominal surgery is transiently impaired, leading to the so-called postoperative ileus (POI). It not only causes patient discomfort, but is also related to abdominal complications and worsening of the nutritional status, as well as increases length of hospital stay and costs[15].

POI is characterized by a transient impairment of bowel function and reduced motility sufficient to disrupt effective transit of intestinal contents[17-19]. It is a transient bowel dysmotility that occurs following abdominal surgery. Multiple factors are thought to contribute to the pathogenesis of POI, including physical manipulation

of the bowel, surgical stress and inflammatory mediators (including endogenous opioids), changes in electrolyte and fluid balance, neural reflex and inflammatory changes, pharmacologic agents such as inhalation anesthetics, and use of opioids for postoperative analgesia[20-22]. In animal experiments, it shows that the pathogenesis of postoperative gastric ileus induced by intestinal manipulation involves viscero-sympathetic pathways. Intestinal manipulation causes impaired gastric motility via inhibitory sympathetic efferent pathway. Feeding may improve the postoperative gastric motility[22]. Chan *et al*[23] suggest that metoclopramide not only prevents prolonged POI at an early postoperative stage, but also can be used as a safe prokinetic drug for post-operative intestinal dysmotility.

Oral intake after intestinal anastomoses has traditionally been prescribed only after the resolution of ileus. de Aguilar-Nascimento JE *et al*[24] suggest the return of oral feeding on the first postoperative day in patients submitted to intestinal anastomoses is safe, not associated with the occurrence of anastomotic dehiscence and moreover, related to a quicker resolution of ileus. A clinical trial showed that gastrografin not only facilitates early oral feeding but also reduces hospitalization after elective colorectal surgery. It can decrease bowel-wall edema and enhance bowel peristalsis[25]. Even, gum-chewing might be beneficial for short hospital stay because it can stimulate bowel motility and should be added as an adjunct treatment in postoperative care[26]. In our study, the time to first passage of flatus ($P < 0.001$), the time to first passage of stool ($P < 0.001$) and the time of postoperative stay ($P < 0.05$) were sooner in the experimental group than in the control group. Our study showed that early oral feeding can be administered safely to patients undergoing major laparotomy for colorectal carcinoma. At the same time, oral feeding can improve the return of gastrointestinal function and shorten hospital stay.

The goal of this study was to determine whether a clinical approach including early removing gastrointestinal decompression and early oral feeding is safe and shows the potential to decrease the length of postoperative stay and reduce complications after colorectostomy. Our data provide preliminary evidence that routine gastrointestinal decompression cannot reduce the pressure of gastrointestinal tract and has no obvious preventing effects upon postoperative complications. Contrary to expectations, routine gastrointestinal decompression may increase the incidence rate of fever, pulmonary infection and pharyngolaryngitis. These strategies of early removing gastrointestinal decompression and early oral feeding in the patients after colorectostomy are feasible and highly safe and are associated with reduced postoperative discomfort and can accelerate the return of bowel function and shorten postoperative stay.

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CASE REPORT

A case of colohepatic penetration by a swallowed toothbrush

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Abstract

Although foreign body ingestion is relatively common, toothbrush swallowing is rare. We report a case of a swallowed toothbrush which passed through the ileocecal valve and perforated the proximal transverse colon, then the liver. To our knowledge, this is the first case to be reported.

Key words: Toothbrush; Colohepatic penetration

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INTRODUCTION

Toothbrush ingestion is uncommon, but requires prompt medical attention. Although 80% of ingested foreign bodies pass spontaneously [1], there are no reports regarding swallowed toothbrushes passing through the pylorus [2]. Here we present an unusual case of a toothbrush swallowing which passed through the ileocecal valve and perforated the proximal transverse colon, then penetrated the liver. To our knowledge, this is the first case to be reported.

CASE REPORT

A 31 year-old man was admitted to the Surgical Department via the Emergency Room with one week history of right upper abdominal pain. He was diagnosed with schizophrenia 13 years earlier and treated at a local hospital. A physical examination revealed tenderness in the right upper quadrant and a temperature of 37 ℃. Laboratory tests showed a white blood cell count in the upper normal range, a slightly elevated C-reactive protein level (23 mg/L) and elevated aspartate aminotransferase/alanine aminotransferase levels (51/99 IU/L).

A plain abdominal radiograph showed a characteristic radiographic image of a toothbrush with parallel rows of short metallic radiodensities in the right upper quadrant (Figure 1). At that time, the patient stated he swallowed a toothbrush 1 year earlier. An abdominal computed tomography scan revealed a metallic density in the ascending colon (Figure 2A) and a low density lesion penetrating the lateral section of the liver (Figure 2B). The presumptive diagnosis was colohepatic penetration by a swallowed toothbrush and a laparotomy was performed. No abnormal ascites fluid was observed. Dense adhesions between the proximal transverse colon and the lateral section of the liver were found. When the proximal transverse colon was mobilized, the shaft of a toothbrush was observed penetrating the colon and liver (Figure 3). The toothbrush was removed and the perforated colonic opening was repaired. The extracted toothbrush was 20 cm long (Figure 4). The patient had an uneventful hospital course and was discharged eleven days after surgery.

DISCUSSION

Ingestion of a foreign body is commonly encountered in the clinic among children, adults with intellectual impairment, psychiatric illness or alcoholism, and dental prosthetic-wearing elderly subjects [1, 3]. However, toothbrush swallowing is rare, with only approximately 40 reported cases [2]. It was reported that a toothbrush shows a characteristic radiographic image with parallel rows of short metallic radiodensities due to the metallic plates that hold the bristles in place [4]. Unlike most other foreign bodies, there are no reports of swallowed toothbrushes passing spontaneously [2]. Thus, prompt intervention is required in order to avoid complications such as pressure necrosis causing gastritis, ulceration and perforation [5]. An initial extraction strategy to consider is endoscopy by a skilled technician, and the first successful performance of this procedure has been reported by Ertan *et al* [6]. If endoscopic removal is not possible and particular complications are not present, a laparoscopic approach may be an alternative to laparotomy [7].

To our knowledge, this is the first report of a swal-

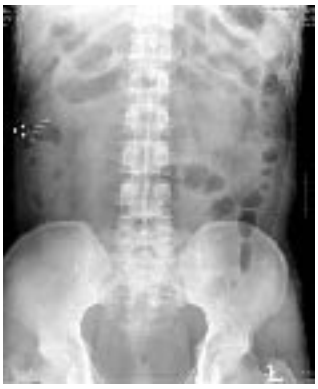

Figure 1 A plain abdominal radiograph showing a characteristic toothbrush image with parallel rows of short metallic radiodensities in the right upper quadrant (arrow).

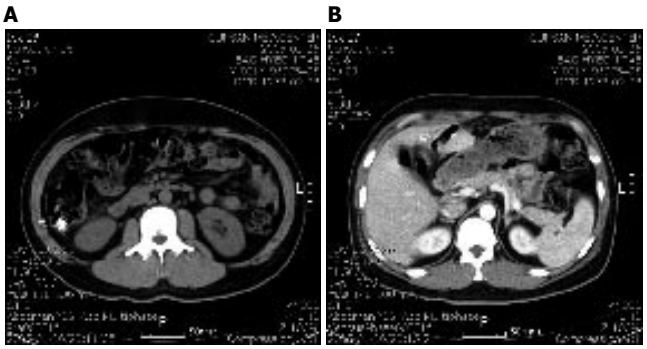

Figure 2 Abdominal computed tomography (CT) imaging. **A**: A metallic density in the ascending colon (arrow); **B**: A low density lesion penetrating the lateral section of the liver (arrow).

Figure 3 Shaft of a toothbrush penetrating the proximal transverse colon and the lateral section of the liver (arrow).

Figure 4 Extracted 20 cm toothbrush.

lowed toothbrush passing through the ileocecal valve and penetrating the colon and liver. Similar to the present case, there are reports of toothpicks penetrating the pyloroduodenal region and migrating to the liver [8]. A Medline search indicates that other similar reports involving toothbrushes are found only in the esophagus and stomach. In the present case, it is highly remarkable that a 20 cm toothbrush could pass through the pylorus and duodenal loop.

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CASE REPORT

Fulminant hepatic failure resulting from small-cell lung cancer and dramatic response of chemotherapy

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Abstract

Prompt treatment in tumor-associated encephalopathy may prolong survival. We describe a 69-year-old male patient who was presented with fulminant hepatic failure, secondary to small-cell lung carcinoma with rapidly progressing encephalopathy. Both symptoms remitted following chemotherapy, suggesting swift diagnosis and administration of chemotherapy to be effective in treatment of fulminant hepatic failure and encephalopathy.

Key words: Small-cell lung carcinoma; Fulminant hepatic failure; Chemotherapy

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INTRODUCTION

Fulminant hepatic failure (FHF) is defined as a liver failure with encephalopathy, developing within eight weeks from the onset symptoms in the absence of pre-existing liver disease. The most common causes of FHF are viral or drug-induced hepatitis[1]. Small-cell lung cancer (SCLC) manifesting as acute hepatic failure resulting from diffuse parenchymal infiltration by metastatic tumor is rare. The prognosis is poor, with death usually occurring within several days[1]. We describe herein a patient with SCLC and FHF that improved after chemotherapy.

CASE REPORT

A 69-year-old male was admitted to our hospital because of a 2-wk history of nausea, anorexia, fatigue, jaundice, and pain in the right upper quadrant of the abdomen. He had no history of blood transfusion, hepatitis, intravenous drug use, or alcohol abuse, though had an average of 2 packs of cigarettes a day for 50 years. Physical examination revealed hepatomegaly and bilateral pretibial edema, but he was alert. Laboratory analyses revealed 1760 U/L alkaline phosphatase, 6695 U/L lactate dehydrogenase (LDH), 470 U/L aspartate aminotransferase (AST), 7.6 mg/dL bilirubin, 85 μg/dL ammonia, prothrombin time level of 63%, and 680 ng/mL neuron specific enolase (NSE) levels. A computed tomography (CT) of the abdomen showed multiple lesions and massive infiltration throughout the liver parenchyma (Figure 1A). A chest roentgenogram and CT scan showed a right hilar mass with pleural effusion (Figure 1B). Bronchoscopy with transbronchial lung biopsy revealed small-cell carcinoma of the lung (Figure 2). On the seventh day of hospitalization, chemotherapy was initiated with carboplatin (area under the curve 4) administered on d 1, and 80 mg/m^2 etoposide from d 1 to 3, with cycles repeated every 3 wk.

After two cycles of chemotherapy, the patient felt symptomatically better, and a CT of the chest and abdomen revealed a marked reduction of tumor size in the primary site and liver metastasis. The laboratory analyses revealed an improvement of hepatic failure. During the 2 months of initial chemotherapy, the patient's condition deteriorated due to progression of hepatic failure (total bilirubin level, 19.5 mg/dL; LDH, 4 699 U/L; AST, 424 U/L; ammonia, 145 μg/dL; glucose, 129 mg/dL; prothrombin time, 38%), the development of ascites, and stage II encephalopathy. A CT scan of the brain revealed no evidence of cerebral metastatic disease. However, the radiological examination revealed the recurrence of SCLC. Therefore, the patient was treated with 35 mg/m^2 amrubicin from d 1 to 3, with cycles repeated every 3 wk. Though grade 4 neutropenia (NCI-CTC ver 2.0) occurred after two cycles of chemotherapy, the patient had a marked improvement of encephalopathy (stage 0), general condition, and laboratory values (LDH, 180 U/L; AST, 45 U/L; ammonia, 42 μg/dL; prothrombin time 89%; and NSE, 9.6 ng/mL). Follow-up CT imaging documented a good partial response of the liver metastases, and a complete disappearance of the right hilar mass. Then,

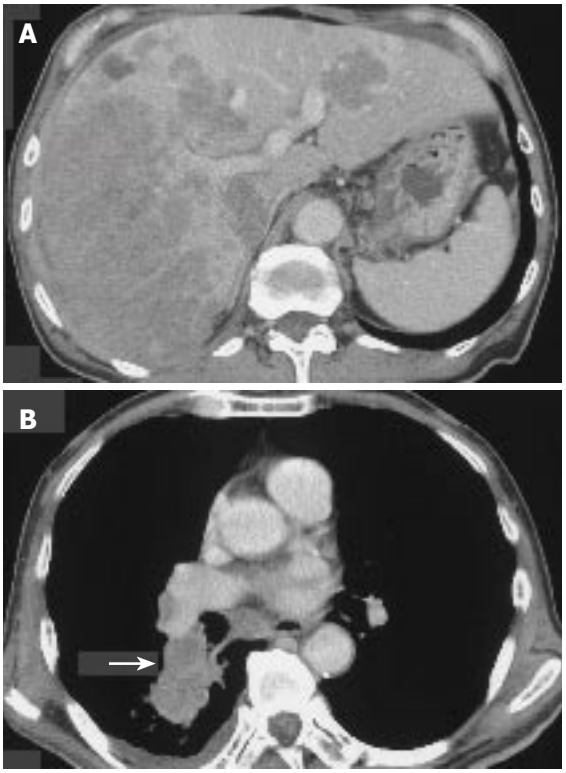

Figure 1 Computed tomography of the patient. **A**: CT of the abdomen showing multiple lesions and massively infiltrative process throughout the liver parenchyma; **B**: CT of the chest showing a right hilar mass (arrow) with pleural effusion.

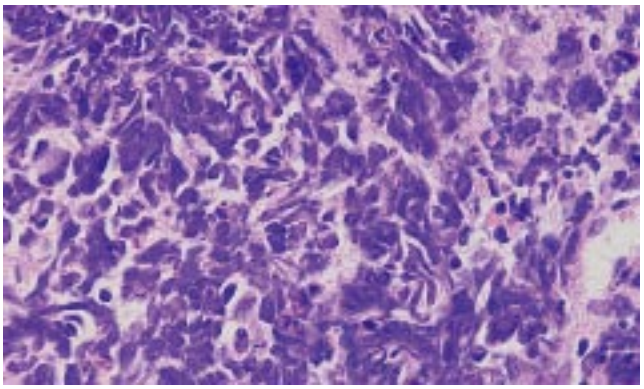

Figure 2 Histological examination of transbronchial lung biopsy specimen showing extensive infiltration small-cell carcinoma.

he was discharged from our hospital. However, the patient died of bacterial pneumonia at six months after presentation. A post-mortem was not done because of refusal of his family.

DISCUSSION

FHF secondary to metastatic SCLC is rare and results in death within days to weeks[1-3]. In literature, there are 21 previously reported cases of acute hepatic failure resulting from metastatic SCLC (Table 1). Two of the 21 reported patients presented an apparent dramatic response of severe tumor-related lactic acidosis to the initiation of chemotherapy (cyclophosphamide, doxorubicin, and vincristine)[3,4]. In most of the reported patients, however, the administration of appropriate chemotherapy was not performed, and no diagnosis was made before death. The rapid deterioration of the patient's condition may account for the difficulty of an accurate diagnosis[1]. The seven patients of acute hepatic failure with encephalopathy, even if metastatic SCLC was diagnosed before death, all died without the administration of chemotherapy because of a rapidly deteriorating course[1,5-8].

Table 1 Summary of 21 previously reported cases of acute hepatic failure due to metastatic small-cell carcinoma

References	Age (yr)	Chemotherapy	Coma	Diagnosis before death	Survival (d)
1	68	No	Yes	No	18
1	66	No	Yes	Yes	1
1	58	No	Yes	No	2
1	51	Yes	No	Yes	19
2	68	No	No	Yes	3
3	56	Yes	No	Yes	>140
4	45	Yes	No	Yes	114
5	66	No	Yes	No	3
6	85	No	Yes	No	8
7	64	No	Yes	No	6
8	46	No	Yes	No	8
12	48	No	No	No	14
12	59	No	No	No	14
12	45	No	No	No	6
13	79	No	No	No	6
14	57	No	No	No	10
14	52	No	No	No	16
15	55	Yes	No	No	5
15	57	NR[1]	NR[1]	No	NR[1]
16	46	NR[1]	NR[1]	No	9
17	65	No	No	No	20
Present case	71	Yes	Yes	Yes	>182

[1]NR: not reported.

This case presents an apparent dramatic response of tumor-related encephalopathy to the administration of chemotherapy. There was no evidence for other, more usual causes of encephalopathy. Although the combination of carboplatin and etoposide as first-line chemotherapy was effective to acute hepatic failure, it failed to exert any effect against rapid encephalopathy. However, the encephalopathy improved dramatically after the administration of amrubicin as second-line chemotherapy.

Amrubicin (SM-5887) is a totally synthetic anthracycline and a potent topoisomerase II inhibitor[9], and is one of the most active agents for SCLC. In a phase II study of amrubicin using a schedule of 45 mg/m^2 on d 1-3 every 3 wk in 33 previously untreated extensive-stage SCLC patients, an overall response rate of 76% and median survival of 11.7 mo have been observed[10]. In this case, amrubicin, as second-line chemotherapy, was effective for the improvement of encephalopathy and liver metastases. However, the efficacy of amrubicin as a salvage chemotherapy remains unknown.

Extremely high serum LDH level represents diffuse replacement of the liver parenchyma and is associated with a higher risk of developing FHF[2]. Several reports have shown correlation between serum LDH levels with survival in SCLC patients with liver metastases, and suggested an accurate relationship between elevated LDH

levels and hepatic dysfunction[1,2,7]. These findings are in agreement with the LDH levels in our patient, who was observed with elevated LDH serum levels prior to chemotherapy, then regressing to normal LDH levels after the improvement of liver metastasis.

In the reports of malignant diseases manifesting as acute hepatic failure complicated by encephalopathy, a non-Hodgkin's lymphoma patient responded to chemotherapy and survived FHF[11]. Once FHF develops secondary to diffuse liver metastases in cancer patients, prognosis is dismal and there is no effective treatment with the exception of chemotherapy. Our case, with supporting evidence from non-Hodgkin's Lymphoma[11] and 2 cases of lactic acidosis[3,4], suggests that early diagnosis and a prompt administration of appropriate chemotherapy may result in the improvement of acute hepatic failure, and thereby improving survival.

Hence, we suggest that in addition to the efficacy of amrubicin as second-line chemotherapy, early diagnosis and prompt initiation of chemotherapy may result in the improvement of FHF, and thereby improving survival.

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CASE REPORT

Bochdaleck's hernia complicating pregnancy: Case report

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Abstract

Diaphragmatic hernia complicating pregnancy is rare and results in a high mortality rate, particularly if early surgical intervention is not undertaken. We report a case in which a woman presenting at 23 wk's gestation was admitted with symptoms of respiratory failure and bowel obstruction due to incarceration of viscera through a left posterolateral defect of the diaphragm (Bochdalek's hernia). Surgery (left thoracoabdominal incision) demonstrated compression atelectasis, mediastinal shift, strangulation and gangrene of the herniated viscera which led to segmental resection of the involved portion of large intestine with re-establishment of bowel continuity by end to end anastomosis. The greater omentum was partly necrotic necessitating resection. The diaphragmatic defect was closed with interrupted sutures. Postoperative period was uncomplicated. Pregnancy was allowed to continue until 39 wk's gestation at which time elective cesarean delivery was performed. It is concluded that symptomatic maternal diaphragmatic hernia during pregnancy is a surgical emergency and requires a high index of suspicion.

Key words: Pregnancy; Diaphragmatic hernia; Incarceration

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INTRODUCTION

Diaphragmatic hernia complicating pregnancy is rare and is associated with a poor or complicated outcome, particularly if early surgical intervention is not undertaken[1]. Such hernias usually involve the left diaphragm through a congenital defect or a previous traumatic rupture. The main life-threatening complications described, include respiratory distress, visceral obstruction, strangulation and gangrene of the herniated viscera, visceral perforation (spontaneous or thoracentesis-induced) and maternal death[2-5].

A case of a patient with left-sided congenital diaphragmatic hernia during pregnancy is described in this report.

CASE REPORT

A 31-year-old woman in her first pregnancy (23 wk's gestation) was admitted to our hospital with a 10-day history of dyspnoea, nausea, persistent vomiting and intermittent sharp epigastric and substernal pain leading to a 3 kg weight loss. On physical examination the patient was afebrile, hypotensive with tachycardia (125 beats/min) and dyspnoea (32 breaths/min). There was distention of the neck veins fullness of the supraclavicular fossa. Chest auscultation demonstrated absence of breath sounds in the left hemithorax. Heart sounds were heard to the right of the sternum. Her abdomen was soft and moderately tender over epigastrium and left hypochondrium with audible bowel sounds. Laboratory findings were remarkable only for a white blood count of 13 800/mL (with 88% neutrophils). Electrocardiogram indicated sinus tachycardia and a T-wave flattening. A nasogastric tube was placed and returned 800 mL bilious fluid.

A chest X-ray suggested diaphragmatic hernia because of air bubbles, air-fluid level and non-homogeneous opacity in the left hemithorax (Figure 1). A right mediastinal shift and loss of the sharp left hemidiaphragm line separating the abdomen from thorax, were also noted.

A chest ultrasound examination demonstrated bowel loops in the left pleural cavity and slight pericardial and pleural effusions and confirmed definitely the diagnosis of diaphragmatic hernia. It is generally considered that the hernia could become manifest on a basis of anatomical weakness possibly evoked by a trauma or increasing abdominal pressure, however, the patient denied any previous trauma.

Due to her unstable condition and because of concerns for ischemia of the herniated viscera, the patient was rushed to surgery. On exploration through a left thoracoabdominal incision, a large segment of right and transverse colon, the greater omentum and the stomach were identified in the left hemithorax. Compression atelectasis, mediastinal shift, strangulation and gangrene of large part of the herniated colon were also noted (Figure 2). The

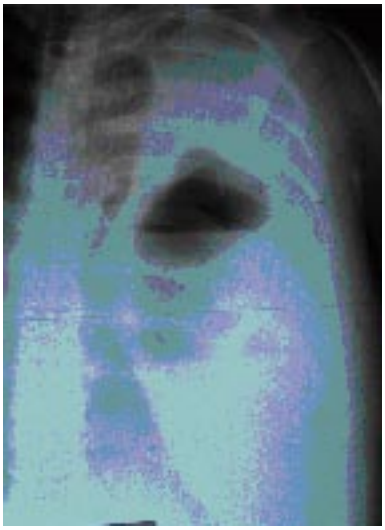

Figure 1 Chest radiograph: Herniation of abdominal viscera in the left hemithorax.

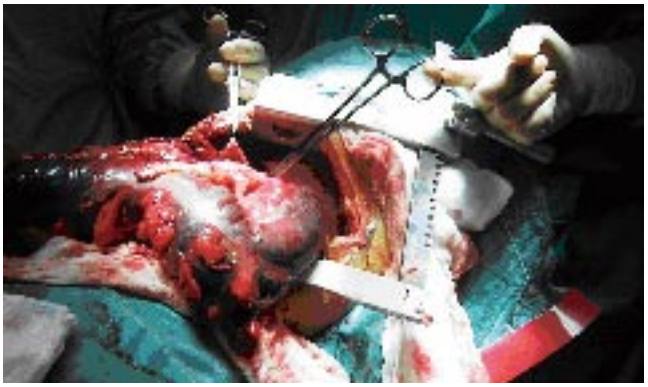

Figure 2 Gangrene of a large part of the herniated transverse colon.

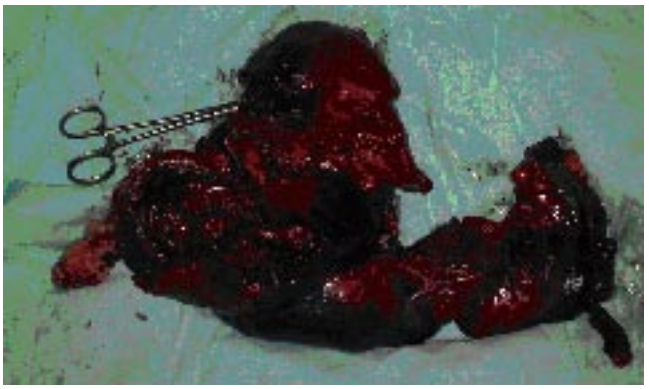

Figure 3 Large part of transverse colon resected due to strangulation and gangrene.

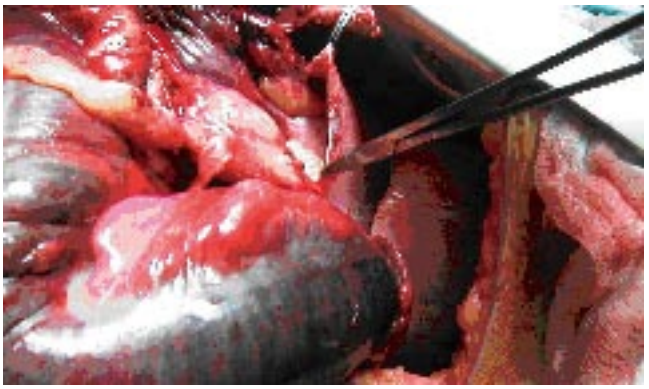

Figure 4 Bochdaleck's defect in the left hemidiaphragm.

viscera had protruded upward through a 4 cm × 7 cm posterolateral foramen of Bochdaleck diaphragmatic defect (Figure 3). The greater omentum seemed to be ischemic. A segmental resection of the involved portion of large intestine with re-establishment of bowel continuity by an end to end anastomosis was mandatory (Figure 4). Stomach was reduced in the abdominal cavity with no evidence of ischemic damage. The diaphragmatic defect was repaired with interrupted sutures. Manipulation of the uterus was avoided during surgery. Fetal well-being was confirmed throughout the procedure by continuous fetal heart rate monitoring.

After surgery, the patient recovered rapidly and her complaints ceased immediately. The postoperative course was uneventful and fetal and uterine activities were monitored daily. The patient was discharged home on postoperative day 10 after she tolerated a regular diet.

Pregnancy was allowed to continue until 39 wk's gestation at which time elective cesarean delivery was performed.

DISCUSSION

The diaphragm develops during the first 8 wk of gestation. In the eighth week this developing diaphragm leaves two channels located posterolaterally (Bochdaleck) and anteriorly (Morgagni) between the thorax and the abdomen. These two channels are closed by a two-layer membrane derived from pleura and peritoneum. When the posterolateral pleuroperitoneal channels fail to close, these are called foramina of Bochdaleck. Usually, it is rare for a defect like this to remain undetected in childhood through to adult life[6]. When the defect becomes manifest during pregnancy life-threatening events may affect both the mother and fetus. This is due to incarceration or strangulation of intraabdominal structures.

There are various symptoms in patients with congenital diaphragmatic hernia. Most of them are due to the effects of abdominal viscera within the pleural cavity and the commonest are chest pain and dyspnoea. Diminished breath sounds on the ipsilateral side are the most common physical finding. There may be signs of high or low mechanical ileus depending on which part of the gastrointestinal tract is herniated. In our patient, intraabdominal viscera herniated into the chest and caused dyspnea due to striking collapse of the left lung. Tachycardia, hypotension and distention of neck veins were the result of mediastinal shift and diminished venous return. Persistent vomitus was a sign of obstruction. However heartburn, nausea, vomiting and malaise are common symptoms during pregnancy. The failure of antacids, antispasmodics and dietary changes to relieve the symptoms especially in women with advanced pregnancy should lead the physician to suspect gastrointestinal pathology[7].

The diagnosis of a congenital diaphragmatic hernia during pregnancy requires a high index of suspicion. As in our case, the key to diagnosis is the chest radiograph. Thoracic ultrasonography, computed tomography scans and magnetic resonance imaging are possible auxilliary diagnostic methods. Pleural effusion or pneumothorax may be mimicked, leading to inappropriate thoracentesis or tube thoracostomy and inadvertent perforation of the herniated viscera[8].

The management of a pregnant patient with symptomatic diaphragmatic hernia is challenging. Traditionally, immediate repair is undertaken if symptoms arise, because further delay might be fatal both to the mother and fetus[5,6] . For asymptomatic patients Kurzel and Naunheim recommended cesarean delivery after fetal lung maturity is documented, with simultaneous hernia repair[6]. They opposed vaginal delivery under any circumstance because of the increased risk of incarceration with subsequent strangulation when the patient is bearing down. They based their recommendation on 17 cases reported in the English literature. In that case series the maternal and fetal morbidity was as high as 55% and 27% respectively, when vaginal delivery was attempted before the diaphragmatic hernia was repaired[6]. Genc *et al* suggested that gastric decompression might improve the clinical condition of the pregnant patient with a diaphragmatic hernia who presents with symptoms and signs of obstruction[9]. Such an improvement can allow surgery to be delayed until the patient is transferred to a tertiary care center or until antenatal corticosteroids are administered. Given this fact, Genc *et al* suggested observation and proposed immediate surgery whenever there is any suspicion of visceral incarceration. In our case, symptoms of cardiorespiratory failure and bowel obstruction led us to urgent repair because further delay could be fatal for both mother and fetus. Pregnancy was allowed to continue until 39 wk's gestation at which time elective cesarean delivery was performed.

The incidence of asymptomatic Bochdaleck's hernia in the adult population was reported to be at least 0.17%, with a female-to-male ratio of 17:5[10]. Despite such a high incidence, the number of pregnancies complicated by unrecognized congenital diaphragmatic hernia is extremely small - 35 cases, including this one, have been reported since 1928[5,9].

In conclusion, the diagnosis of congenital diaphragmatic hernia complicating pregnancy requires a high index of suspicion. Symptoms like abdominal pain, nausea, vomiting and dyspnoea should be investigated adequately. Signs of bowel obstruction and respiratory compromise are indications for a surgical emergency.

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Meetings

MAJOR MEETINGS COMING UP

Digestive Disease Week
107th Annual of AGA, The American Gastroenterology Association
20–25 May 2006
Loas Angeles Converntion Center, California

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24–25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3–8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26–30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14–16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1–5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4–5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6–7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

ILTS 12th Annual International Congress
3–6 May 2006
Milan
ILTS
www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006–1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15–18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006–1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6–8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

7th World Congress of the International Hepato-Pancreato-Biliary Association
3–7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

Society of American Gastrointestinal Endoscopic Surgeons
26–29 April 2006
Dallas – TX
www.sages.org

Digestive Disease Week 2006
20–25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25–26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3–7 June 2006
Seatlle - Washington
www.fascrs.org

EVENTS AND MEETINGS IN 2006

10th World Congress of the International Society for Diseases of the Esophagus
22–25 February 2006
Adelaide
isde@sapmea.asn.au
www.isde.net

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24–25 March 2006
Sydney - NSW
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symposia@falkfoundation.de

10th International Congress of Obesity
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www.ico2006.com

Easl 2006 - the 41st annual
26–30 April 2006
Vienna, Austria
Kenes International

VII Brazilian Digestive Disease Week
19–23 November 2006
www.gastro2006.com.br

International Gastrointestinal Fellows Initiative
22–24 February 2006
Banff, Alberta
Canadian Association of Gastroenterology
cagoffice@cag-acg.org
www.cag-acg.org

Canadian Digestive Disease Week
24–27 February 2006
Banff, Alberta
Digestive Disease Week Administration
cagoffice@cag-acg.org
www.cag-acg.org

Prague Hepatology 2006
14–16 September 2006
Prague
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12th International Symposium on Viral Hepatitis and Liver Disease
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Falk Seminar: XI Gastroenterology Seminar Week
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European Multidisciplinary Colorectal Cancer Congress 2006
12–14 February 2006
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Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
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Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6–7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

14th United European Gastroenterology Week
21–25 October 2006
Berlin
United European Gastroenterology Federation
www.uegw2006.de

World Congress on Controversies in Obesity, Diabetes and Hypertension
25–28 October 2006
Berlin
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codhy@codhy.com
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Asia Pacific Obesity Conclave
1–5 March 2006
New Delhi
info@apoc06.com
www.apoc06.com/

ILTS 12th Annual International Congress
3–6 May 2006
Milan
ILTS
www.ilts.org

XXX Panamerican Congress of Gastroenterology
11–16 November 2006
Cancun
www.panamericano2006.org.mx

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22 July 2006–1 August 2006
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15–18 March 2006
Rio Grande
Office of Continuing Medical Education
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Hepatitis 2006
25 February 2006–5 March 2006
Dakar
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mangosee.com/mangosteen/hepatitis2006/hepatitis2006.htm

World Congress on Gastrointestinal Cancer
28 June 2006–1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6–8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

5th International Congress of The African Middle East Association of Gastroenterology
24–26 February 2006
Sharjah
InfoMed Events
infoevent@infomedweb.com
www.infomedweb.com

7th World Congress of the International Hepato-Pancreato-Biliary Association
3–7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

13th International Symposium on Pancreatic & Biliary Endoscopy
20–23 January 2006
Los Angeles - CA
laner@cshs.org

2006 Gastrointestinal Cancers Symposium
26–28 January 2006
San Francisco - CA
Gastrointestinal Cancers Symposium Registration Center
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Digestive Disease Week 2006
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71st ACG Annual Scientific and Postgraduate Course
20–25 October 2006
Venetian Hotel, Las Vegas, Neveda
The American College of Gastroenterology

AASLD 57th Annual – The Liver Meeting™
27–31 October 2006
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AASLD

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13–16 December 2006
New York
www.nysge.org

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Barcelona
Imedex
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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 Xiaohua Zazhi* 1999; 7: 285-287

Books and other monographs (list all authors)

4 **Sherlock S,** Dooley J. Diseases of the liver and billiary 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

Electronic journal (list all authors)

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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World Journal of Gastroenterology ISSN 1007-9327

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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
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6	2 hs	2 h	After Arabic numerals
7	hr, hrs,	h	After Arabic numerals
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10	Ten yr	Ten years	At the beginning of a sentence
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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
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22	10 μM	10 μmol/L	
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26	15 year experience	15- year experience	
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29	6900	6 900	
30	1000 rpm	1 000 r/min	
31	sec	s	After Arabic numerals
32	1 pg $\cdot L^{-1}$	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
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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
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43	One g/L	One microgram per liter	At the beginning of a sentence
44	*A*260 nm	A_{260} nm	*A* should be in italic.
	^{b}P<0.05	^{a}P<0.05	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 there 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: ^{e}P<0.05, ^{f}P<0.01.
45	**F*=9.87, $^{\S}F$=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	$10\times10\times10cm^3$	10 cm×10 cm×10 cm	
66	N·km	KN·m	moment
67	$x\pm s$	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 *et al.*	
72	EcoRI	*Eco*RI	*Eco* in italic and RI in positive. Restriction endonuclease has its prescript form of writing.
73	Ecoli	*E.coli*	Bacteria and other biologic terms have their specific expression.
74	Hp	*H pylori*	
75	Iga	*Iga*	writing form of genes
76	*igA*	IgA	writing form of proteins
77	-70 kDa	~70 ku	

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EDITORIAL

Multimodal pain stimulation of the gastrointestinal tract

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Abstract

Understanding and characterization of pain and other sensory symptoms are among the most important issues in the diagnosis and assessment of patient with gastrointestinal disorders. Methods to evoke and assess experimental pain have recently developed into a new area with the possibility for multimodal stimulation (e.g., electrical, mechanical, thermal and chemical stimulation) of different nerves and pain pathways in the human gut. Such methods mimic to a high degree the pain experienced in the clinic. Multimodal pain methods have increased our basic understanding of different peripheral receptors in the gut in health and disease. Together with advanced muscle analysis, the methods have increased our understanding of receptors sensitive to mechanical, chemical and temperature stimuli in diseases, such as systemic sclerosis and diabetes. The methods can also be used to unravel central pain mechanisms, such as those involved in allodynia, hyperalgesia and referred pain. Abnormalities in central pain mechanisms are often seen in patients with chronic gut pain and hence methods relying on multimodal pain stimulation may help to understand the symptoms in these patients. Sex differences have been observed in several diseases of the gut, and differences in central pain processing between males and females have been hypothesized using multimodal pain stimulations. Finally, multimodal methods have recently been used to gain more insight into the effect of drugs against pain in the GI tract. Hence, the multimodal methods undoubtedly represents a major step forward in the future characterization and treatment of patients with various diseases of the gut.

Key words: Pain; Gut; Experimental; Allodynia; Hyperalgesia; Neurophysiology

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INTRODUCTION

Abdominal pain is very common in the general population[1], and pain is the most prevalent symptom in the gastroenterological clinic[2]. Consequently, characterization of gut pain is one of the most important issues in the diagnosis and assessment of organ dysfunction. However, in clinical practice, the different symptoms of the underlying diseases confound the characterization of pain. These confounders may include complaints relating to psychological, cognitive and social aspects of the illness, as well as systemic reactions, such as fever and general malaise[3]. Furthermore, treatment with analgesics often causes sedation and other side effects. This will invariably bias the clinical evaluation of the pain-related symptoms. Hence, the patients tend to interpret other effects of the medication, e.g. an effect on the anxiety and depression relating to the disease, as a relief of pain[4]. Because of these confounding factors experimental pain models are often advantageous. Using these models, the investigator can control the experimentally induced pain (including the nature, localization, intensity, frequency and duration of the stimulus), and provide quantitative measures of the psychophysical, behavioral or the neurophysiological responses[3,5,6].

Experimental models have been used in different animal species. Here the investigators can study the neuronal activity in anesthetized or spinalized animals directly with invasive techniques or with assessment of behavior[7]. However, the neurobiology of the pain system differs between the animal species. This limits to a high degree the interpolation of findings from animal studies to man. Pain is the net effect of complex multidimensional mechanisms including intensity coding, affective, behavioral and cognitive components that involve most parts of the central nervous system. Furthermore, in humans, pain is closely related to linguistic terms and expressions. Thus, it is a complex sensory experience which is difficult to quantify with simple neurophysiological and/or behavioral methods. Therefore, animal experiments can only to some degree reflect the experience of clinical pain in humans and the interest in human experimental pain studies has increased rapidly during the last decade[3,8].

The primary advantages of experimental pain

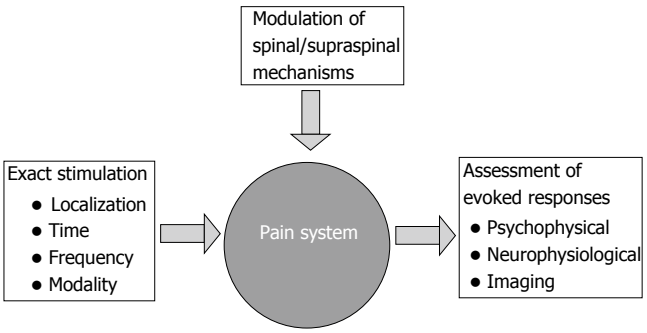

Figure 1 The concept for experimental induction, assessment and modulation of experimental gut pain in man.

approaches are that the stimulus can be controlled, delivered repeatedly and modulated, and that the responses can be assessed quantitatively with psychophysical and/or neurophysiological methods (Figure 1). Depending on the experimental model, different central mechanisms and conditions mimicking pathological pain can be studied. These are increased sensation to normal physiologic/non-painful and painful stimuli (allodynia and hyperalgesia, respectively). Experimental approaches can be applied in the laboratory for basic studies in healthy subjects and in patient groups, or used for preliminary screening of drug efficacy[9]. The methods can also be used in the clinic to characterize patients with sensory dysfunction and pain in organic and functional diseases[5,10-12]. The methods have been widely used in the skin and muscle[6]. However, due to the difficulties with access to the organs in the gastrointestinal (GI) tract, experimental pain testing is much more difficult than stimulation of the skin. The risk of perforation and other complications also limit the possibilities. Thus, most previous studies have relied on relative simple mechanical or electrical stimuli. These methods are easy to apply, but unless advanced modeling is used they have several limitations[3]. Most importantly, as pain is a multidimensional perception, it is obvious that the reaction to a single stimulus of a given modality can represent only a limited fraction of the entire pain experience. The possibility for combining different methods to stimulate the gut and evoke hyperalgesia will approximate the clinical situation, and give more comprehensive and differentiated information about the nociceptive system[3]. Multimodal models have clearly shown their value in testing of analgesics where a single stimulus has been inadequate assessing effects of specific drugs. Hence, Enggaard *et al*[13] showed that tricyclic antidepressants, which are valuable in treatment of functional pain disorders of the gut, increased the pain threshold to electrical stimuli, but did not reduce cold pressor pain. More sophisticated methods using a vide battery of tests will therefore be able to select the best test procedures to explore different basic aspects of pain as well as pharmacological modulations[9].

In this review, we outline the recent developments into test systems allowing standardized multimodal stimulations of the GI tract and their applications.

The rationale for multimodal stimulations of the gastrointestinal tract

The ideal experimental stimulus to elicit gut pain in man should be natural, minimally invasive, reliable in test-retest experiments and quantifiable[14]. The response to the stimulus should increase with increasing stimulus intensity and preferably the pain should mimic the observations in diseased organs by evoking phenomena, such as allodynia and hyperalgesia[8]. The different methods for pain stimulation of the human GI tract are electrical, mechanical, chemical, thermal and ischemic stimulation; further detailed informations regarding the advantages and shortcomings of these methods are explained elsewhere[3]. The ischemic stimuli are difficult to quantify in man and is normally not used as a direct stimulus. One of the major limitations of the different models is that they may not mimic clinical pain. Hence, they are relative short-lasting without the inflammation and subsequent activation of the many peripheral and central nervous mechanisms that are typically activated during diseases. Therefore, the basic neurobiological mechanisms in clinical pain may be different from those relating to an experimental stimulus[5,10]. For comprehensive experimental studies mimicking the clinical situation, a multimodal testing approach must therefore be used. A test battery where multimodal stimuli are used will increase the probability for activation of a range of relevant nervous mechanisms. Especially, if the stimulation is relatively long-lasting and includes modalities known to evoke peripheral as well as central sensitization, the likelihood that the model will mimic clinical pain is high despite the non-harmful nature of the stimulation.

In the GI tract, technical limitations of the currently available models have until now made a multimodal stimulation approach difficult. Some authors have combined mechanical and electrical stimuli[15,16] or used electrical stimuli combined with sensitization to acid[17]. The Center for Visceral Biomechanics and Pain in our department has recently introduced a multimodal pain model where mechanical, electrical, cold and warmth stimuli were combined. A summary of the findings in healthy subjects and patients is shown in Tables 1 and 2. Table 2 should be interpreted with caution as many data are still unpublished. In the multimodal model, mechanical stimulation is achieved with bag distension. Quantifications of the bag pressure and cross-sectional area are typically done by means of impedance planimetry or ultrasonography. Thermal stimulation is achieved by re-circulating fluid inside the bag with concomitant measurement of temperature. Chemical and electrical stimulation is done using side-holes placed proximal to the bag and by electrodes on the outside of the bag[18]. Sensitization with acid was added to the protocol to evoke allodynia and hyperalgesia together with increased referred pain areas (Figure 2)[19]. The multimodal approach gives the possibility for a differentiated stimulation of receptors in the superficial and deep layers of the gut. The possibility for induction of hyperalgesia and evoking central phenomena such as summation, allodynia and referred

Table 1 Multimodal comparison of clinical experimental data obtained in the esophagus from healthy volunteers

Group	Mechanical stimuli	Heat stimuli	Cold stimuli	Electrical stimuli	Sensitization with acid
Basic data	Differences between the sensations and referred pain areas was evoked by the stimulus modalities[18]. Reliability demonstrated[19,60] The sensation to mechanical stimulations was unaffected by relaxation of the smooth muscle[40,61]. Evidence for low and high threshold mechanoreceptors[22].	Reliability demonstrated. Stimulus-response functions obtained[18,19,60].	Reliability demonstrated. Stimulus-response functions obtained[18,19,60].	Reliability demonstrated. Stimulus-response functions obtained[18,19,60].	Allodynia and hyperalgesia evoked[19,26], although not consistent for mechanical stimuli (see text)[26,41]. Increased referred pain and amplitude of the nociceptive reflex indicating central hyperexcitability[19,26,46]. Acid perfusion sensitizes the oesophagus to heat but not cold, indicating sensitization of peripheral TRPV1 receptors[28,46]. Remote hyperalgesia was seen in the rectum after acid perfusion of the esophagus[20]. Hyperreactivity of contractions in esophagus, but tone was unaffected[26,46].
Gender differences	Males were more sensitive to stimulations, but an increased referred pain area was seen in females, reflecting sex differences in central pain processing[27,46].	No differences in sensation, but the referred area was larger in females[27,46].	As heat stimuli	Males less sensitive to single and repeated stimuli (Staahl et al., unpublished).	In females, the referred pain area increased to heat after acid sensitization, but no changes were seen to mechanical and cold stimulations[46].
Pharmacologic modulation	Oxycodone was better than morphine (and placebo) in attenuating mechanical pain[59].	Oxycodone was better than morphine (and placebo) in attenuating heat pain[59].	Not done	Both morphine and oxycodone attenuated the electrically evoked pain, but there were no differences between the opioids[59].	Not done

pain makes the models clinically relevant with respect to increasing the knowledge about peripheral and central pain mechanisms. The model have mostly been used in the esophagus, but recently also in the duodenum[20]. The pain assessment should ideally also be multimodal and, for example, include quantitative and qualitative sensations, assessment of referred pain and neurophysiological measurements[19].

Multimodal stimulation and peripheral receptors

The multimodal approach has given valuable information about the receptors in the gut wall. Theoretically, the thermal stimulation activates preferentially the receptors in the mucosa, the electrical stimulation penetrates into deeper layers of the gut and the mechanical stimulations affect predominantly receptors in the muscle layers (Figure 3).

It is generally believed that most visceral afferents are polymodal and respond to a wide range of stimuli including thermal stimuli[21]. However, sub-populations of these receptors exist and recently, we combined controlled distension with statistical modeling to demonstrate the existence of low and high threshold mechano-receptors in the human esophagus[22]. Most data on the sensation to thermal stimuli of the human viscera relate to few and relatively old studies[23-25], although some new studies have recently been published[18,19,26-29]. These studies point towards the existence of sensory pathways for thermal stimuli in the human GI tract. The thermal energy spreads from the superficial layers into the deeper layers of the gut depending on the temperature and conductance of the tissue[4]. However, as thermal stimuli are rather short-lasting in man mainly receptors in the mucosa are thought to be activated. Chemical stimulation with acid or capsaicin also activates receptors in the mucosa. Pedersen *et al*[28] used a multimodal approach to combine acid and heat stimuli of the esophagus. In this study, sensitization with acid resulted in a significant increase in the sensation to heat stimuli. The TRPV1 receptor is a polymodal detector of potential harmful stimuli, including noxious heat and protons[30]. Hence, it was suggested that TRPV1 receptors (or receptors with the same characteristics) were sensitized with acid and

Table 2 Multimodal comparison of clinical experimental data obtained in the esophagus from patient with different GI diseases

Patient group	Mechanical stimuli	Heat stimuli	Cold stimuli	Electrical stimuli	Sensitization with acid
Non-cardiac chest pain[41]	No differences to single stimuli, but increased pain to repeated stimuli and increased referred pain area, reflecting central hyperexcitability.	Not done	Not done	Not done	Increased sensation to mechanical stimulations after acid in patients only.
Esophagitis[31]	Patients were hyposensitive but with larger and more widespread referred pain. The distension induced more reactive contraction.	Patients were hypersensitive probably via increased activation of TRPV1 receptors.	No differences	Not done	Not done
Non-erosive reflux disease (Reddy *et al* unpublished data)	Patients were hyposensitive to mechanical stimuli. The distensions induced more reactive contractions in the esophagus in the patients and they had larger referred pain areas. Patients with pathological 24-h pH-measurement were more hyposensitive than the patients with normal pH profile.	The patients were hypersensitive to heat with increased referred pain areas to this modality.	No differences between patients and controls	Not done	Patients had a higher sensitivity score to acid perfusion.
Diabetes (Frøkjær *et al*, unpublished data)	Patients had hyposensitivity to distension, but increased referred pain areas, reflecting peripheral neuropathy and central hyperexcitability. Increased stiffness of the gut wall in diabetes.	As mechanical stimulations	Not done	As mechanical stimulations	Not done
Chronic pancreatitis (Dinmcevski *et al*, unpublished data)	No differences in sensation. No differentiated effect on morphine and oxycodone in attenuation of mechanical pain.	No differences in sensation Oxycodone attenuated heat pain better than morphine.	Not done	Larger referred pain area in the patients. Opioids were not better than placebo in attenuating electrical pain	Not done

subsequently resulted in increased firing of the afferents to heat stimulation. The role of this receptorsystem in the clinic was addressed in another study where selective hyperalgesia to heat but not cold stimuli was found in patients with reflux and grade B esophagitis[31]. The evidence for receptor-specific activation pattern in the experimental studies was supported in a recent study where the TRPV1 receptor was demonstrated in the human esophagus, and especially the receptor was up-regulated in esophagitis[32]. Thus, the multimodal approach gave valuable quantitative *in vivo* information about the receptor characteristics and pain mechanisms in healthy subjects as well as in patients with acid-evoked inflammation.

Multimodal stimulation and primary afferents

Electrical stimuli bypass the receptors and although all fiber populations (nociceptive as well as fibers mediating physiologic/non-nociceptive sensations) are excited by electrical stimuli, the relative proportion of activation depends on the stimulus intensity. With normal bipolar electrical stimulation, the current is believed first to activate receptors in the mucosa and submucosa, whereas the deeper layers are activated with increasing current[3]. The depth of activation also depends on the stimulation method and frequency[4]. In the gut, electrical stimulation is thought preferentially to activate thinly myelinated (Aδ) fibers[33]. Chemical and thermal stimuli, on the other hand, activate mainly non-myelinated (C) fibers and the terminals of mechanosensitive fibers are mainly localized in the muscle layers or have intraganglionic nerve endings[34,35]. Roughly speaking, the different modalities may therefore activate different fiber populations and, for example, may thermal and mechanical stimulation activate fibers in the mucosa and muscle layers, respectively. However, the difference between the stimulation paradigms may be of minor importance as Hobson *et al*[36] showed that the evoked brain potentials to mechanical and electrical stimulation of the gut were similar, reflecting that the same pathways were activated. The mechanical stimulation protocol may also be important. Hence, in the human

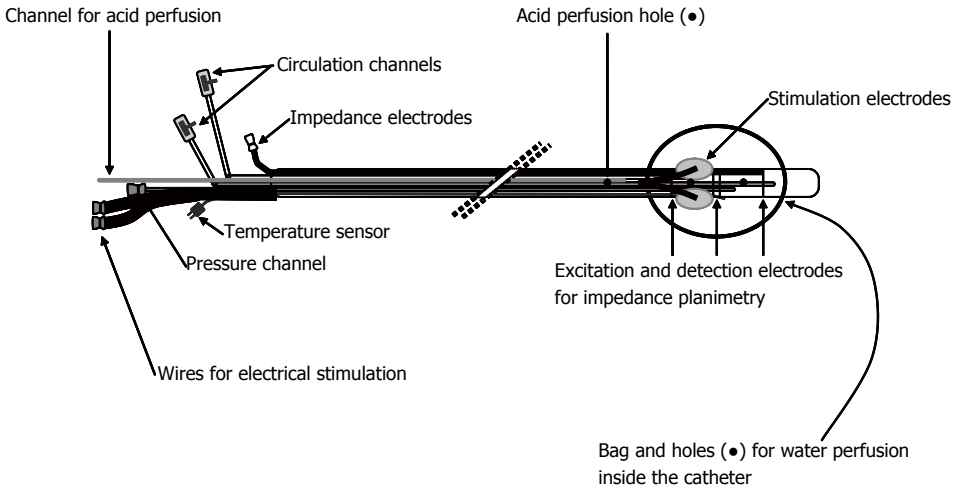

Figure 2 Schematic illustration of the probe used for multi-modal (electrical, mechanical, cold and warmth stimuli) of the esophagus.

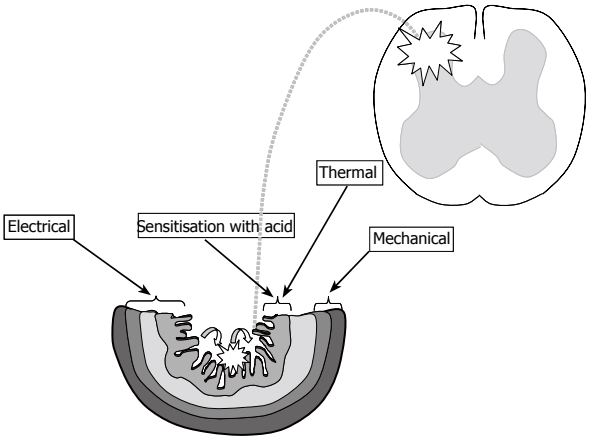

Figure 3 Schematic illustration of the proposed gut layers which are preferentially affected with: (1) Thermal stimuli (mucosa - dark grey and submucosa - light grey); (2) Mechanical stimuli (circular muscle layer - hatched grey and longitudinal muscle layer - prickled grey); (3) Electrical stimuli (all layers depending on stimulus intensity). Perfusion of the esophagus with acid (curved arrows) gives the possibility to evoke peripheral and (mainly) central sensitization (illustrated with stars).

rectum phasic distensions were shown preferentially to stimulate spinal pathways thought to mediate pain, whereas slow tonic stimuli mainly affect parasympathetic nerves[37]. We recommend a slowly ramp distension as it is more physiological and allows the subjects to assess the pain continuously. We also strongly recommend to precondition the tissue by two-three distensions until the stress-strain relationship becomes reproducible[38]. For advanced muscle analysis, we normally use butylscopolamine to relax the smooth muscle, and this does not seem to modify the sensation *per se* [20,39,40].

The multimodal approach has recently been used to compare the response to mechanical stimuli before and after chemical stimulation with acid in patients with non-cardiac chest pain[41]. In these patients, there is a normal sensory response to mechanical stimuli at baseline. However, after acid the evoked hyperalgesia resulted in a marked increase of the sensory response in the patients (Figure 4). Although peripheral sensitization may be important, the findings gave evidence for an amplification of central pain mechanisms manifested as allodynia, hyperalgesia, and increased and widespread referred pain areas to the mechanical stimulations. Mechanical stimulation together with advanced muscle analysis has also been used to explain the symptoms in patients with systemic sclerosis. In these patients, the contraction amplitude was smaller and there was an evidence for a stiffer gut wall in the small intestine[42]. The pain evoked by a controlled strain of the gut was increased and this may explain many of the symptoms reported in the clinic. In patients with diabetes, we also found evidence for increased stiffness in the duodenum using the multimodal approach (Frøkjær *et al*, unpublished data). This may reflect the increase in collagen deposition seen in these patients and may (together with autonomic neuropathy) explain the motor abnormalities seen in these patients.

Multimodal stimulations and central pain mechanisms

In diseases of the GI tract, central sensitization and neuroplastic changes are probably of major importance to understand the sensory response as manifested by pain and hyperalgesia. Central pain mechanisms may be evoked by multiple stimuli (either temporal or spatial summation), resulting in central amplification of the response. The response is comparable to early phase of the frequency-dependent "wind-up" seen in animal experiments. In practice the central integration can be evoked by repeated electrical stimulation above 0.5 Hz[43,44], resulting in increased local and referred pain. Recently, the multimodal probe was used to give repeated mechanical stimulation in patients with non-cardiac chest pain. In this study, the number of stimuli tolerated was significantly lower in the patients compared with healthy controls, reflecting central hyperexcitability as a key to understanding the symptoms in these patients[41].

Sensitization of the esophagus with acid is an another possibility to evoke central (and peripheral) sensitization. Previously, it was shown that acid perfusion of the distal esophagus resulted in an amplified response to electrical, mechanical and thermal stimuli[19,26]. The central changes were documented in an experiment where there was an amplification of the nociceptive reflex[19]. The reflex was evoked by stimulation of the sural nerve, resulting in

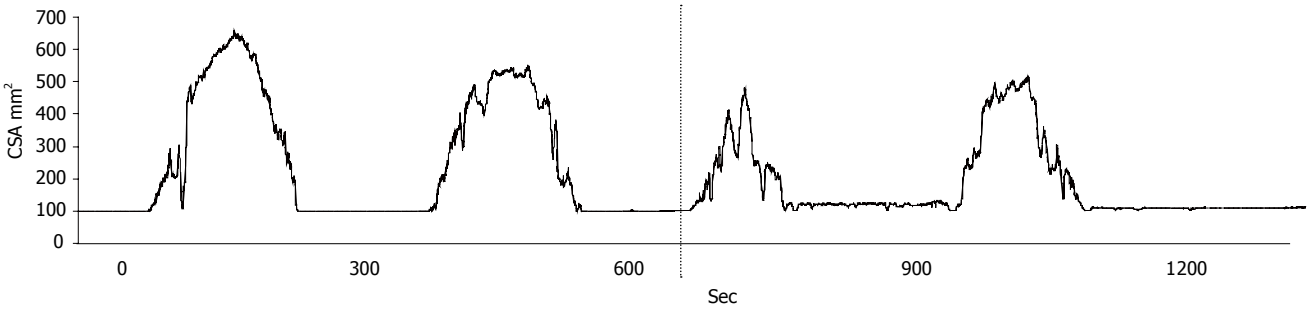

Figure 4 Cross-sectional area (CSA) at moderate pain during two distensions of the esophagus in a patient with non-cardiac chest pain. The distensions were performed before and after perfusion of the distal esophagus with acid (illustrated with the stippled line). A clear reduction in the tolerated mechanical stimulus was seen after acid perfusion.

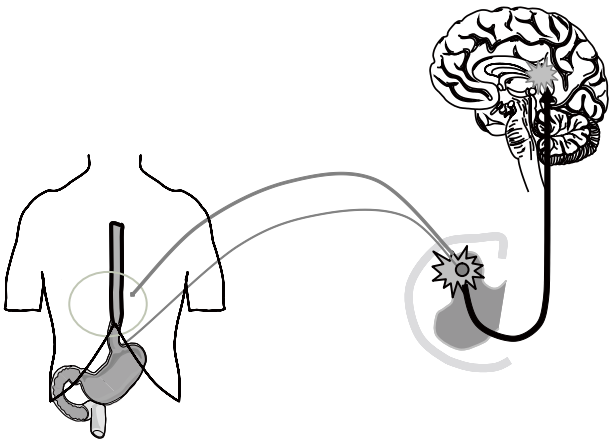

Figure 5 Referred pain in the somatic tissues is believed to be generated by central mechanisms, where visceral and somatic nerves converge on nerves in the same area of the spinal cord or at supraspinal centers. The phenomena also includes unmasking of latent connections and focal central hyperexcitability of the neurons.

activity of the biceps muscle of the thigh. The connection from the primary afferents to the motor neurons is a polysynaptic spinal pathway, which can be modulated by other afferent input, spinal neuronal excitability, and activity in descending control systems[5]. The reflex was evoked together with a painful mechanical stimulus of the esophagus. In the experiment, an amplification of the reflex was seen after sensitization of the esophagus with acid, reflecting central changes at the spinal cord level. Evidence for central changes to acid perfusion was also demonstrated by Sarker *et al*[17] who demonstrated a decreased pain threshold to electrical stimulation of the proximal esophagus after acid perfusion of the distal part. As the proximal esophagus was not affected by the acid, only central changes would explain the findings.

Referred somatic pain to visceral stimuli is regarded as a phenomenon generated by central mechanisms due to visceral nerves terminating in the same area of the spinal cord as somatic afferents[45] (Figure 5). Assessment of the referred pain area to electrical, mechanical and thermal stimulation can be used to determine the central response to these differentiated modalities[19]. The referred pain area to electrical, mechanical, cold and heat pain differs in size and localization reflecting the different peripheral (and hence central) nerves that are activated[18]. The increase in referred pain areas after acid perfusion is also an evidence for central sensitization caused by the chemical stimulation[26,46]. Recently, Pedersen *et al*[28] showed that the referred pain to heat but not cold stimulation of the esophagus increased after acid perfusion of the organ. This has also been shown in a more recent study[46]. As discussed previously, selective sensitization of the TRPV1 receptors by acid could result in an increased afferent barrage after a heat stimulus, which again was manifested as an increase in the referred pain area. The changes in local and referred pain to mechanical stimulations may, however, be difficult to determine as the acid also evokes secondary contractions that may squeeze the bag and influence the stimulus parameters[26]. On the other hand, an increase in the referred pain after acid perfusion is typically seen to mechanical stimulation of the esophagus in healthy subjects[19,26]. Increased referred pain to mechanical stimulations was also seen in patients with esophagitis and in non-erosive reflux disease, reflecting that facilitation of central pain mechanisms is important in the understanding of these diseases[31]. In patients with diabetes and chronic pancreatitis, we found hypoalgesia to peripheral stimulation, whereas there was a significant increase in the referred pain area. These data were interpreted as a descending inhibition of the afferent input counterbalancing central hyperexcitability (Frøkjær *et al*, unpublished data). Hence, the multimodal approach may be used explaining the symptoms in these patients, and may be used to evaluate the stage of disease in a more mechanism-based manner. Central changes may also result in allodynia and hyperalgesia to stimulation of other viscera[47]. Such changes are regarded important in the understanding of functional gut disorders where abnormal sensation to physiologic stimuli, such as feces or air in the gut, may contribute to the symptoms (allodynia). Recently, a multimodal approach was used to assess the sensation of the proximal esophagus, duodenum and rectum after sensitization of the distal esophagus with acid[20]. In this study, an increased sensitivity to mechanical stretch in the three gut segments was seen after acid perfusion. This was mainly due to increased sensitivity in the rectum being very remote from the experimentally inflamed esophagus.

Neuroplastic changes at the cortical level may also be shown by multimodal stimulations of the gut. Thus, Sarker *et al*[48] showed changes in the evoked brain potentials to

electrical stimulation of the proximal esophagus after acid perfusion of the distal segment. Recently, we showed that acid perfusion resulted in neuroplastic changes at the cortical level reflected. Reduction in latency and a backward shift of the electrical dipole in the anterior cingulate dipole were observed to electrically evoked pain in the esophagus after acid perfusion of the organ (Sami *et al*, unpublished data). Such changes were also found when the gut was electrically stimulated in patients with irritable bowel syndrome[49]. The backward shift in the cingulate activation after sensitization with acid in healthy subjects may, therefore, represent the central nervous system change corresponding to the allodynia and hyperalgesia to gut stimuli found in patients with functional disorders of the gut.

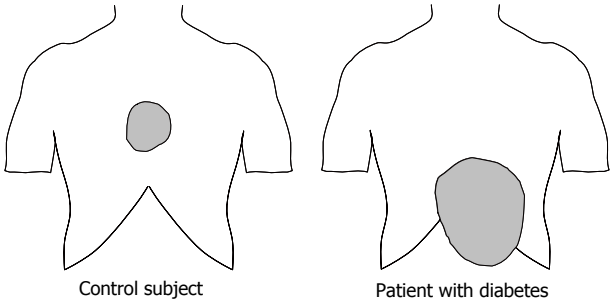

Figure 6 Referred pain area to mechanical distension of the esophagus in a typical healthy subject, and in a patient with diabetes mellitus and autonomic neuropathy. The patient complained of severe nausea and pain in the epigastrium. The referred pain area in the diabetic patient was larger and abnormally localized.

Gender differences to multimodal pain stimulations

Women are diagnosed more frequently with chronic visceral pain disorders than men[50]. Extensive evidence indicates that females and males differ in their nociceptive processing, although it seems modality- and tissue-specific. The reason for the female predominance is not known, but sex differences are found in basic GI functions, such as gallbladder emptying and colon transit[51,52]. For most studies using experimentally delivered somatic pain stimuli, females have lower thresholds, less tolerance, and higher pain ratings than males[53]. However, few studies have focused on sex-related differences in visceral pain in man, and these have been contradictory with respect to sex differences[51]. The multimodal probe was recently used to investigate any differences to mechanical and thermal stimuli of the esophagus. The results were somewhat ambiguous, but in general males seemed to be more sensitive to the stimuli[27,46]. However, a greater size of the referred pain areas to the different stimuli was seen in women. After acid perfusion, the males were also more sensitive than females to distensions, but no differences were found in response to the thermal stimuli[46]. In the females, only the referred pain area was increased to heat stimulations after sensitization with acid. The bigger referred pain areas may thus reflect that the central processing of pain to visceral stimuli differs between males and females as previously shown by our group and by Kern *et al* [54]. Thus, the multimodal stimulations revealed a differentiated response to peripheral and central pain mechanisms, which may explain the sex-related differences seen in several gastrointestinal disorders.

Multimodal stimulations outside the esophagus

Accarino *et al*[15] used multimodal stimulation (mechanical and electrical) of the jejunum. The verbal response to electrical stimuli and distension was compared, and no differences in the evoked response were found. This led the authors to conclude that the practical differences between the two modalities may probably be of minor importance. Recently, we used thermal and mechanical stimuli of the duodenum in healthy subjects[20] and in patients with diabetes and autonomic neuropathy (Frøkjær *et al*, unpublished data). The diabetes patients showed hypoalgesia to mechanical and electrical stimuli, whereas no changes were found to heat stimulation compared with controls. Furthermore, the referred pain area in the abdomen was enlarged in the patients (Figure 6). Such data may enhance our knowledge about peripheral and central pain mechanisms in these patients with implications for the treatment.

Mechanical stimulation of the rectum is one of the most used methods in experimental visceral research. Electrical and thermal stimulations have also been used in the rectum[3], but combinations of the methods were not done. However, combinations of mechanical and electrical stimulations have been used in assessment of evoked brain potentials[55] and to assess the effect of viscero-visceral hyperalgesia[20]. The stomach and other parts of the digestive system have not yet been studied with multimodal stimulations. Although the complicated anatomy, nervous innervation and function of these organs should be taken into account, multimodal models are obviously highly warranted.

Multimodal stimulations in drug research

Experimental models are widely used in research of the effect of analgesics. The differentiated information of the drug effect can be used as "proof-of concept", dose-efficacy analysis, and for designing further clinical trials. An approach to mimic the clinical situation is the use of multimodal tests, where different receptor types and mechanisms are activated. The multimodal model has clearly shown its value in somatic pain testing, where a single stimulus has been inadequate to test, for example, pathophysiological changes and effects of specific drugs[9]. Hence, differentiated effects could reflect how the drugs can modify different disease mechanisms. In the esophagus, Sarkar *et al*[56] recently used a model where the upper esophagus was stimulated following sensitization of the distal segment with acid. The secondary hyperalgesia in the proximal part was reduced with a prostaglandin inhibitor, demonstrating the preferentially central action of prostaglandins in this model. Recently, we used a multimodal (and multi-tissue) approach to test the effect of opioids. Opioids are widely used in treatment of visceral pain despite the many side effects. Opioids preferentially attenuate nociceptive responses produced by central integration (spinally amplified signals) to tonic activation

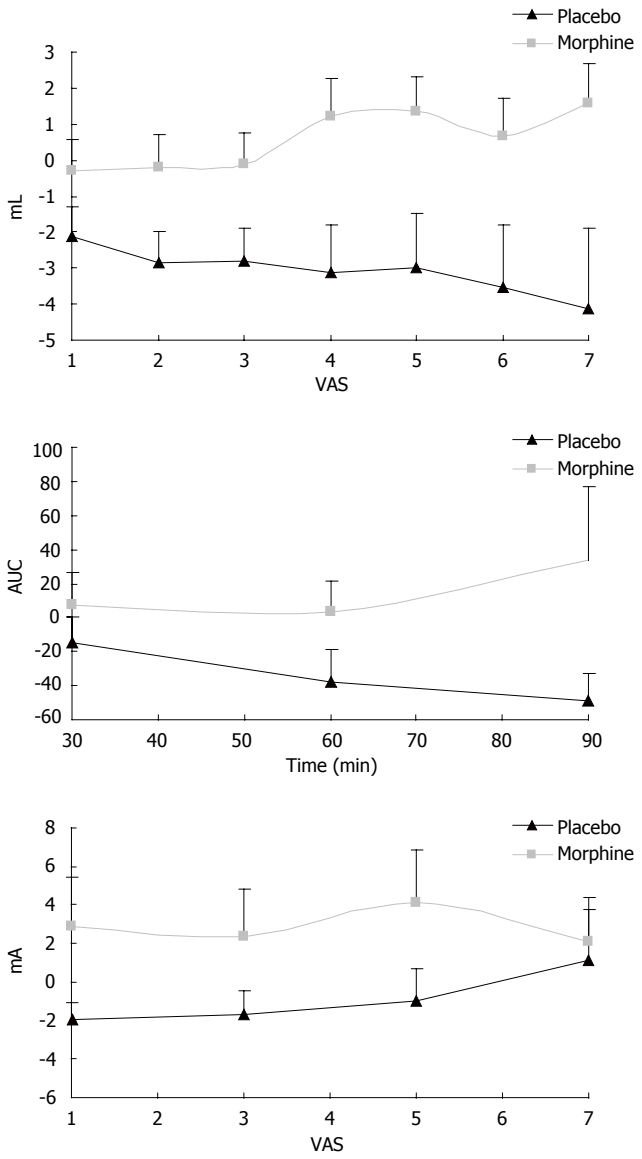

Figure 7 Change in sensory rating compared with baseline recordings for oral morphine and placebo using multimodal stimulation of the esophagus. Top picture: Morphine attenuates mechanically evoked pain better than placebo 60 min after drug intake ($P < 0.01$). VAS on the X-axis denotes the sensory rating at a visual analogue scale with 5 as the pain threshold. ML at the Y-axis denotes the bag volume. Middle picture: Morphine attenuates heat pain better than placebo ($P < 0.05$). Here the X-axis illustrates time after drug intake (min) and AUC on the Y-axis denotes the area under the temperature curve used as a proxy for the thermal energy. Bottom picture: Morphine also worked better than placebo ($P < 0.05$) on electrical stimulation 60 min after drug intake.

of unmyelinated fibers[4,5,7]. Therefore, evaluation of the anti-nociceptive effects of opioids may be clearer using slow rates of temperature or tonic pressure. In the viscera typically only one modality (pressure) has been used in the testing of analgesics[58]. However, Staahl *et al* (unpublished data) recently compared the effects of morphine and placebo on the pain thresholds to multimodal stimulation of the esophagus. A clear effect of morphine in attenuating heat, electrical and slow-ramp pressure stimulations was found (Figure 7). Morphine can definitely attenuate GI pain in the clinical situation and the model, therefore, proved its validity. The model was also used to differentiate between morphine and oxycodone, the latter was believed also to affect κ-opioid receptors thought to be predominant on visceral afferents. In equipotent doses, oxycodone was better than morphine in attenuating visceral pain, whereas there were no differences between the drugs on pain evoked in the muscle and skin[59]. The study thus demonstrated a different pharmacological profile of oxycodone compared to morphine, and therefore oxycodone may be a useful alternative to morphine in the treatment of visceral pain syndromes. We recommend that future studies evaluating analgesics in the GI tract should use a multimodal approach to get the necessary insight into visceral pain mechanisms and the effect of drugs in the gut. This will facilitate the design of subsequent clinical (phase III) studies. Hence, a substitution of the current "trial and error design" with a more mechanisms-based approach will reduce the economic and human burden in the development of new drugs targeted against pain in the GI tract.

CONCLUSION

Multimodal pain stimulation in the human GI tract is a newly developed experimental approach that mimics the clinical pain to a higher degree than previous models. The method has been used to gain more insight into basic peripheral and central pain mechanisms as well as characterizing patients with different diseases of the GI tract. Together with the possibility for pharmacological testing, the models represent a major step forward in the experimental characterization and treatment of patients with gastroenterological diseases.

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REVIEW

Diagnostic criteria for autoimmune chronic pancreatitis revisited

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Abstract

Autoimmune chronic pancreatitis (AIP) is increasingly being recognized worldwidely, as knowledge of this entity builds up. Above all, AIP is a very attractive disease to clinicians in terms of its dramatic response to the oral steroid therapy in contrast to ordinary chronic pancreatitis. Although many characteristic findings of AIP have been described, definite diagnostic criteria have not been fully established. In the year 2002, the Japan Pancreas Society published the diagnostic criteria of AIP and many clinicians around the world use these criteria for the diagnosis of AIP. The diagnostic criteria proposed by the Japan Pancreas Society, however, are not completely satisfactory and some groups use their own criteria in reporting AIP. This review discusses several potential limitations of current diagnostic criteria for this increasingly recognized condition. The manuscript is organized to emphasize the need for convening a consensus to develop improved diagnostic criteria.

Key words: Autoimmune chronic pancreatitis; Diagnostic criteria

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INTRODUCTION

Autoimmune chronic pancreatitis (AIP) can be defined as a chronic inflammation of the pancreas due to an autoimmune mechanism; autoimmunity is responsible for producing the pancreatic lesion[1,2]. AIP is a distinctive type of chronic pancreatitis that shows reversible improvement of pancreatic morphology and function with oral steroid therapy, in comparison to other types of chronic pancreatitis which hardly respond to various treatments[1-5].

AIP is increasingly being recognized to be a worldwide entity[6-8]. The sudden increment in cases reported probably reflects the growing awareness of the entity, rather than a rise in the true incidence. The diagnosis of AIP is, however, still challenging. Many groups have cited the diagnostic criteria proposed by the Japan Pancreas Society (Table 1)[9], whereas some groups have used their own criteria in reporting AIP[1,2,4,5,10]. This makes it difficult to compare studies from different centers, judge the relevance of comparisons and establish evidence on AIP. Above all, the largest problem was that a substantial portion of patients revealed clinical findings compatible to AIP and even responded to the steroid, yet failed to fulfill the Japanese diagnostic criteria[11,12]. This review revisits currently used diagnostic criteria of AIP focusing on the Japanese ones, discusses the potential limitation of diagnostic criteria and raises some important issues related to the diagnosis of AIP. In view of our experiences of a relatively small cohort of 28 patients, we propose a new revision that may help physicians diagnose AIP. By revising the diagnostic criteria of AIP, more patients may benefit from this diagnosis and be spared from burdensome surgery.

OUR EXPERIENCES

We reviewed the clinical, radiologic, laboratory and histologic features of 28 patients with AIP who responded to the oral corticosteroid. The response to the steroid was defined as improvement in clinical symptoms, negative conversion of detected autoantibodies, normalization of elevated level of IgG or IgG4, and reversion of abnormal pancreatic imaging including CT and endoscopic retrograde pancreatogram.

The diagnosis of AIP in our hospital was made by the criteria as shown in Table 2. For the diagnosis of AIP, imaging criterion (CT and ERCP findings) was an essential component. If the patient fulfilled the imaging criterion (diffuse enlargement of pancreas and diffuse or segmental irregular narrowing of main pancreatic duct) together with laboratory and/or histopathologic criteria, the patient was diagnosed of AIP. Even though a patient fulfilled imaging criterion only, in other words, the laboratory criterion and histopathologic criterion were incompatible

Table 1 Diagnostic criteria for autoimmune pancreatitis by the Japan Pancreas Society

	Diagnostic criteria
I	Imaging criterion: Diffuse narrowing of the main pancreatic duct with irregular wall (more than 1/3 length of the entire pancreas) and enlargement of the pancreas
II	Laboratory criterion: Abnormally elevated levels of serum gamma-globulin and/or IgG, or the presence of autoantibodies
III	Histopathologic criterion: Marked lymphoplasmacytic infiltration and dense fibrosis
For diagnosis, criterion I must be present, together with criterion II and/or III	

Table 2 Diagnostic criteria for autoimmune pancreatitis in Asan Medical Center

Inclusion Criteria
Criterion I. Pancreatic imaging (essential)
(1) CT: Diffuse enlargement (swelling) of pancreas
and (2) ERCP: Diffuse or segmental irregular narrowing of main pancreatic duct
Criterion II. Laboratory findings
(1) elevated levels of IgG and/or IgG4
or (2) detected autoantibodies
Criterion III. Histopathologic findings
Fibrosis and lymphoplasmacytic infiltration
Criterion IV. Response to the steroid
Definite diagnosis: Criterion Ⅰ and any of criterion Ⅱ-Ⅳ

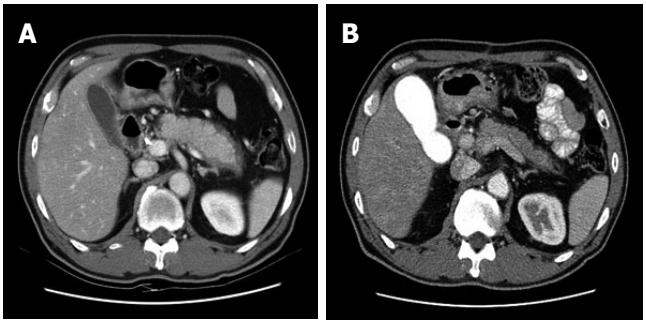

Figure 1 Abdominal CT findings. **A:** Diffusely enlarged pancreas without any calcification or stones in a 59 year-old man. A capsule-like low-density rim can also be seen around the pancreas; **B:** After steroid therapy, the pancreas returned to its normal size and the rim disappeared.

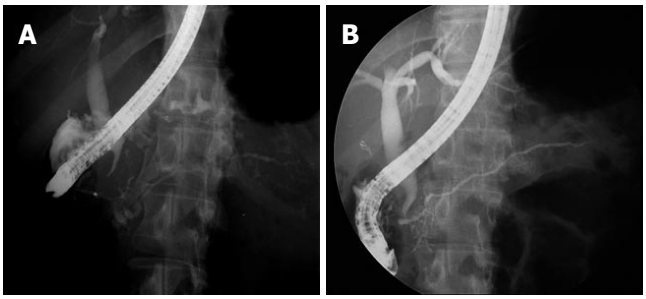

Figure 2 ERCP findings. **A:** Diffuse irregular narrowing of more than 2/3 of the entire length of main pancreatic duct noted in the 49 year-old woman; **B:** After steroid therapy, evidently widening of the main pancreatic duct.

or not available, steroid was administered and the patient was diagnosed as AIP if the response to the steroid was shown.

The patients' mean age was 55.3 years (range, 32-78 years) and they were comprised of 22 males and 6 females. None of the patients had a history of alcohol abuse or other predisposing factors for chronic pancreatitis.

On CT, all the patients revealed a diffusely enlarged pancreas with no or mild peripancreatic fat infiltration. All the patients had no any typical findings of ordinary chronic pancreatitis, such as multiple parenchymal calcifications or pancreatic ductal stones. Capsule-like low-density rim surrounding the pancreas was shown in five (18%) patients (Figure 1). On direct pancreatogram, 8 (29%) patients showed diffuse irregular narrowing of main pancreatic duct which involved entire main pancreatic duct or at least more than 2/3 of entire length of main pancreatic duct (Figure 2). In seven patients with segmental irregular narrowing, total length of ductal involvement was less than 2/3 of the entire length of main pancreatic duct and it was between 1/4 and 1/3 (Figure 3).

The IgG level was increased (>1 800 mg/dL) in 14 (50%) patients (Table 3). The IgG4 level was measured in 23 patients and it was increased in 15 (65%) patients. In 4 patients, the IgG4 level was increased without elevation of IgG level. Autoantibody was detected in 15 (68%) patients.

Pancreatic tissue specimens were obtained from 19 patients. Percutaneous ultrasound-guided core biopsy with an 18-gauge needle was performed in 17 patients and open biopsy in 2 patients. The biopsy specimen of 14 (74%) patients showed marked inflammatory infiltrates mostly of lymphocytes and plasma cells and dense fibrosis of the pancreatic tissue. However, one patient showed predominant eosinophilic granulocytes infiltrating into the pancreatic parenchyma rather than lymphoplasma cell infiltration. Overall, the biopsy findings were non-diagnostic, that is, either lymphoplasma cell infiltration or fibrosis was minimal or absent, in 5 of 19 (26%) cases.

Seven of 28 (25%) patients who responded to the steroid did not satisfy the Japanese imaging criterion because the extent of ductal narrowing was less than one third of entire length of main pancreatic duct. Another two patients showed normal IgG level, negative results of autoantibody measurements and non-diagnostic pancreatic histopathology. Taken together, 9 of 28 (32%) patients did not meet the Japanese diagnostic criteria for AIP, yet responded to the steroid.

DIAGNOSTIC CRITERIA

The diagnosis of an autoimmune disease is always an impetus to the clinician. Autoimmune conditions often lack pathognomonic findings on histopathology. The sensitivity and specificity of serologic markers also leave controversies in the diagnosis. To overcome these problems, combinations of common clinicopathologic findings are often used to guide physicians in diagnosing autoimmune diseases.

During the past decade, Japanese investigators have described many common clinicopathologic findings of AIP by reporting over three hundred cases domestically.

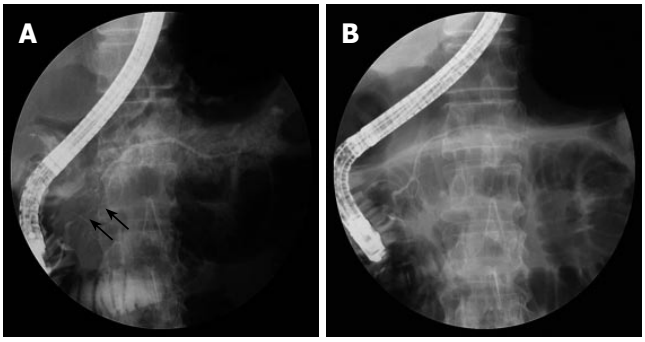

Figure 3 ERCP findings. **A:** Segmental narrowing (arrows) of the main pancreatic duct noted at the pancreatic head. The extent of irregular narrowing is less than 1/3 of the entire length of main pancreatic duct; **B:** Direct pancreatogram revealing a normal-appearing main pancreatic duct following steroid therapy.

Based on this experience, the Japan Pancreas Society published the "Diagnostic Criteria for Autoimmune Pancreatitis" in the year 2002[9]. The criteria are constituted of 3 components: (1) Pancreatic imaging: diffuse irregular narrowing of the main pancreatic duct with irregular wall (more than 1/3 length of the entire pancreas) and diffuse enlargement of the pancreas; (2) Laboratory data: elevated levels of serum gammaglobulin and/or IgG, or the presence of autoantibodies; and (3) Histopathologic findings: fibrotic changes with lymphocyte and plasma cell infiltration. For the diagnosis of AIP, criterion 1 must be present, together with criterion 2 and/or 3 (Table 1). Interestingly, the criteria does not include symptoms or common laboratory findings, as they are not specific to AIP[13,14]. The condition commonly manifests as obstructive jaundice, weight loss, and recent-onset diabetes in elderly men. None of the patients diagnosed as AIP have a history of alcohol abuse or other predisposing factors for chronic pancreatitis. In contrast to other types of pancreatitis, severe abdominal pain is infrequently encountered. These features are similar to that of pancreaticobiliary malignancies, which are the most difficult and important entities to differentiate from AIP[15].

Imaging criterion

Radiologic imaging of the pancreas is an essential component of the Japanese criteria where it is mentioned as a "must" for diagnosing AIP. The criterion is stated as diffuse narrowing of the main pancreatic duct with irregular wall (more than 1/3 length of the entire pancreas) and diffuse enlargement of the pancreas which can be identified with ERCP (endoscopic retrograde cholangiopa ncreatography) and CT, respectively[9]. Although abdominal US is most frequently performed imaging method for the screening of pancreatic disease, overlying bowel gas or obesity often hinders the sonographic visualization of the pancreatic gland, rendering the examination limited in scope and quality.

On CT, most cases of AIP reveal a diffusely enlarged pancreas with no or mild peripancreatic fat infiltration. Phlegmonous changes of the pancreas, peripancreatic fluid collection or pseudocysts formation are rare. Diffuse enlargement of the pancreas correlates with the pathology of marked stromal edema, which manifests as diffuse swelling on gross examination[16,17]. Surprisingly, CT does not reveal any typical findings of chronic pancreatitis, such as multiple parenchymal calcifications or pancreatic ductal stones. Rather, AIP resembles mild form of acute pancreatitis according to Balthazar classification[18]. Some cases of AIP reveal peculiar CT findings, such as a capsule-like low-density rim surrounding the pancreas (Figure 1)[19]. This rim is thought to correspond to the histologic findings of an inflammatory process that contains fibrous changes involving the peripancreatic adipose tissue[20]. Delayed enhancement of the pancreatic parenchyma is another distinguishing feature of AIP. On arterial enhanced phase, the pancreas appears hypodense when compared to the spleen. On the delayed phase, attenuation increases when compared to early images. This reflects fibrosis with an associating inflammatory process. These characteristic patterns observed on CT are important in differentiating AIP from pancreatic cancer[15].

The term "enlargement" of the pancreas can be subjective and vague. The size of the pancreas may be affected by many factors, including body mass, ethnic group, gender and age. There are individual variations in the size of the gland, with smaller atrophic glands seen in older individuals[21,22]. While a pancreas may seem normal for a large young man, the same size can be described as "enlarged" for an elderly patient with small body mass. Nishino *et al*[23], therefore, describe that the pancreas is considered to be enlarged when the width of the pancreatic body or tail was more than two thirds of the transverse diameter of the vertebral body or the width of the pancreatic head is more than the full transverse diameter of the vertebral body.

The Japanese imaging criterion has described the ductal pathology as diffuse narrowing of the main pancreatic duct with irregular wall (more than one third of the length of the entire pancreas), which can be observed on direct pancreatogram[9]. However, we were confronted with confusion when applying this criterion. This may be because the terms used were imprecise and vague in the meaning. First, in the previous international symposium on chronic pancreatitis[24,25], pancreatic lesion was classified as focal, segmental, or diffuse according to the extent of involvement. In this international classification, "diffuse" is used when a process involves the entire pancreatic duct or at least more than two-thirds of the entire length of the main pancreatic duct, whereas lesions that are not continuous and involve the head, body or tail are defined as "segmental" (Figures 2 and 4)[24,25]. In Japanese criteria, the terms "diffuse" and "at least one third of the entire length" in the same sentence are contradictory to each other. Thus, it may be more appropriate that the term "diffuse" irregular narrowing of main pancreatic duct in Japanese imaging criterion (Table 1) is changed to "diffuse or segmental" irregular narrowing. In AIP cases of segmental involvement, the intervening normal-appearing duct upstream to the stricture shows no or minimal dilatation in spite of the long stricture (Figure 4). These findings differentiate AIP from pancreatic cancers, which reveal stricture associated with marked upstream ductal dilatation[26,27]. Second, "narrowing" of the main pancreatic duct is a term based on subjective assessment.

Table 3 Clinical characteristics of 28 patients with autoimmune chronic pancreatitis

Age/sex	Other autoimmune diseases	IgG (mg/dL)	IgG4 (mg/dL)	Auto antibody	ERCP	CT findings	Pancreas pathology	Response to steroid
54/M	+	3570	1764	-	S	DE	N-C	Y
60/M	+	4500	810	+	S	DE	N-C	Y
32/M	-	974	13	-	S	DE	ND	Y
68/M	-	2010	350	-	S	DE	Diagnostic[b]	Y
52/M	+	1440	32	+	S	DE	ND	Y
59/M	-	1880	324	-	S	DE	Diagnostic	Y
53/M	+	1990	150	+	S	DE	Diagnostic	Y
56/F	-	2730	1464	+	S[a]	DE	N-C	Y
74/M	-	2210	N-C	-	S	DE	N-C	Y
52/M	-	2100	N-C	-	D	DE	N-C	Y
53/F	-	1240	29	-	S[a]	DE	Diagnostic	Y
45/M	-	1470	N-C	+	D	DE	Diagnostic	Y
58/M	-	4100	780	+	S	DE	Diagnostic	Y
63/F	-	1200	10	-	D	DE	Diagnostic	Y
61/M	-	1500	190	-	S[a]	DE	Diagnostic	Y
63/M	-	1780	658	+	S[a]	DE	Diagnostic	Y
49/F	-	2000	445	+	D	DE	N-C	Y
59/M	-	1230	840	+	D	DE	Diagnostic	Y
51/M	-	1570	310	-	D	DE	Diagnostic	Y
68/M	+	3550	1360	+	S	DE	N-C	Y
33/M	-	1350	66	-	S[a]	DE	N-C	Y
36/M	-	1060	50	+	D	DE	ND	Y
60/M	-	1930	N-C	+	S[a]	DE	Diagnostic	Y
64/F	-	1470	N-C	+	S	DE	ND[c]	Y
70/M	+	1820	310	+	D	DE	Diagnostic	Y
44/M	-	1110	36	-	S	DE	Diagnostic	Y
34/F	+	1070	11	-	S	DE	ND	Y
78/M	-	4570	1580	+	S[a]	DE	N-C	Y

S: Segmental irregular narrowing of main pancreatic duct; D: Diffuse irregular narrowing of main pancreatic duct; DE: Diffuse enlargement of pancreas; N-C: Not checked; ND: Non-diagnostic; [a]: Extent of irregular narrowing was less than 1/3 of the entire length of main pancreatic duct; [b]: Lymphoplasma cell infiltration and fibrosis; [c]: Eosinophilic infiltration.

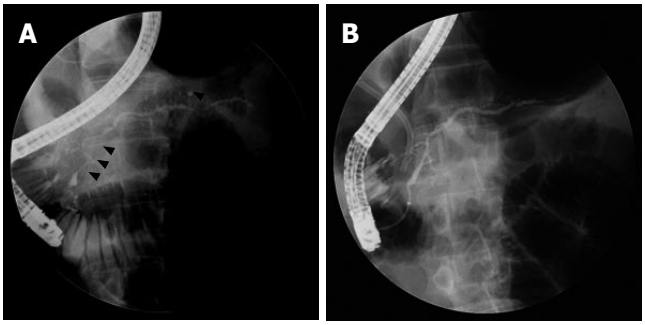

Figure 4 ERCP findings. **A:** Segmental irregular narrowing (arrow heads) of the main pancreatic duct that involves the pancreatic head and tail portion is noted in a 53-year-old man; **B:** After steroid therapy, the narrowing sites on preceding ERCP were resolved. Biliary stenting for narrowing of intrapancreatic common bile duct is noted.

At the present time, there are no references for how thin "narrowing" is. Like the size of pancreatic gland, duct diameter also varies by age, gender, and size of patient, *etc*. In the cases of AIP, most patients are elderly, which complicates matters because the diameter of the main pancreatic duct physiologically increases with age[28,29]. In patients with AIP, the ductal diameter widens to normal caliber after steroid therapy (Figures 2 and 4).

Some authors suggested that as a Japanese length criterion of direct pancreatogram, "more than 1/3" of ductal narrowing (Table 1) should be changed to "more than 1/4" because the extent of ductal narrowing was between 1/4 and 1/3 of the entire length of main pancreatic duct in some cases of AIP[30]. Actually, in our series, the extent of irregular narrowing was less than 1/3 of the entire length of main pancreatic duct in 7 of 28 (25%) patients (Figure 3, Table 3). Moreover, a variant form of AIP that is characterized by focal parenchymal swelling with a localized short stenosis of the main pancreatic duct and evident upstream dilatation has been reported[31]. Because this focal type is a rare form of AIP and mainly diagnosed after laparotomy, further discussion is beyond the scope of this review. However, studies reported that segmental narrowing progressed to diffuse narrowing on serial ERCPs without steroid treatment[32,33]. In other words, "focal" and "segmental" types can evolve into "diffuse" with time, which implies that the total length of the stricture can be less than one third at an earlier stage of the disease[32,33]. Last, "irregular wall" is another confusing term. While Japanese criteria use the term of "irregular" to portray the marginal irregularity of the narrowed ductal wall[30], we have experienced AIP patients with direct pancreatograms that reveal smooth margins not uncommonly. This may be related to the fact that pathologic specimens often reveal inflammation confined to the subepithelial space with an intact ductal epithelial lining[34,35]. Therefore, the term "irregular wall" may not always be appropriate for AIP. On the other hand, the Cambridge classification for chronic pancreatitis uses

"irregular" (*i.e.*, 'irregular dilatation' of main pancreatic duct) to describe the overall contour of the main pancreatic duct[24]. These pancreatographic findings may be explained by the heterogenous pattern of inflammatory infiltrates and fibrosis noted in pathology[34].

The intriguing point of imaging is that CT findings resembling mild acute pancreatitis are quite unusual when associating ERCP findings, which reveal diffuse or segmental irregular narrowing of the main pancreatic duct, are considered. Narrowing of the main pancreatic duct with or without intrapancreatic common bile duct stenosis on ERCP is a finding that is rarely seen in acute pancreatitis, but often in chronic pancreatitis[36]. While diffuse enlargement without calcification or stone is common in acute pancreatitis, this finding is rare in chronic pancreatitis and parenchymal atrophy is more frequently observed in ordinary chronic pancreatitis. CT findings have no evident chronic parenchymal changes in contrast to the ductal pathology detected on ERCP[37]. Coexistence of such contradicting radiologic findings (CT and ERCP) in the same patient was a rare yet peculiar association that was consistently observed in patients with AIP[38].

We believe that clinicians should vigorously obtain direct pancreatograms when patients show unusual clinical features or atypical clinical manifestation in patients suspected of pancreaticobiliary malignancies. Although direct pancreatogram holds a critical position in diagnosing AIP, hardships follow in obtaining it. Due to the possible risk related to the procedure, the visualization of pancreatic duct is not always carried out when the main pancreatic duct is not dilated on US or CT in patients with diffuse pancreatic swelling alone[39,40]. There are also pitfalls in interpreting pancreatograms. Because the main pancreatic duct is extrinsically compressed, it is technically difficult to attain whole ductal images and, sometimes, sufficient amount of contrast is not introduced into the main pancreatic duct. An "abrupt cutoff" of the main pancreatic duct may conceal a true "irregular narrowing" when ERCP is performed inadequately. Deep cannulation of catheter up to the tail portion guided by a thin guide wire can help circumvent this problem. While magnetic resonance cholangiopancreatography (MRCP) is replacing diagnostic ERCP in many pancreaticobiliary diseases, it seems to be limited in delineating the detailed pathology of main pancreatic duct associated with AIP. AIP is basically a "narrow-duct" disease and the resolution of MRCP is inferior to that of ERCP in this aspect[41,42].

Laboratory criterion

Abnormally elevated levels of serum gamma globulin and/or IgG or the presence of autoantibodies are described as the laboratory criterion of AIP by the Japan Pancreas Society[9]. In general, these features are known facets of autoimmune diseases, which provide laboratory evidence of an autoimmune pathogenesis[43]. By measuring various autoantibodies, a number of candidates have emerged for clinical usage in AIP. Detection rates for autoantibodies, however, have varied among reports[44,45]. This difference may be accounted by the number of measured autoantibodies, which can affect detection rates of autoantibodies; rates tend to increase as more autoantibodies are checked[46]. Among the conventional autoantibodies that are commonly investigated in autoimmune diseases, anti-nuclear antibody and rheumatoid factor are more frequently detected in AIP. Other markers, including anti-smooth muscle antibody and anti-mitochondrial antibody, have failed to show detection rates above 10%. On the other hand, anti-lactoferrin antibody (ALF) and anti-carbonic anhydrase II antibody (ACAII) are relatively organ-specific autoantibodies, which reveal the highest detection rates (over 50%) among autoantibodies in AIP[47]. Lactoferrin is normally present in the pancreatic acinus and carbonic anhydrase II in the ductal cells of the pancreas. ALF and ACA II, however, require a special laboratory for measurement that is unavailable to many clinicians. And, even if ALF and ACA II are measured, these markers are not 100% sensitive[48,49]. This implies that although carbonic anhydrase II and lactoferrin are considered to be the most likely target antigen for AIP, cases without ALF and ACAII, despite a good response to steroid therapy, also exist. ALF and ACAII are not specific to AIP as they may be elevated in other diseases[48,49]. Elevated levels of ACAII were also reported in patients with pancreatic cancer or ordinary chronic pancreatitis[10,50,51].

Elevated IgG and hypergammaglobulinemia are not always seen in patients with AIP. IgG4, a subtype of IgG, levels have been reported to be able to distinguish AIP from other pancreatic disorders with a high sensitivity (95%) and specificity (97%)[52]. Moreover, some cases of AIP show elevated serum levels of IgG4 in spite of normal IgG levels[53]. We, therefore, believe that both IgG and IgG4 levels should be measured in all patients suspected with AIP. In Japanese criteria, however, the increased IgG4 level was not included in the laboratory criteria and only mentioned in the appendix that there are cases with elevated levels of IgG4[9]. The Japanese criteria for laboratory data states that IgG levels should be higher than 1 800 mg/dL (normal range, 614-1295 mg/dL[54]). Although cutoff value of IgG was set at 1 800 mg/dL in Japanese criteria, another group uses different cutoff value (1 700 mg/dL) of IgG for the diagnosis of AIP[55]. If we set cutoff value of IgG at higher level, the specificity of IgG increases but the sensitivity decreases. More evidence on sensitivity and specificity according to each cutoff value should be provided for IgG before cutoff level is established.

While serologic markers have provided a base in understanding AIP, there are still controversies. As autoantibodies and IgG can rise non-specifically during the course of various injuries and diseases, the mere increment can not indicate a cause-and-effect relationship. Moreover, elevation of IgG4 level has been reported in patients suffering from pancreatic carcinoma and other types of chronic pancreatitis[50]. A number of groups have tried to find other laboratory indicators of AIP. HLA may identify patients susceptible to AIP. One report mentioned that frequencies of DRB1*0405 and DQB1*0401 were significantly higher in patients with AIP when compared with chronic calcifying pancreatitis[56]. At the present time, however, further studies are required to evaluate the value of each laboratory indicator and find a more reliable one.

Histopathologic criterion

The Japanese criteria described the histopathologic findings of AIP as marked inflammatory infiltrates mostly of lymphocytes and plasma cells and dense fibrosis of the pancreatic tissue[9]. In one report, however, lymphoplasma cell infiltration was minimal in one third of AIP cases. In general, marked infiltration of inflammatory cells is noted in early stage of an autoimmune disease, but fibrosis becomes predominant as the disease progresses. This suggests that degree of inflammatory cell infiltrates and fibrosis and predominance of either one may be dependent on the stage of AIP[57]. The characteristic lymphoplasmacytic infiltration and fibrosis may not be pathognomonic to AIP. Alcoholic chronic pancreatitis (ACP) also showed an abundant amount of lymphoplasma cell infiltration and fibrosis[58]. Although periductal inflammation and fibrosis were observed in both ACP and AIP, the detailed histologic patterns differed. While ACP mainly revealed dense interlobular fibrosis with a cirrhosis-like appearance, AIP showed loose fibrosis in both interlobular and intralobular region[58-60]. AIP showed more severe and diffuse acinar atrophy than ACP. As for extracellular matrix proteins, collagen type IV, a component of the intact basement membrane that ensures accurate regeneration of tissue, was preserved in cases associated with AIP, whereas ACP showed a significant loss of this collagen subtype[58]. Not only may this help differentiate AIP from ACP, but also aid our understanding of the regeneration of acinar structures and regression of fibrosis following steroid treatment. More specific histologic features, *i.e.*, the pattern of fibrosis and inflammation, should be supplemented for histopathologic criterion of AIP.

Periductal fibrosis and inflammatory infiltrates surrounding the duct like a cuff compress the ductal lumen into a star-like structure[61]. Intrapancreatic portion of common bile duct is often involved and the biliary involvement in AIP develops by the same mechanism as the pancreatitis[23]. Some reports of AIP show predominant neutrophilic or eosinophilic granulocytes infiltrating into the pancreatic parenchyma rather than lymphoplasma cell infiltration[62]. This acute inflammatory component of AIP is characterized by focal detachment, disruption and destruction of the duct epithelium due to invading granulocyte, which has been named "granulocytic-epithelial lesions" of the ducts. The extension and severity of chronic and acute changes in AIP vary from case to case, and even from area to area within the same pancreas.

Many clinicians come across difficulties in obtaining adequate pancreatic specimens for histologic evaluation[48]. Fine-needle aspiration biopsy often fails to gain sufficient amounts of pancreatic tissue. Large-bore needle biopsies may be used to yield adequate amounts for pathologic examinations. However, this is at the cost of increasing risks of procedure-related complications. Because of the patchy nature of inflammation seen in AIP, percutaneous biopsy may not be diagnostic due to sampling error problems[13]. Another problem is the potential risk of tumor seeding during biopsy in patients in whom cancer can not be omitted. Due to this reason, endoscopic ultrasonogram (EUS)-guided biopsy is recommended for patients in whom pancreatic cancer can not be excluded[63]. This approach is useful because the pancreatic head is the most frequently involved portion in AIP. One recent paper has reported the usage of EUS-guided trucut biopsy, which may help surmount the above problems and allow optimal histologic examination in AIP[64].

Although histologic evaluation offers a gold standard in diagnosing many disease entities, its role maybe a little different in autoimmune conditions, as seen in primary sclerosing cholangitis and autoimmune hepatitis[65,66]. In these conditions, the histopathologic findings are not disease-specific. Biopsy is often performed to exclude other entities that coexist or show resemblance rather than to make a diagnosis. Taken together, the role of histopathologic examination in patients suspected of AIP may be the exclusion of other diseases, such as malignancy, rather than the confirmation of diagnosis.

Response to the steroid

Although the dramatic response to the steroid is a well-known phenomenon in AIP, a detailed steroid schedule has not been fully established at the present time. Prednisolone is usually initiated at 30-40 mg per day and tapered after confirmation of the response to the steroid. The response to the steroid is defined as improvement in clinical symptoms, negative conversion of detected autoantibodies, normalization of elevated levels of IgG, and reversion of abnormal pancreatic imaging, including CT and endoscopic retrograde pancreatography[58]. In cases of obstructive jaundice associated with intrapancreatic common bile duct narrowing, however, biliary stenting is often needed additionally. The dose of oral corticosteroid may be tapered by 5 mg each 2-4 wk. Eventually, the steroid is completely discontinued or maintained at a dose of 2.5-10 mg/d according to the preference of the doctor[50,52,67].

The response to the oral steroid provides a circumstantial evidence of underlying autoimmune pathogenesis[68]. Among the responses to the oral steroid therapy, recovery of pancreatic ductal narrowing is top priority in the differential diagnosis of AIP. For the recovery of pancreatic ductal narrowing, histologic recovery including periductal fibrosis should be accompanied. We already reported histologic recovery, especially regression of pancreatic fibrosis in patients with AIP after steroid therapy[69]. Relief of pancreatic ductal narrowing by steroid administration is a unique and specific finding that can not be seen in any other type of chronic pancreatitis or pancreatic cancer (unpublished observation, Myung-Hwan Kim, MD). And, because marked improvement of pancreatic ductal narrowing can be observed at as early as 2 wk after steroid therapy[32,50], steroid trial may be a practical diagnostic tool that has clinical impact, especially when differentiation from cancer is an issue. This is analogous to autoimmune hepatitis where steroid treatment is justified when the entity is highly suspected, and the response to treatment is incorporated into the diagnostic scoring system[66]. If steroid therapy fails to show clinical improvement, imaging studies should be performed again to differentiate AIP from pancreatic cancer. There are opposes against

including the response to the steroid into the diagnostic criteria of AIP. They argue that steroid is not usually prescribed in any other type of chronic pancreatitis and one has to strongly suspect AIP in the first place to ever consider steroid therapy. To observe the response to the oral steroid in patients suspected of AIP, however, may be a diagnostic trial as well as therapeutic trial. Actually, in one Japanese university hospital and Italian group, they use the response to the steroid as one diagnostic criterion of AIP[50,70].

There are also concerns of the possibility of cancer progression during a trial of steroid therapy in an operable patient[6]. Despite this risk, we believe that a trial with steroids can be used to guide diagnosis when used in a proper fashion. If a patient shows typical pancreatic images of AIP, a short course (about 2 wk) of steroid may differentiate AIP from pancreatic cancer due to the fact that pancreatic ductal images of malignancy do not change[32,50]. And if the results are equivocal or do not favor AIP, the diagnosis of AIP should undergo reevaluation and the possibility of laparotomy should be considered. By including the response to the steroid into the diagnostic criteria, we can overcome the fact that there are patients who do not satisfy laboratory data and histologic findings, yet reveal typical images and an excellent response to the steroid.

Association of other postulated autoimmune diseases

Autoimmune diseases tend to cluster, and one patient may have more than one autoimmune conditions[43]. This is also the case in AIP which is frequently associated with Sjögren's syndrome, retroperitoneal fibrosis, primary sclerosing cholangitis, primary biliary cirrhosis, and inflammatory bowel disease[71]. This association may be explained by the fact that carbonic anhydrase II and lactoferrin are present in the salivary gland and biliary duct as well as the pancreas. An autoimmune response against these common antigens may result in "autoimmune exocrinopathy" which describes an autoimmune disease that involves multiple exocrine organs[72]. On the basis of the absence or presence of a systemic autoimmune diseases, Okazaki *et al*[14] divided AIP into 2 major groups: Primary or secondary forms of the disease. In contrast to the Japanese criteria, Italian group has included the association of other postulated autoimmune disease into their diagnostic criteria of AIP (Table 4)[50].

The prevalence of other postulated autoimmune diseases, however, varies among papers with some reporting rates that exceed 50%[50]. Comorbidities are manifested at various time points on the natural course of AIP; some manifest before AIP and some simultaneously and others after remission[32]. Thus, primary form of AIP may need to be changed to secondary form in cases where other autoimmune diseases are not recognized at diagnosis of AIP but develop later. For patients with AIP, the presence of an associated autoimmune disease may help the diagnosis, but if not present at the onset of the disease, we must carefully search for it.

In addition, pancreatitis in patients suffering from other autoimmune diseases does not always indicate that the cause of pancreatitis is autoimmunity. For example, when pancreatitis occurs in patients with systemic lupus erythematosus, pancreatitis can be related to various causes, such as vasculitis or medications[73]. This also adds confusion in reporting AIP in patients with underlying autoimmune conditions. In these cases, the remarkable response to steroids can help identify autoimmunity as the cause of pancreatitis[74].

Table 4 Diagnostic criteria for autoimmune pancreatitis by Italian group

Diagnostic criteria
1 Histology and cytology
2 The association with other postulated autoimmune disease
3 Response to the steroid therapy

REVISED DIAGNOSTIC CRITERIA FOR AIP: OUR PROPOSAL

The Japan Pancreas Society proposed diagnostic criteria in 2002 (Table 1). In our experience, a considerable number of cases of AIP is diagnosed confidently when these criteria are applied. Clinicians, however, reported cases that benefited with steroid therapy that did not satisfy the criteria[11]. In our study, 9 of 28 (32%) patients with AIP did not fulfill Japanese criteria, yet responded to the steroid (Table 3). In other words, clinicians may miss a substantial portion of patients suffering from AIP when the diagnosis is confined to those who satisfy the criteria proposed by the Japan Pancreas Society.

On the basis of a single institute experience of 28 patients, we introduce the following system for the diagnosis of AIP (Table 5). We have designed a system where patients are stratified by evidence strength for AIP into "definite", "probable" and "possible". We respect the diagnostic criteria proposed by the Japan Pancreas Society and believe that it contains the strongest findings that support the diagnosis of AIP. Some of the original descriptions create confusion and the diagnostic criteria are not completely satisfactory. Descriptions have been, therefore, rephrased and the diagnostic criteria have been expanded to include more patients who can benefit from this diagnosis. While "definite" AIP is almost same as the original Japanese criteria, those who only reveal typical pancreatic images of AIP are diagnosed as "possible" AIP. This is because the combination of pancreatic imaging (CT and ERCP) seen in AIP is quite distinctive and rarely seen in any other disease entity. When other postulated autoimmune diseases are associated with typical pancreatic images of AIP, the suspicion index becomes higher and patients are designated as "probable" AIP. The diagnosis of AIP is confirmed by the unique response to steroid that can be observed by the dramatic resolution of pancreatic ductal narrowing, and then patients are reassigned to "definite".

We believe that patients who present with typical pancreatic images of AIP deserve a short course of steroids before undergoing surgical resection, despite the lack of any serologic markers of autoimmunity or

Table 5 A proposal of revised diagnostic criteria for autoimmune pancreatitis

Diagnostic Criteria
Criterion I. Pancreatic imaging (essential)
(1) CT: Diffuse enlargement (swelling) of pancreas
and (2) ERCP: Diffuse or segmental irregular narrowing of main pancreatic duct
Criterion II. Laboratory findings
(1) elevated levels of IgG and/or IgG4
or (2) detected autoantibodies
Criterion III. Histopathologic findings
Fibrosis and lymphoplasmocytic infiltration
Criterion IV. Association of other postulated autoimmune disease
Definite
I+II+III+Ⅳ or I+II +III or I+II or I+III
Probable, I+IV; Possible, I only: Rediagnosed as "definite" if "response to the steroid" is present

Table 6 General features of autoimmune diseases

General features of autoimmune diseases
1 Elevated levels of serum gammaglobulin and/or IgG, or detected autoantibody
2 Lymphoplasma cell infiltration and fibrosis at lesional site
3 Association with other autoimmune diseases
4 Response to the steroid

pathognomonic histopathologic findings. The results of serologic markers and histologic examination in patients with AIP may be closely related to the disease activity (active *vs* inactive) or the stage (early *vs* late) of the disease. In our proposal, therefore, a diagnostic trial of steroid can be initiated even though serologic markers or pathologic findings do not fulfill the Japanese criteria or are not available, providing that pancreatic images are typical to AIP.

The Japan Pancreas Society emphasizes imaging (CT and ERCP) criterion and serologic abnormalities as important and relatively specific markers for this entity, while they do not use "the response to the steroid" and "association of other postulated autoimmune diseases" as diagnostic criteria (Table 1). However, Italian experience does not believe imaging findings and serologic abnormality are specific (Table 4). In our revised diagnostic criteria (Table 5), pancreatic imaging (CT and ERCP) is also essential and the findings are almost same as Japanese criteria. Instead, we abolished the condition "more than 1/3 of the entire length of main pancreatic duct" and added "segmental" irregular narrowing. In the laboratory criterion, we newly inserted elevated serum IgG4 level. Consequently, more patients may benefit from oral steroid therapy and can avoid unnecessary major operation. In addition to pancreatic imaging, we included the response to the steroid and association with other postulated autoimmune diseases in the diagnostic criteria, in order to reduce cases that might be occluded by the Japanese criteria. It is already well known that the general features of autoimmune diseases include detected serum autoantibodies or elevated IgG, lymphoplasmacytic infiltration and fibrosis at the lesional site, response to the steroid and association with other autoimmune diseases (Table 6)[68]. Among the general features of autoimmune diseases, one feature does not have more evidence of strength to be superior to the others in the diagnosis of AIP. We, therefore, used all aforementioned features of autoimmune disease for the diagnosis of AIP.

CONCLUSION

Autoimmune chronic pancreatitis (AIP) is a relatively new disease entity to many physicians, yet because of the clinical impact, they must vigilantly look for it. This review discusses several potential limitations of current diagnostic criteria for this increasingly recognized condition. The manuscript is organized to emphasize the need for convening a consensus to develop improved diagnostic criteria. These efforts will refine the diagnosis of AIP, which will lead to less inter-observer variation and provide a strong base for the research of this treatable condition.

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REVIEW

Anastomotic disruption after large bowel resection

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Abstract

Anastomotic disruption is a feared and serious complication of colon surgery. Decades of research have identified factors favoring successful healing of anastomoses as well as risk factors for anastomotic disruption. However, some factors, such as the role of mechanical bowel preparation, remain controversial. Despite proper caution and excellent surgical technique, some anastomotic leaks are inevitable. The rapid identification of anastomotic leaks and the timely treatment in these cases are paramount.

Key words: Colon; Colectomy; Anastomosis; Surgical complications

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INTRODUCTION

Anastomotic leakage following colorectal resection and primary anastomosis is a major clinical problem. The increased morbidity and mortality following anastomatic leakage are considerable, and lead to prolonged hospital stay. Leakage after partial colectomy with primary anastomosis may result in abscess formation, sepsis, multiple procedures and death. Despite vast improvements in surgical technique and devices, anastomotic leakage continues to be a clinical problem. The prevalence of intraperitoneal anastomotic leak varies in the literature between 0.5% and 30%, but is generally between 2% and 5%[1-3]. The double staple anastomotic technique does not appear to increase the risk for anastomotic leak, which has been reported to be 2.7%[4].

There are many factors that contribute to anastomotic leakage. Certainly, poor surgical technique can lead to an anastomotic leak. However, even when the operation is done technically well, anastomotic leaks are inevitable. Hence, a great deal of research has been done to elucidate the factors, which may decrease the rate of anastomotic leaks. Several factors have been identified that may impact on anastomotic leakage: adequacy of blood flow to the anastomoses, contamination, anastomotic technique, the presence of a pelvic drain, anastomotic tension, absence of active disease or distal obstruction, and the distance from the anal verge[5].

Numerous different techniques have been used to fashion a colorectal anastomosis. These techniques can be divided into 2 categories: hand sewn or stapled anastomosis. Hand sewn techniques include single-layer interrupted or continuous with either absorbable or nonabsorbable sutures, or various double layer techniques. The advent of stapling devices in the last century has made a significant contribution to colorectal surgery. Stapling devices have been widely accepted by surgeons performing gastrointestinal surgery. Numerous studies have been conducted comparing the various anastomotic techniques. Debates have been raised comparing single *versus* double-layered closure, absorbable *versus* nonabsorbale sutures, sutures *versus* staples, and inverting *versus* everting techniques. None of the various methods of anastomosis has been proven to be superior to the others.

HISTORY

During the early 19th century, while writing on intestinal injuries, Travers stressed the uniform contact of cut ends of intestine utilizing everting sutures. Later that century, Lembert countered this idea, instead advocating inverting sutures with serosal to serosal contact. Halsted noted that the submucosal layer was the strength-bearing layer in intestinal anastomoses. By the time that Treves published "A System of Surgery" in 1895 "Lembert Sutures" were recommended in intestinal anastomoses. The first acclaimed mechanical device to create a non-sutured anastomosis was Murphy's button introduced in 1892. It consisted of two mushroom shaped pieces, which were secured within bowel ends by purse string sutures. The pieces were then joined together. The bowel would heal as an inverted anastomosis. The excess inverted tissue would slough and the intact "button" would pass per rectum. Murphy's button gained considerable acceptance for several decades.

Circular end-to-end stapling devices were developed in the Soviet Union during the 1950s. The KT, PKS and SPTU instruments were bulky and unwieldy but served as the prototypes for today's end-to-end staplers[6]. A Soviet instrument was brought to the United States by Ravitch in 1958. Subsequently, such devices have been manufactured in the United States but did not attain widespread use until the 1970s. Thus, today's surgeon has the option of suturing or stapling intestinal anastomoses.

RISK FACTORS

Numerous risk factors have been implicated as predisposing for anastomotic leaks. Schrock *et al*[7] performed a large retrospective analysis of factors relating to leakage of colonic anastomoses. Factors that were found to correlate with an increased leakage rate were older age, anemia, prior radiation therapy, intraperitoneal infection and anatomic level of anastomosis. Conversely, steroid use, nutritional status and experience of the operating surgeon did not significantly influence the anastomotic leak rate. Rullier *et al*[8] analyzed factors associated with leakage and reported male sex and level of anastomosis as independent risk factors. In the same study, low anastomoses in obese patients were reported as associated with higher risk of leak. A higher leak rate with low pelvic anastomosis has also been reported by other investigators[9].

In a more recent study, Makela *et al*[10] compared 44 patients with anastomotic leaks to 44 control patients matched for age, gender and indications for surgery. They found that malnutrition, weight loss, alcohol intake, lengthy operative times, peritoneal contamination, and blood transfusions were independent predictors for leaks. In addition, the presence of multiple risk factors increased the risk for anastomotic leaks.

Law *et al*[11] performed a prospective study to identify risk factors for anastomotic leak in 196 patients undergoing total mesorectal excision for rectal cancer ranging from 3 cm to 12 cm from the anal verge. The overall leakage rate was 10.2%. The leakage rate was significantly higher in men (13.4%) as compared with women (5.2%) ($P=0.049$). As expected, the presence of a proximally diverting stoma significantly decreased the leakage rate especially in patients with risk factors for anastomotic dehiscence and low pelvic anastomosis. Interestingly, ages, level of anastomosis, stage of disease, or techniques of anastomosis were not significant predictors of anastomotic leak.

Mechanical forces

Investigators have used strength measurements to assess colonic healing using either breaking strength or bursting strength[12]. The breaking strength represents the uniaxial force required to break a wound *in vitro* and is a test of the entire anastomotic line. The bursting strength is a multiaxial test that measures the weakest point of an intestinal anastomosis which is the most likely location of an anastomotic leak[13].

The mechanical strength of an anastomosis is related to whether an anastomotic leak occurs. The strength of an anastomosis is dependent on the deposition of collagen. The measurement of tissue collagen content is another tool used in experimental models[14]. Martens *et al*[15] demonstrated that increased production of collagen at the anastomotic site was present 12 h after surgery. Brasken *et al*[16] showed that large amounts of type I and III collagen were present on postoperative day 4 in the anastomosis. In support of Halsted's observations, it is generally appreciated that the ultimate strength of an anastomosis depends on the collagen content in the submucosa.

Nutrition

Bowel rest (with a low-residue diet) lowered the bursting strength of non-operated colon in rats[17]. Interestingly, however, it did not impair the strength of a healing anastomosis. Dietary protein depletion impairs colonic strength in healing rat colon. Data regarding the duration of protein depletion needed to impair colonic healing in rats is conflicting. While some studies suggest that as little as one week of protein depletion has a detrimental effect, others suggest that at least 7 wk of protein restriction are needed[18,19]. In a comparison of alimentation means, Kiyama *et al*[20] showed that colonic anastomoses in rats were stronger after enteral nutrition compared to parenteral nutrition.

Bowel preparation

The role of preoperative bowel preparation has become a matter of controversy. Poth EJ[21] in 1953 proposed use of neomycin and sulfathalidine for intestinal antisepsis with reduction in the postoperative complications. Nichols RL and Condon R[22] also suggested a historic reduction in mortality and morbidity with the use of bowel preparation in a collective review of literature. In a 1973 retrospective study, Irvin TT and Goligher JC[23] reported a significant decrease in anastomotic dehiscence with the use of mechanical bowel preparation than that without mechanical bowel preparation (7% *vs* 24%). Most of the reports favoring the use of mechanical bowel preparation are based on retrospective data. However, some randomized trials have reported significant differences in outcomes with use of oral antibacterial agents and mechanical preparation. Matheson *et al*[24] reported a significant reduction in the incidence of wound sepsis and anastomotic dehiscence using both a mechanical and antibiotic preparation.

In contrast, recent literature suggests no significant advantage utilizing aggressive mechanical preparations. To assess the need for mechanical preparation to decrease the rate of anastomotic leaks in elective colorectal surgery, a number of prospective randomized trials have been completed[25-28]. Recently, Guenaga *et al*[29] conducted a meta-analysis on the existing clinical trials which studied the effect of mechanical bowel preparations on the rate of anastomotic leaks. A total of 1204 patients were enrolled in the various studies. Patients were divided into 2 groups: Group 1 ($n=595$) which received a mechanical bowel preparation; and group 2 ($n=609$) without a mechanical bowel preparation. They showed that the rate of anastomotic leaks in group 1 was obviously higher (5.5%)

compared to group 2 (2.9%) ($P=0.02$). Clearly, controversy exists on whether mechanical bowel preparations influence the rates of anastomotic leaks in elective colorectal surgery. Recent meta-analysis and prospective trials have questioned the usefulness of mechanical bowel preparations and do not support its use.

Chemo-radiation

Preoperative chemo-radiation has been used in patients with rectal carcinoma and reductions in tumor size can be achieved with its use. Chemo-radiation may predispose to anastomotic problems in patients having colon surgery, particularly in patients with anastomosis in the pelvis. Many surgeons perform a temporary diverting stoma to minimize the consequences of anastomotic disruption in patients who have had pelvic radiation therapy[30]. Anastomotic leak and radiation therapy may contribute to the formation of pelvic fibrosis, rendering the neorectum stiff and noncompliant. After reconstruction, patients may suffer from tenesmus and fecal incontinence[31].

Since many colectomies are performed for cancer, the effects of common chemotherapeutic agents and external beam irradiation on colonic healing are of interest. Immediate post-operative administration of intravenous 5-flourouracil (5-FU) in rats undergoing colectomy resulted in more conflicting data. While 4-8 mg/(kg.d) for 10 d impaired breaking strength of rat colon[32], 20 mg/(kg.d) for 5 d had no significant effect compared to controls[33]. A third study on rats showed that 600 mg/m^2 of 5-FU in the early postoperative period had no effect on colon anastomotic bursting strength[34].

In a study on rats, preoperative vitamin A supplementation protected against impaired colonic healing caused by preoperative radiation therapy[35].

Del Rio *et al*[36] showed that chronic steroids (time released via subcutaneous route) impaired colonic anastomotic strength in rats. In contrast, a large retrospective review in humans suggested no steroid effect[7].

Surgical technique

The technique used to fashion a colorectal anastomosis is largely based on surgeon preference. In order to achieve an adequate colonic anastomosis with a low rate of post-operative anastomotic leak or stricture formation, certain basic surgical principles must be met. First, the technique utilized for the anastomosis must assure an adequate lumen. Second, an adequate blood supply must be maintained for both the proximal and distal colon after resection. Finally, the anastomosis must be performed so that there is no tension to pull it apart (i.e., the surgeon must assure adequate mobilization of the proximal and distal colon). Considerable investigation has been conducted during the last century to determine the best technique for colonic anastomoses. An intestinal anastomosis may be constructed by a variety of techniques, including single layered suture, double layered suture, interrupted or continuous sutures, absorbable or nonabsorbable sutures, stapling devices or with use of a biofragmentable ring. To date, no single technique, single layer suture, double layer suture or stapling has ever been definitely demonstrated to be superior in preventing anastomotic leaks[37,38].

Table 1 Prospective randomized trials comparing the effect of mechanical bowel preparation *versus* no preparation on anastomotic leaks in elective colorectal surgery (*n*, %)

Investigators	Number of patients	Bowel preparation group (leak rate)	No bowel preparation group (leak rate)	*P*
Miettinen *et al*[25]	267	4%	2%	0.28
Zmora *et al*[26]	380	3.7%	2.1%	0.50
Santos *et al*[27]	149	10%	5%	0.52
Burke *et al*[28]	186	7.8%	11%	0.90

Surgical technique has been extensively studied in animal models. When comparing inverting *versus* everting sutured techniques, the everting technique produced less inflammation and less stricture but the inverting technique was less likely to disrupt[39,40]. (Table 1) This was also supported by the work of Irvin *et al*[41] in both animal as well as human studies. In addition, they reported no difference in the two layered *versus* single layer inverting anastomosis technique when doing intestinal anastomosis[41,42]. With disruption being the most serious problem, the inverting technique is more commonly used.

Stapled *versus* various sutured anastomoses have been compared numerous times in animal models. In a detailed study in dogs, Chung *et al*[43] showed a single layered sutured anastomosis resulted in the least reduction in anastomotic blood flow. Stapled anastomoses reduced blood flow the most. Conversely, Kozol *et al*[40] showed that early anastomotic edema was greater in two layered sewn anastomoses than in stapled. It should be noted that in some clinical circumstances, the surgeon's choice of technique is limited. For example, it is generally accepted that for low pelvic colo-rectal anastomoses stapled techniques are easier to perform.

Numerous clinical studies have been performed to define the anastomotic leak rate using sutures (Table 2). The largest of these studies was conducted by Max *et al*[44] in 1000 patients. A retrospective study was performed in 1000 consecutive patients who underwent a single layer continuous polypropylene colorectal anastomosis. The clinical anastomotic leak rate was only 1%[44-47].

Similarly multiple studies have been performed utilizing the stapled technique for colorectal anastomoses (Table 3). The leak rates from these studies ranges from 1.5% to 11%[4,48-54]. The largest of these studies was conducted by Detry *et al*[48]. A prospective study was performed in 1 000 consecutive patients undergoing stapled colorectal anastomosis by a single surgical team. The clinical leak rate was 3.5%. Also, Hansen *et al*[53] performed a large prospective study in 615 patients who underwent stapled colorectal anastomoses by a total of 18 surgeons, showing only 1.5% clinical leak rate.

Specific studies have been performed comparing stapled and sutured colorectal anastomoses (Table 4)[55-58]. Docherty *et al*[55] conducted a randomized prospective multicenter trial in 732 patients undergoing either hand-sewn ($n=321$) or stapled ($n=331$) colorectal anastomoses. The location of the anastomosis included ileocolic, colocolic, colorectal, and colostomy closures. There was no difference in the stapled or sutured group with regards to rate of anastomotic leakage. Demetriades *et al*[58] conducted

Table 2 Clinical studies utilizing sutures for fashioning colorectal anastomosis

Investigators	Number of patients	Types of suture	Continuous *vs* interrupted	Leak rate (%)
Max *et al*[45]	1 000	Non-absorbable	Continuous	1
Mann *et al*[46]	320	Absorbable	Interrupted	3.4
Flyger *et al*[47]	105	Absorbable	Continuous	1
Deen *et al*[48]	26	Absorbable	Interrupted	3.9

Table 3 Clinical studies utilizing staples for fashioning colorectal anastomosis

Investigators	Study design	Number of patients	Leak rate (%)
Detry *et al*[49]	Prospective	1 000	3.5
Griffen *et al*[50]	Prospective	75	2.7
Cohen *et al*[51]	Prospective	26	3.8
Laitinen *et al*[52]	Prospective	39	5.3
Baran *et al*[53]	Retrospective	104	2.8
Karanjia *et al*[54]	Prospective	276	11
Hansen *et al*[55]	Prospective	615	1.5
Memon *et al*[56]	Prospective	218	3

Table 4 Comparison of stapled versus sutured colorectal anastomoses

Investigators	Number of patients	Staple technique leak rate (%)	Suture technique leak rate (%)	*P*
Docherty *et al*[57]	732	4.7	4.3	0.93
Fingerhut *et al*[58]	113	13	18.7	0.05
Everett *et al*[59]	100	0	2	NS
Demetriades *et al*[60]	207	6.3	7.8	0.69

NS = not significant.

a prospective multicenter trial comparing hand-sewn to stapled colonic anastomosis in the emergent penetrating trauma setting. A total of 207 patients were enrolled in the study from 19 different centers. All patients underwent colon resection with primary anastomosis. There were 128 hand-sewn anastomoses and 79 stapled anastomoses. The demographics of both groups were similar with respect to age, gender, mechanism of injury, associated injuries, and fecal contamination. They demonstrated that there was no statistically significant difference in the 2 groups with respect to anastomotic leaks.

Surgeons have attempted several intraoperative techniques in hopes of lowering anastomotic leak rates. One is "omentoplasty" which involves wrapping the anastomosis with omentum. This was prospectively studied by the French Associations for Surgical Research[59]. In their randomized study of 705 patients, omentoplasty did not decrease the anastomotic leak rate or the clinical severity of anastomotic leaks compared to the patients without omentoplasty. Some surgeons have routinely placed a pelvic drain after low anterior resections. In a prospective, randomized study of 319 patients, the same French investigators showed that routine pelvic drainage did not lower the rate or severity of anastomotic leaks[9,60].

Many surgeons utilize intraoperative air/water testing of colon anastomoses. With this technique, after completing the anastomosis, the patient is placed in reverse Trendelenburg position. The pelvis is filled with sterile saline solution and an assistant places a sigmoidoscope (flexible or rigid) into the rectum, below the anastomosis. The colon is then insufflated with air, and the surgeon views the pelvic saline bath for bubbling (a sign of an inadequate or leaky anastomosis). If bubbling is seen, the leak is identified and repaired with sutures. There are at least two studies of the efficacy of this technique. In a study of 145 patients, Beard *et al*[61] were able to lower the "radiologic" leak rate from 29% to 11% using air/water testing in order to plan the placement of additional sutures as needed. In a study of 82 patients, Pritchard *et al*[62] found the air/water test helpful in higher anastomoses but unreliable in very low anastomoses. This may be due to the difficulty in suture repairing very low anastomoses.

In many series, the leak rates were higher for anastomoses below the peritoneal reflection[8,63,64]. One large study revealed a 12.7% leak rate in colorectal anastomoses compared to 2.9% in colo-colonic anastomoses[65]. Anastomotic leak can be a serious complication of resection for low rectal resection. Several studies have been conducted to identify risk factors that contribute to anastomotic dehiscence in patients undergoing low anterior resection (LAR) and proctectomy with coloanal anastomosis. Leaks after coloanal anastomoses are no more frequent than with colorectal anastamoses with a range of 6% to 8%[66,67]. Certain risk factors are more frequently associated with rectal resection. Meade *et al*[68] reported that a distance of less than 5 cm from anal verge, male sex, alcoholism and smoking were the risk factors for anastomotic breakdown after low rectal resections. Similar results were reported by Rudinskaite *et al*[69].

Law *et at.* investigated operative results and oncological outcomes of anterior resection for rectal and rectosigmoid cancer. They reported a significantly higher leak rate (8.1%) in patients who underwent a total mesorectal excision than those who underwent partial mesorectal excision (1.3%). Additionally, they reported that higher anastomotic leakage rate was associated with the male gender, absence of stoma, and increased blood loss[70]. Recently, Matthiessen *et al*[71] reported similar results, but they did not report any advantage of performing a temporary stoma. It should be noted that the creation of a proximally diverting stoma to protect a low pelvic or technically inadequate anastomosis does not alter the risk for dehiscence but does ameliorate the septic effects of the leak[7,71,72].

Recently, emphasis on the quality of surgical care offered has increased tremendously. There is an increasing awareness of the outcomes of surgical care as a marker of quality. Dimick *et al*[73] reported lower mortality rates in patients undergoing surgery for colorectal cancer when these procedures were performed in high volume centers. Similarly, Hannan *et al*[74] suggested an inverse relationship between in-hospital mortality rates and case volume for patients undergoing certain procedures. They reported that individual physician volume has more significant influence on the mortality rates for certain procedures. The same

authors[75] recently reported a significant reduction in mortality of patients who underwent colectomy when these procedures were performed by high-volume surgeons at high-volume centers. Conversely, the data reported by Urbach *et al*[76] did not support superior outcomes when colon operations were done at high volume centers.

CLINICAL PRESENTATION AND DIGNOSIS

Anastomotic leakage typically occurs between the 3rd and the 6th post-operative days. The clinical manifestation of anastomotic dehiscence varies in magnitude from failure to thrive to profound sepsis. The presentations in a given patient depend, in part, on the location and magnitude of the leak, and whether any adjacent tissues such as omentum or small intestine contain the leak. Indeed, a less severe leak may be walled off by adjacent organs or omentum and may present with vague abdominal pain, failure to thrive, temperature elevation, tachycardia, prolonged ileus, diarrhea or intestinal obstruction. Recognition of this situation may be delayed as the nonspecific symptoms can be attributed to delayed recovery from a major operation rather than to an anastomotic failure. However, the physician must have a high index of suspicion to make an early diagnosis. Most patients with anastomotic dehiscence will have prolonged ileus, increased postoperative abdominal pain, fever, and leucocytosis. However, the spectrum can include sepsis, peritonitis and/or hemodynamic instability. Longo *et al*[77] described the initial symptoms in 56 patients with postoperative pelvic abscess that developed after colon surgery, showing that 93% had intestinal dysfunction and 4% were in shock.

The presence of the aforementioned risk factors should raise the index of suspicion for leaks. Suspicion of a leak should lead to diagnostic imaging. A gastrografin enema is a quick and inexpensive way to evaluate the integrity of a colonic anastomosis. A gastrografin enema is less useful for right colonic anastomoses because it becomes too dilute to accurately define the anastomosis. A CT scan with intravenous, oral and rectal contrast material may also be obtained in those patients with suspected anastomotic leak and should demonstrate any abscess or extravasation of contrast from the intestine. Barium enema should not be used in this circumstance because of the increase in morbidity and mortality associated with barium-induced peritonitis. Indium-labeled leukocyte scans are occasionally helpful to identify abdominal abscesses that are suspected but not seen using conventional imaging.

MANAGEMENT

The specific method of management of an anastomotic dehiscence depends on the manifestation of the leak and the clinical condition of the patient. As many as 36% to 49% of patients with a pelvic anastomosis will have a leak demonstrated when gastrograffin enemas are routinely used during the first postoperative week[78,79]. Most of these are "subclinical" leaks. In a patient with evidence of low-grade sepsis and documentation of a contained anastomotic leak with abscess, drainage of the abscess and broad-spectrum parenteral antibiotic therapy are required and may be sufficient therapy. Drainage of an abscess can be accomplished percutaneously or operatively. A CT scan of the abdomen and pelvis with intravenous, oral, and rectal contrast medium is advocated whenever an anastomotic leak and abscess is suspected. CT scan is highly sensitive and accurate (95%) in determining the presence of abdominal or pelvic abscess[80]. CT-guided percutaneous drainage is successful in as many as 85% of appropriately selected patients[81]. For a low colorectal anastomosis, abscess drainage can be accomplished through the anastomosis if the dehiscence is readily apparent at endoscopic examination. The defect can be gently enlarged to allow better drainage, and transrectal drains can be placed in the cavity for continuous or intermittent irrigation. Transvaginal and transperineal drainage can also be performed.

Clinically ill patients with sepsis, pain and tenderness will require reoperation. Creation of a proximal colostomy or ileostomy plus peritoneal lavage and placement of drains are indicated. Some studies have advocated proximal diversion without resection if the anastomosis has been used with good results[82,83].

Gross peritonitis requires laparotomy, resection of the anastomosis with end colostomy and mucous fistula or Hartmann pouch. Diversion alone without resection of the leaking anastomosis is not ideal because of persistent sepsis from the leaking anastomosis. In such cases, wide drainage of the anastomosis should be performed. Repair of the anastomosis, either alone or in combination with a proximal stoma, is not recommended because of the high risk of recurrent anastomotic failure and/or anastomotic stricture in the presence of intra-abdominal sepsis.

Unrecognized anastomotic leaks may present as enterocutaneous fistulas. A fistulogram and/or CT scan should be obtained to determine the site of the defect in the intestine and whether any undrained collection of pus is present. Any adjacent fluid collection should be drained to facilitate closure of the fistula. After control of the source of sepsis and in the absence of distal bowel obstruction or a foreign body, the majority of colocutaneous fistulas will close without operative intervention. Other important management guidelines include correction of anemia and fluid and electrolyte abnormalities, excellent wound care, and adequate nutrition. Bowel rest and total parenteral nutrition may be necessary to facilitate closure. A late manifestation of unrecognized anastomotic leaks is anastomotic stricture. Strictures may require endoscopic dilation. Refractory strictures will require surgical revision or resection and reanastomosis.

CONCLUSION

In summary, surgeons should be aware of risk factors for colonic anastomotic leaks. The ideal is avoidance of a colonic anastomotic leak by use of proper surgical technique. In fashioning a colorectal anastomosis, some basic surgical techniques must be followed to have an acceptable result. These include the presence of adequate blood flow to the anastomosis, minimal

contamination, absence of anastomotic tension, absence of active disease, and no distal obstruction. The utility of preoperative mechanical bowel preparation in decreasing the anastomotic leak rate has been questioned by findings from several recently performed randomized prospective studies. The use of sutures or staples to create a colorectal anastomosis has never been shown to significantly alter the anastomotic leak rate.

Even when excellent surgical technique is used, a small percentage of leaks are inevitable. Characteristics, such as male gender, obesity, level of anastomosis, peritoneal contamination, age, operative time and blood transfusions, have all been implicated as potential risk factors for anastomotic leakage in various studies[84]. The clinicians must have a high index of suspicion to diagnose an anastomotic leak early. If a leak occurs, it must be identified and treated expediently. Treatment is based on the patient's conditions and the magnitude of the leak.

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Pulmonary complications in patients with chronic obstructive pulmonary disease following transthoracic esophagectomy

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Abstract

AIM: To investigate the incidence of various types of postoperative pulmonary complications (POPCs) and to evaluate the significance of perioperative arterial blood gases in patients with esophageal cancer accompanied with chronic obstructive pulmonary disease (COPD) after esophagectomy.

MEHTODS: Three hundred and fifty-eight patients were divided into POPC group and COPD group. We performed a retrospective review of the 358 consecutive patients after esophagectomy for esophageal cancer with or without COPD to assess the possible influence of COPD on postoperative pulmonary complications. We classified COPD into four grades according to percent-predicted forced expiratory volume in 1 s (FEV1) and analyzed the incidence rate of complications among the four grades. Perioperative arterial blood gases were tested in patients with or without pulmonary complications in COPD group and compared with POPC group.

RESULTS: Patients with COPD (29/86, 33.7%) had more pulmonary complications than those without COPD (36/272, 13.2%) ($P < 0.001$). Pneumonia (15/29, 51.7%), atelectasis (13/29, 44.8%), prolonged O_2 supplement (10/29, 34.5%), and prolonged mechanical ventilation (8/29, 27.6%) were the major complications in COPD group. Moreover, patients with severe COPD (grade ⅡB, FEV1 < 50% of predicted) had more POPCs than those with moderate(grade ⅡA, 50%-80% of predicted) and mild (grade Ⅰ ≥ 80% of predicted) COPD ($P < 0.05$). PaO_2 was decreased and $PaCO_2$ was increased in patients with pulmonary complications in COPD group in the first postoperative week.

CONCLUSION: The criteria of COPD are the critical predictor for pulmonary complications in esophageal cancer patients undergoing esophagectomy. Severity of COPD affects the incidence rate of the pulmonary complication, and percent-predicted FEV1 is a good predictive variable for pulmonary complication in patients with COPD. Arterial blood gases are helpful in directing perioperative management.

Key words: Chronic obstructive pulmonary disease; Arterial blood gas; Esophageal cancer; Complication

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INTRODUCTION

Radical esophagectomy remains the most effective method in patients with esophageal cancer, and the five-year survival rate of 40% or higher can be achieved[1-3]. However, esophagectomy may be one of the greatest surgical operations. Postoperative pulmonary complications (POPCs) are common after esophagectomy[4,5].

Chronic obstructive pulmonary disease (COPD) is a common fatal disease in China. Postoperative pulmonary complication after thoracotomy is the major complication in patients with COPD[6-8].

COPD is considered as an important risk factor for pulmonary complications due to low cardiopulmonary reserve[9-12]. However, the incidence of each type of complications and its relationship with percent-predicted FEV1 after esophagectomy in patients with COPD are unclear.

Our study was to document the effect of COPD on complication rates and the incidence of various types of pulmonary complications in esophageal cancer patients with or without COPD following esophagectomy, to elucidate the relationship between percent-predicted FEV1 and pulmonary complications and to evaluate the change and significance of arterial blood gases after operation in patients with COPD.

MATERIALS AND METHODS

A total of 358 patients who underwent esophagectomy at Beijing Friendship Hospital and Peking University First Hospital between July of 2001 and March of 2005 were included in this study. Eighty-six of the 358 patients were diagnosed having COPD.

We divided COPD into 4 grades: grade Ⅰ: percent predicted FEV1%p ≥ 80 and FEV1/FVC< 70%(45 cases); grade Ⅱ A: 50% ≤ FEV1%p < 80%, FEV1/FVC < 70% (32 cases); grade Ⅱ B: 30% ≤ FEV1%p < 50%, FEV1/FVC < 70% (9 cases); gradeⅢ: FEV1%p < 30%, FEV1/FVC < 70% (0 case).

Table 1 summarizes the patient characteristics. The two groups were similar in terms of age, sex, presence of other medical conditions, type and duration of operation, cancer stage, anastomosis site, blood loss and serum albumin. All patients underwent radical resection of tumors in the middle or lower third thoracic esophagus which were confirmed to be squamous cell cancers of esophagus after surgery. In our study, patients with tumor of the cervical and upper third thoracic esophagus were excluded because of different oncological characteristics and treatment protocols.

Basic hematological and biochemical tests, pulmonary function tests, electrocardiograph, chest CT scan, abdominal ultrasonography, barium contrast study and endoscopy were carried for all patients.

Patients were advised to stop smoking and to quit of alcohol two weeks prior to operation. Patients with hypercapnia and pulmonary hypertension were excluded. All patients in our study did not receive preoperative chemotherapy and chemoradiotherapy.

Esophagectomy via a left thoracotomy approach or cervical left thoracotomy approach was performed. Reconstruction of intestinal continuity was restored with a stomach placed in the left thoracic cavity or via orthotopic route when the anastomosis was carried out in the neck. The circular stapler was used when anastomosis was performed in the thoracic cavity and a hand-sewn anastomosis was done when it was in the neck. All patients received intravenous nutrition and continuous gastrointestinal decompression after esophagectomy.

All patients were followed up after surgery and complications occurring during the patient hospitalization were recorded. For this study, pulmonary complications were defined to include: pneumonia (manifesting fever, productive cough, increased white blood cell count, and marked infiltration on chest roentgenogram), atelectasis (manifesting segmental or lobar's atelectasis on chest roentgenogram without bronchial stenosis), pulmonary abscess (displaying intrapulmonary air containing space on chest roentgenogram and purulent exudation in the pleural cavity with fever and increased white blood cell count requiring drainage and antibiotic therapy), prolonged O_2 supplement (protracted supplemental oxygen≥14 d), acute respiratory distress syndrome(ARDS; PaO_2:FiO_2 ratio less than 250 for more than 24 h with pulmonary infiltrates, without clinical suspicion of volume overload, deterioration of respiratory status needing mechanical ventilatory support), and prolonged mechanical ventilation ≥2 d[4,6,9,13]. Postoperative pulmonary complications studied included pulmonary parenchyma but not complications of pleural cavity such as hemothorax, pneumothorax, thoracic abscess, chylothorax, pleural effusion, mediastinal emphysema.

Table 1 Baseline characteristics of study population with or without COPD undergoing transthoracic esophagectomy

Characteristics	COPD (*n* = 86, %)	Non-COPD (*n* = 272, %)	*P* value
Age, yr	61.3±5.5	63.2±7.1	0.522
Sex (male:female)	63/23	191/81	0.638
Smoking history	79 (91.9)	214 (78.7)	< 0.01
Past medical history			
Hypertension	16 (18.6)	48 (17.6)	0.840
Cardiac disease	13 (15.1)	35 (12.9)	0.594
Diabetes	10 (11.6)	33 (12.1)	0.900
Cancer stage			
Ⅰ	17 (19.8)	56 (20.6)	0.869
Ⅱ	48 (55.8)	145 (53.3)	0.685
Ⅲ	21 (24.4)	71 (26.1)	0.115
Ⅳ	0	0	-
Site of anastomosis			
Neck	32 (37.2)	108 (39.7)	0.679
Chest	54 (62.8)	164 (60.3)	0.679
Duration of operation (min)	162.75±51.05	185.15±66.24	0.239
Blood loss (mL)	365.50±219.36	434.00±232.48	0.344
Low serum albumin (<35g/L)	25 (29.1)	89 (32.7)	0.526
Spirometry			
FEV1 (L)	1.6±0.3	2.3±0.5	< 0.01
FEV1,% predicted	51.5±10.5	80.4±13.1	< 0.01
FEV1/FVC,%	57.6±8.9	73.3±6.8	< 0.01
DLCO,% predicted	83.7±13.4	85.0±13.9	0.774

FEV1 = forced expiratory volume in 1 s; FVC = forced vital capacity; DLCO = diffusion capacity of the lung for carbon monoxide.

Arterial blood gases were tested daily from first preoperative day to the seventh postoperative day at 4-5pm. Patients with respiratory failure or mechanical ventilation 7 d prior to operation were excluded in order to avoid intervention. Statistical analysis was performed using *t-test*, *ANOVA* and *chi-square test*. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using the SPSS statistical package (version 10.0, SPSS Inc., Chicago, IL).

RESULTS

Postoperative pulmonary complications such as pneumonia and atelectasis occurred early after operation. Pulmonary complications in the 358 patients with or without COPD are shown in Tables 2 and 3. Patients with COPD (29/86, 33.7%) had more pulmonary complications than those without COPD (36/272, 13.2%) ($P < 0.01$), Pneumonia (15/29, 51.7%), atelectasis (13/29, 44.8%), prolonged O_2 supplement (10/29, 34.5%), prolonged mechanical ventilation (8/29, 27.6%) were the major complications in COPD group.

The rate of mortality was 3.5% (3/86) in the COPD group and 66.7% (2/3) of deaths were due to respiratory failure. In patients without COPD, the rate of mortality was 1.5% (4/272) and two patients (50%) had respiratory failure as their cause of death.

Table 2 Pulmonary complications occurring in patients with or without COPD undergoing transthoracic esophagectomy

Pulmonary complications	COPD (n = 86)	Non-COPD (n = 272)	P value
Pneumonia	15	19	<0.01
Atelectasis	13	15	<0.01
Pulmonary abscess	4	5	0.227
Prolonged O_2 supplement	10	11	<0.01
Prolonged mechanical ventilation	8	10	<0.05
Acute respiratory distress syndrome	5	4	<0.05

Table 3 Degree of COPD and outcomes in patients with or without POPC in COPD group

Severity of COPD	POPCs (n = 29)	Non-POPCs (n = 57)	P value
FEV1,%predicted			
Grade Ⅰ (>80%)	10	35	<0.05
Grade Ⅱ A (50%-80%)	13	19	
Grade Ⅱ B (30%-50%)	6	3	
GradeⅢ (<30%)	0	0	

POPCs = postoperative pulmonary complications.

Table 4 Comparison of PaO_2 changes in the first postoperative week in patients with or without POPCs in COPD group (mean±SD)

Time	POPCs	Non-POPCs	P
1 preoperative day	82.17±7.37	82.75±4.45	0.758
1 postoperative day	68.38±5.24	76.00±8.28	<0.01
2 postoperative day	61.83±7.03	69.05±7.78	<0.01
3 postoperative day	61.75±6.14	67.20±7.35	<0.05
4 postoperative day	62.71±4.93	70.15±9.34	<0.01
5 postoperative day	61.13±5.62	72.75±10.30	<0.001
6 postoperative day	61.83±6.49	76.55±9.62	<0.001
7 postoperative day	61.63±6.31	80.45±7.50	<0.001

PaO_2 = partial pressure of oxygen.

Table 5 Comparison of $PaCO_2$ changes in the first postoperative week in patients with or without POPCs in COPD group (mean±SD)

Time	POPCs	Non-POPCs	P
1 preoperative day	42.85±4.10	42.37±6.03	0.766
1 postoperative day	44.42±4.61	41.24±4.93	<0.05
2 postoperative day	45.35±4.97	41.38±5.19	<0.05
3 postoperative day	46.02±4.88	42.45±5.62	<0.05
4 postoperative day	46.01±5.56	39.10±3.59	<0.001
5 postoperative day	44.63±5.31	40.62±5.44	<0.05
6 postoperative day	45.95±6.56	42.03±5.60	<0.05
7 postoperative day	45.18±5.80	40.49±6.13	<0.05

$PaCO_2$ = partial pressure of carbon dioxide.

Table 6 Comparison of SaO_2 changes in the first postoperative week in patients with or without POPCs in COPD group (mean±SD)

Time	POPCs	Non-POPCs	P
1 preoperative day	94.48±1.93	94.44±1.51	0.940
1 postoperative day	94.56±1.18	93.78±1.80	0.095
2 postoperative day	94.01±1.94	94.30±1.39	0.572
3 postoperative day	95.39±1.15	94.56±1.75	0.077
4 postoperative day	94.65±1.27	95.31±1.15	0.079
5 postoperative day	94.94±1.39	94.26±1.81	0.176
6 postoperative day	94.88±1.60	94.96±1.74	0.868
7 postoperative day	95.02±1.16	94.61±1.90	0.399

SaO_2 = arterial oxygen saturation.

Moreover, patients with severe COPD (Grade Ⅱ B, 30% ≤ FEV1%p < 50%) had more POPCs than patients with moderate (grade Ⅱ A, 50% ≤ FEV1%p < 80%) and mild (grade Ⅰ, FEV1%p ≥ 80%) COPD (P < 0.05).

The perioperative changes in arterial blood gases in patients with or without POPCs in COPD group are listed in Tables 4-6. In non-POPC group PaO_2 decreased in the first three days after operation and then gradually returned to its normal level. Values for $PaCO_2$, SaO_2 and pH were in normal range. However, in POPC group, PaO_2 dropped significantly, recovered more slowly, and failed to return to normal at the end of the first week compared with non-POPC group. $PaCO_2$ in POPC group was significantly higher than that in non-POPC group in the first 7d, reaching more than 6Kpa in first postoperative week. In addition, there was no significant difference in SaO_2 and pH between the two groups.

DISCUSSION

Transthoracic esophagectomy displays a remarkable effect on pulmonary function, including lung and chest wall compliance reduction, ventilation function reduction, increase of oxygen consumption[14]. Pulmonary complication is considered as one of the most serious and threatening complications after esophagectomy, and is associated with poor short- and long-term outcomes[9,15]. Postoperative pulmonary complications occur frequently after transthoracic esophagectomy for esophageal cancer, accounting for 7.3%-50%[9,13,16-18]. In addition to surgical techniques and perioperative management strategies, different definition criteria for pulmonary complications, patient selection and willingness of surgeons to undertake high-risk cases may influence the outcomes[4,19,20].

COPD is considered as a postoperative pneumonia risk index and is significantly associated with the occurrence of pulmonary complications[21,22]. According to our definition, a higher rate of pulmonary complication is associated with esophageal resection for esophageal cancer with COPD, particularly in patients with percent-predicted FEV1 less than 50%. Pneumonia, atelectasis' prolonged O_2 supplement and prolonged mechanical ventilation are the major complications after esophagectomy. The incidence rate of pulmonary complication in COPD group was higher than that in non-COPD group. The incidence rates of acute respiratory distress syndrome and pulmonary abscess were lower. Moreover, pulmonary complications are associated with postoperative mortality and regarded as the most common cause of operation death.

To assess the independent effect of COPD, we matched

patients with severe COPD to comparison groups of patients with moderate and mild COPD. The rate of pulmonary complication increased along with percent-predicted FEV1 reduction, suggesting that percent-predicted FEV1 is a good risk factor for anticipating postoperative pulmonary complications after esophagectomy.

Previous studies indicate that various factors predispose to pulmonary complications[4,10,23-27], including advanced age, history of smoking, cirrhosis and diabetes, abnormal chest radiograph or lung disease, blood loss and low serum albumin, preoperative chemoradiotherapy, general performance status, inadequate postoperative analgesia and stage of disease. Some cell factors have a relation with pulmonary complications after esophagectomy, such as secretary leukocyte protease inhibitor and angiotensin-converting enzyme[28,29]. Our analysis also demonstrated a correlation between percent-predicted FEV1 and pulmonary complication in patients with esophagectomy.

The changes of arterial blood gases coincided well with the timing of pulmonary complications in our patients which occurred in the first postoperative week. The pathophysiologic feature of this group was a further depression and patients had a poor prognosis. However, pH values and oxygen saturation had no remarkable change in the two groups.

Our study was not designed to determine if perioperative care could decrease the rate of pulmonary complications in patients with COPD after esophagectomy, but we believe that aggressive treatment is important in improving the outcome of pulmonary complication so that patients with COPD benefit from radical procedure. It was reported that effective treatment can reduce pulmonary complications[13]. Rehabilitation training, proper antibiotics and eliminating phlegm's drugs, atomization, stopping smoking, nutritional supplementation may have some benefits to patients with COPD before operation[30-32]. Regulation of intravenous transfusion volume can effectively prevent postoperative pulmonary edema and improve oxygenation[33].

In conclusion, chronic obstructive pulmonary disease is the critical factor for the occurrence of postoperative pulmonary complications in esophageal cancer patients undergoing esophagectomy. Pulmonary complications go up along with the severity of COPD. Percent-predicted FEV1 is a good predictor for postoperative pulmonary complications. Arterial blood gases are helpful in directing perioperative management.

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GASTRIC CANCER

Extremely well-differentiated adenocarcinoma of the stomach: Clinicopathological and immunohistochemical features

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Abstract

AIM: Minimal deviation carcinoma of the uterine cervix, otherwise known as extremely well-differentiated adenocarcinoma (EWDA), is characterized by its benign microscopic appearance in contrast to its aggressive behavior. In order to elucidate the clinicopathological features and biological behavior of the gastric counterpart of EWDA, we, using immunohistochemistry, analyzed nine lesions for the phenotypic expression, proliferative activity, and the expression of oncogene-associated products.

METHODS: Clinicopathological features, including preoperative biopsy diagnosis, were reviewed. Using immunohitstochemistry, Ki-67 labeling index and expression of p53 and c-erbB-2 protein in the gastric lesions were detected.

RESULT: Locations in the middle or upper third of the stomach and polypoid macroscopic features are characteristic of EWDA of the stomach. Although 4 of the 9 lesions showed only focal lymphatic or venous invasion, lymph node metastasis was not present and none of the patients died of the lesions (mean follow-up period, 56 mo). All 9 cases of EWDA could be classified into gastric phenotype (5 lesions) and intestinal phenotype (4 lesions). The former resembled gastric foveolar epithelium, mucous neck cells or pyloric glands, but their papillary structures were frequently elongated and the tumor cells and their nuclei were slightly larger and more hyperchromatic compared to normal epithelium. The latter resembled intestinal metaplasia with minimal nulcear atypia and irregular glands; two of these lesions demonstrated complete intestinal phenotype, while two demonstrated incomplete intestinal phenotype. Ki-67 labeling index was low and none of the cases revealed over-expression of p53 and c-erbB-2 protein.

CONCLUSION: Unlike minimal deviation carcinoma of the cervix, these findings suggest that EWDA of the stomach is a lesion of low-grade malignancy. This favorable biological behavior is supported by the data of a low Ki-67 labeling index and a lack of p53 or c-erbB-2 protein over-expression. Because of its resemblance to normal gastric mucosa or mucosa with intestinal metaplasia, EWDA is often misdiagnosed. To prevent the misdiagnosis of such lesions, the clinical and pathologic characteristics should be taken into consideration.

Key words: Stomach neoplasms; Extremely well-differentiated adenocarcinoma; Ki-67; p53; c-erbB-2

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INTRODUCTION

Silverberg and Hurt proposed the term "minimal deviation carcinoma" for extremely well-differentiated adenocarcinoma (EWDA) of the uterine cervix[1], which has a benign microscopic appearance yet shows an aggressive behavior[1-3]. This carcinoma is characterized by mucinous glands which resemble normal endocervical glands but invade the cervical stroma. Several similar cases of adenocarcinomas which show deceptively benign appearance have been reported in the stomach[4-12]. In those reports, the difficulty with histological diagnosis based on biopsy specimens is well discussed in detail; however, the biological behavior of the lesions still remains unclear.

Based on Lauren classification[13], gastric carcinomas

have been classified into two types, intestinal-type and diffuse-type. Following recent advances in mucin histochemistry and immunohistochemistry, it has been clarified that differentiated adenocarcinoma can be classified into two subtypes, these being gastric and intestinal phenotypes[14-20]. With regard to EWDA of the stomach, Endoh *et al*[11] reported eight cases of EWDA mimicking complete-type intestinal metaplasia, confirmed by phenotypic investigation using immunohistochemical methods. Most reported cases of EWDA of the stomach seem to be intestinal-type carcinomas, resembling complete or incomplete intestinal metaplasia[4-10]. In addition, we have encountered a few reports of cases of EWDA mimicking normal gastric mucosa, where the cases were considered to be lesions of the gastric phenotype. However, in these cases, there was very little objective investigation of the phenotypic expression.

In order to elucidate the characteristics of EWDA including its biological behavior, we describe herein the clinicopathological features of nine cases, including phenotypic expression, proliferative activity and expression of some oncogene-associated products.

MATERIALS AND METHODS

Patients

EWDA is defined as neoplastic lesions composed of highly differentiated neoplastic epithelium which mimicks the normal gastric mucosa or intestinal metaplastic mucosa with mild nuclear atypia, but has the ability to invade the gastric wall. We retrospectively reviewed 3106 cases from our old consecutive files that had been diagnosed as well-differentiated adenocarcinoma of the stomach, and found three (0.1%) cases of EWDA. Other 6 collected cases of EWDA were added, making a total of 9 cases for this study. One of the reported cases[7] was included in this study. Although we encountered some similar lesions restricted to the mucosa, these were excluded because of difficulty in diagnosing them as malignant.

The clinicopathological findings were principally based on the General Rules for Gastric Cancer Study as outlined by the Japanese Research Society for Gastric Cancer[21]. Eight specimens were obtained by surgery, and one was endoscopically resected. The resected specimens were fixed in 100 mL/L buffered formalin. The early lesions were cut many times throughout the entire tumors, whereas the advanced lesions were cut only once through their center. The sections were then embedded in paraffin. Then 4-μm thick sections were routinely stained with hematoxylin and eosin stain (H&E). In addition, pre-operative biopsy specimens were also reviewed.

Immunohistochemistry

The monoclonal antibodies against human gastric mucin (45M1, Novocastra, Newcastle-upon-Tyne, UK, diluted 1:50) as a marker for gastric foveolar cells[22,23], MUC6 (Novocastra, Newcastle-upon-Tyne, UK, diluted 1:200) as a marker of gastric mucous neck cells and pyloric glands[24,25], MUC2 (Novocastra, Newcastle-upon-Tyne, UK, diluted 1:200) as a marker for intestinal goblet cells[26-28], CD10 (Novocastra, Newcastle-upon-Tyne, UK, diluted 1:200) as a marker for the small intestinal brush border[29-31], Ki-67 (MIB-1, dilution 1:100; Immunotech, Marseille, France), p53 (PAb 1801, dilution 1:100; Oncogene Research Products, Cambridge, Massachusetts, USA) and c-erbB-2 (dilution 1:200; Nichirei, Tokyo) were used. Immunohistochemical staining was carried out using streptavidin-biotin-peroxidase complex method (Histofine SAB-PO Kit, Nichirei, Tokyo, Japan) following antigen retrieval with microwave heating (citrate buffer, 30 min; phosphate-buffered saline, 10 min, respectively) utilizing an H2800 Microwave Processor (Energy Beam Sciences, Agawan, Massachusetts, USA) at 800 W. Sections were visualized with diaminobenzidine (DAB) and counterstained with methyl-green or hematoxylin. The negative controls consisted of substituting mouse normal serum for the primary antibodies.

Table 1 Phenotypic classification by immunohistochemical stains

		Human gastric mucin or MUC6	
		(-)	(+)
CD10 (+)		C - type	
CD10 (-)	MUC2 (+)	I - type	
	MUC2 (-)	U - type	G - type

C: complete intestinal, I: incomplete intestinal, G: gastric, U: unclassified.

Evaluation

The positivities of human gastric mucin (HGM), MUC6, MUC2 and CD10 were estimated as being significantly positive when more than 10% of the area was positive-stained. According to the combination of their expression, phenotypes were classified into four types: gastric type (G-type), incomplete intestinal type (Incomp. I-type), complete intestinal type (Comp. I-type), and unclassified type (Table 1)[20].

The Ki-67 (MIB-1) labeling index (LI) was defined as a percentage of MIB-1-positive nuclei, and was evaluated in the invasive areas. The MIB-1 LI was determined by counting at least 1000 nuclei in the selected fields at x400 magnification. p53 immunoreactivity was defined as positive when distinct nuclear staining was recognized in at least 10% of the cells, since most of the previously published studies employed this as the cut-off level. Cases with less than 10% positive cells were regarded as negative. c-erbB-2 was regarded as positive when there was membranous staining in more than 10% of the area of the tumor.

RESULTS

Histologic findings and phenotypic expression

All the EWDA had invaded the submucosa or even deeper; four were restricted to the submucosa, two had invaded the muscularis propria, and three had reached the subserosa beyond the muscularis propria. The EWDAs were classified into gastric phenotype (HGM+ or MUC6+/MUC2-/CD10-) containing 5 cases and

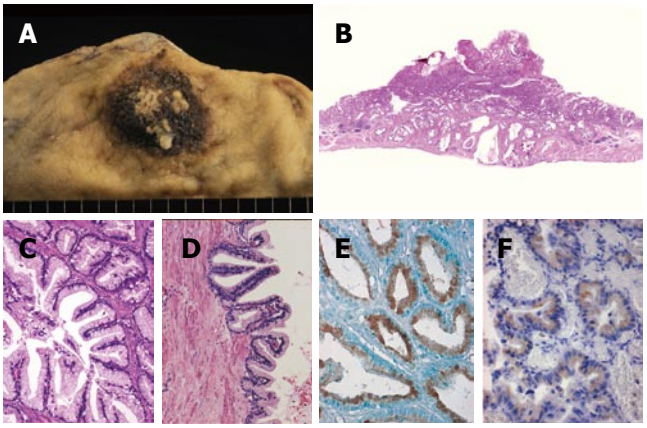

Figure 1 Extremely well-differentiated adenocarcinoma of the stomach, gastric-type (case 4). **A:** Macroscopic view showing a polypoid lesion with an irregular surface; **B:** cancer invasion of the whole thickness of the gastric wall (low-power view); **C:** carcinoma mimicking the normal gastric foveolar epithelium with basally located small nuclei (hyperchromatic nuclei) and abundant mucin; **D:** papillary projections occasionally seen in the carcinomatous glands; **E:** diffuse positive staining of human gastric mucin in carcinomatous glands; and **F:** focally positive staining of MUC6 in carcinomatous glands.

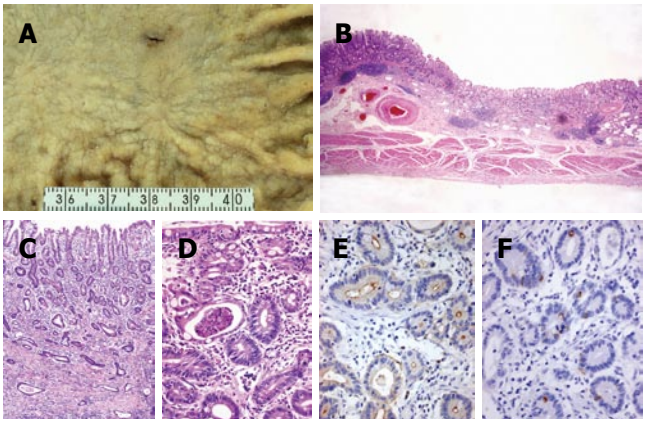

Figure 2 An early lesion of extremely well-differentiated adenocarcinoma of the stomach, complete intestinal-type (case 8). **A:** Macroscopic view showing a shallow depressed lesion with an irregular margin; **B:** carcinoma invasion to the submucosal layer (low-power view); **C** and **D:** carcinoma mimicking the intestinal metaplasia of complete-type with basally located small nuclei, eosinophilic cytoplasm and scattered goblet cells. Note the irregular arrangement of glands and intraluminal debris; **E:** CD10 positivity of carcinomatous glands along the luminal surfaces; and **F:** MUC2 positivity of scattered goblet cells.

intestinal phenotype containing 4 cases. The intestinal phenotype cases were further classified into complete intestinal phenotype (HGM- / MUC6- / MUC2 + / CD10+) and incomplete intestinal phenotype (HGM- / MUC6- / MUC2 + / CD10-), each phenotype contained 2 cases. With regard to MUC6 expression which indicates differentiation to the pyloric glands, MUC6 expression was only detected in 2 of 5 cases of the gastric phenotype.

The five lesions classified as gastric phenotype were composed of well-differentiated epithelium mimicking foveolar epithelium, mucous neck cells or pyloric glands with abundant clear cytoplasm and basally situated nuclei. With careful observation, the nuclei were seen to be slightly larger than those of normal gastric mucosa, and to be markedly hyperchromatic. The superficial area

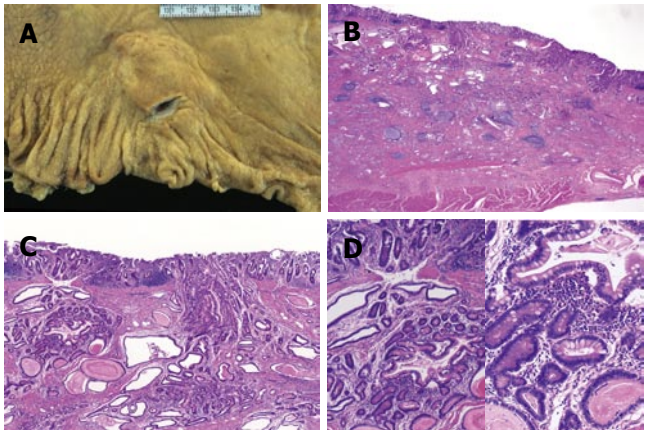

Figure 3 An advanced lesion of extremely well-differentiated adenocarcinoma of the stomach, complete intestinal-type (case 9). **A:** Macroscopic view showing a polypoid mass with an irregular surface, but unclear margin; **B:** cancer invasion of the whole thickness of the gastric wall (low-power view); **C:** carcinomatous gland infiltrating into the submucosa; **D:** carcinoma mimicking the intestinal metaplasia of complete-type with basally located small nuclei, eosinophilic cytoplasm and scattered goblet cells.

tended to resemble the foveolar epithelium while the deep area tended to resemble mucous neck cells or pyloric glands. Two lesions mainly showed remarkable papillary proliferation. The epithelium was lined with a single layer of columnar cells with abundant clear cytoplasm with basally situated nuclei. The glands in this phenotype showed intraluminal papillary projections with or without a fibrous core. In the invasive area, one of five revealed marked desmoplastic reaction, however, other four revealed only slight desmoplastic reaction. One of the cases of gastric-type EWDA is shown in Figure 1.

The four lesions classified as intestinal phenotype were composed of intestinal-type glands with various amounts of goblet cells and Paneth cells, focally showing an irregular shape. Brush border-like structures were occasionally seen, and were confirmed by CD10 staining in the two cases classified as complete intestinal phenotype. We found difficulty in diagnosing these lesions as neoplastic in the mucosa because their glands were somewhat regular in shape and their cytologic atypia was minimal. However, their glands were of varying sizes and showed irregular branching in the deep portion of the mucosa and the submucosa. The glands in the submucosa or proper muscle layer were surrounded by an acute, chronic inflammatory infiltrate with lymphoid follicles. Occasionally, cystically dilated gland was seen in the submucosa, and mucous which had partially leaked out into the stroma owing to destruction of the glands, was seen in these three lesions. In the invasive area, all the four cases revealed marked desmoplastic reaction. Two cases of intestinal-type EWDA, early and advanced, are shown in Figures 2 and 3.

Regarding the background mucosa of the tumors, the surrounding mucosa could not be examined in one gastric phenotypic lesion because the lesion had been endoscopically resected. In another gastric phenotypic lesion, no intestinal metaplasia was seen. As for the other seven cases, various degrees of intestinal metaplasia were

Table 2 Clinicopathological data

Case	Age	Sex	Loc	Size	Macro	Depth	ly	v	LN	Prognosis	Biopsy
Gastric phenotype											
1	81	m	M	5.5	0-I	sm	(+)	(-)	NA	5 mo, alive	benign
2	51	m	U	2.5	0-IIa	sm	(-)	(-)	(-)	23 mo, alive	NA
3	63	m	M	8	1	ss	(-)	(-)	(-)	66 mo, alive	benign
4	76	m	U	3.5	1	ss	(-)	(+)	(-)	30 mo, alive	Ca, susp
5	57	m	U	5	1	ss	(+)	(-)	(-)	48 mo, alive	NA
Intestinal phenotype (incomplete intestinal-type)											
6	65	f	M	1.5	0-IIa	sm	(+)	(-)	(-)	38 mo, alive	Ca, susp
7	45	m	M	2.4	1	mp	(-)	(-)	(-)	129 mo, alive	Ca, susp
(complete intestinal-type)											
8	54	m	M	2.2	0-IIc	sm	(-)	(-)	(-)	136 mo, alive	Ca, susp
9	65	m	M	4	1	mp	(+)	(+)	(-)	30 mo, alive	Ca, susp

Loc: location (U: upper third, M: middle third), Macro: macroscopic feature, Depth: depth of invasion (sm: submucosa, mp: muscularis propria, ss: subserosa), ly: lymphatic permeation, v: venous invasion, LN: lymph node metastasis, NA: not assessed, Ca, susp: carcinoma, suspected.

Table 3 Previously reported cases of gastric EWDA

Case	Author		Age	Sex	Macro	Location	Size	Depth	ly	v	n	Prognosis	Phenotype	Biopsy
1	Araki	(1984)	50	m	1	M	45	ss	1	0	0	?	Incomp-I?	benign
2	Satoh	(1987)	65	m	1	U	40	mp	0	0	0	?	Comp-I?	benign
3	Yaosaka	(1989)	53	m	1	M	80	mp	2	0	1	?	Comp-I?	benign
4	Matsunaga	(1995)	42	m	0-I	L	45	sm	2	0	0	?	?	reg. Atypia
5	Kobayashi	(1999)	55	m	1	M	20	ss	0	0	0	?	Comp-I	Ca, susp
6	Endoh	(1999)	60	f	0-IIa+IIc	M	10	sm	0	0	0	?	Comp-I	Ca
7	Endoh	(1999)	68	f	0-IIc+IIb	M	20	sm	0	0	0	?	Comp-I	Ca
8	Endoh	(1999)	70	m	0-IIb	M	27	sm	0	0	0	?	Comp-I	NA
9	Endoh	(1999)	62	f	0-IIc+IIa	M	15	sm	0	0	0	?	Comp-I	NA
10	Endoh	(1999)	59	m	0-IIa	M	15	sm	0	0	0	?	Comp-I	Ca
11	Endoh	(1999)	74	m	0-IIa+IIc	L	18	sm	0	0	0	?	Comp-I	NA
12	Endoh	(1999)	70	m	0-IIa+IIc	M	25	sm	0	0	0	?	Comp-I	benign
13	Endoh	(1999)	65	m	1	M	55	se	0	0	0	?	Comp-I	benign
14	Adachi	(2000)	54	f	0-I	M	40	sm	?	1	0	?	I?	benign
15	Sato	(2004)	50	m	2	M	48	ss	2	1	0	?	Mixed	benign

Incomp-I: incomplete intestinal, Comp-I: complete intestinal, I: intestinal, reg. atypia: regenerative atypia (benign), NA: not assessed, Ca: carcinoma, Ca, susp: carcinoma, suspected, Macro: macroscopic type Location (U: upper third, M: middle third, L: lower third) Depth: depth of invastion (sm: submucosa, mp: muscularis propria, ss: subserosa).

seen in the surrounding mucosa of both the gastric and intestinal phenotypes.

Patient characteristics

The clinicopathological findings of the nine patients with EWDA of the stomach are summarized in Table 2. The patients included eight men and one woman with ages ranged from 45 to 81 (average 62) years. There were no patients who were diagnosed as Peutz-Jeghers syndrome. None of the patients died or suffered recurrence during the follow-up periods which ranged from 5 to 136 (average, 56) mo.

Macroscopic findings

The tumors had a maximum diameter of 1.5 to 8 (average, 3.6) cm. Of the nine lesions, three tumors were located in the upper third of the stomach, the remaining six were located in the middle third. No lesions were present in the lower third. Among the four early lesions (restricted to the submucosa), two lesions were of superficial elevated (Type 0-IIa) type while the others were of superficial depressed (Type 0-IIc) type or protruding (type 0-I) type. All the advanced lesions (invading the muscularis mucosa and/or the subserosa) were of polypoid type (Type 1).

Pre-operative biopsy

Pre-operative biopsy specimens could be evaluated only in seven cases because of unavailability of specimens in two cases. Two of the seven cases were diagnosed as benign lesions, and the remaining five were initially suspected as being carcinomas, although there was difficulty in distinguishing whether they were neoplastic or regenerative lesions. Only one lesion could be finally diagnosed as a definite carcinoma through repeated biopsy (Case 9).

Ki-67, p53 and c-erbB-2 expressions

None of the cases of EWDA revealed over-expression of p53 or c-erbB-2. Regarding the proliferating activity, the mean value of Ki-67 LI of the EWDAs was 8.7% (range, 0.5%-23.9%).

DISCUSSION

Gastric carcinomas, based on Lauren classification, have been divided into two histologic types by standard hematoxylin and eosin (H&E) staining, such as

"intestinal" and "diffuse" types[13]. It has been considered that intestinal-type carcinoma is almost equivalent to differentiated type carcinoma and that diffuse-type carcinoma is almost equal to gastric or undifferentiated type carcinoma. However, gastric carcinomas are currently classified according to the expression of gastric or intestinal phenotypes, using immunohistochemical or mucin-histochemical methods[14,18]. Accordingly, we divided gastric carcinomas into three phenotypes (complete-intestinal type, incomplete-intestinal type and gastric type) according to the type of intestinal metaplasia, as suggested in previous studies[19,20], using immunohistochemical methods for CD10 (CALLA) which is considered to be expressed by the brush border of the small intestine[29,31], MUC2 which is considered to be expressed by intestinal goblet cells[26,28] and human gastric mucin (HGM) which is considered to be expressed by the gastric foveolar epithelial mucin [22,23]. Some authors have also reported that the phenotypic expression is related to the tumor growth pattern and aggressiveness[32,33], and that this classification is clearly in a good accordance with that of the background mucosa[19]. In this study, the phenotype of EWDA of the stomach was investigated using not only these three antibodies, but also MUC6 as a marker of gastric mucous neck cells and pyloric glands[24,25]. Our nine cases of EWDA could be classified into three phenotypes (complete-intestinal type, incomplete-intestinal type and gastric type).

Clinicopathologically, EWDA of the stomach had several characteristic features, in comparison with the previously reported cases of EWDA listed in Table 3[4-6,8-12]. The location and macroscopic features of the tumors are characteristic. Usually, more than 40% of the gastric carcinomas are located in the distal part of the stomach and polypoid type is rare (3.3%) among advanced gastric carcinomas, as reported by our previous study [34]. All EWDA in our study and most (13/15) ones of the previous reports were located in the middle and upper stomach. Macroscopically, the early lesions of EWDA were flatly elevated (0-IIa) or depressed (0-IIc), while all the advanced lesions were polypoid. The same tendency was also seen in the previous reports. This finding implies that the EWDA arises as a flat lesion but latter grows into a polypoid mass due to massive infiltration of carcinoma cells beneath the mucosa.

The gastric phenotype of gastric EWDA is more likely to be confused with normal gastric mucosa or hyperplastic polyps, whereas the intestinal phenotype of gastric EWDA is more likely to be confused with intestinal metaplastic epithelium. The high degree of differentiation and mild cellular atypia of these lesions result in frequent diagnostic difficulties especially with regard to biopsy specimens prior to surgery. In fact, pre-operative biopsies were negative in eight of 12 cases in previous reports and in two of our current seven cases. These highly differentiated lesions of the stomach have received relatively limited attention. Although EWDA of the stomach is very rare, it is important to take it into consideration when making a differential diagnosis of neoplastic or dysplastic lesions in the stomach.

The histological features of EWDA with regard to pre-operative biopsy specimens and surgical specimens were retrospectively reviewed. The most useful histological feature in diagnosing the intestinal phenotype of EWDA is the irregularity of the tubules in the deep portion of the mucosa and the submucosa. It is therefore important to obtain biopsy specimens from these areas. Moreover, endoscopic mucosal resection by means of which we can examine the entire thickness of the mucosal layer may be a suitable diagnostic procedure in diagnosing intestinal phenotypic lesions. In the cases of gastric phenotype of EWDA, the neoplastic epithelium resembled gastric surface mucous cells or pyloric glands. Their papillary structure was similar to that of normal foveolar epithelium and hyperplastic polyps, although some of them strikingly elongated. In addition, the individual cells and nuclei were obviously larger and their nuclei were more hyperchromatic than those in normal foveolar epithelium. Since the cellular atypism is minimal, it is important to compare their size and the amount of chromatin with that in the surrounding normal epithelium.

There have been no reports about the biological behavior of EWDA, although a low incidence of lymphovascular invasion and lymph node metastasis has been noted (Table 3). As for our cases, all the patients are currently alive. Three lesions revealed only focal venous or lymphatic invasion, but no lymph node metastasis was seen in our cases of EWDA. These findings suggest a favorable prognosis for EWDA of the stomach unlike the prognosis for minimal deviation adenocarcinoma of the uterine cervix, although it needs to be noted that our series was small with limited follow-up data. With regard to the correlation between phenotypes and clinicopathological features, there was no significant difference between the two except for tumor location. Three of the five EWDAs of gastric phenotype were located in upper third of the stomach, whereas all the EWDAs of intestinal phenotype were located in the middle third.

The proliferative compartment in normal gastric mucosa is known to be restricted to the middle layer of the mucosa. Several reports have indicated the relationship between a high Ki-67 LI and poor prognosis in cases of gastric carcinoma[35,36]. In our present study, the Ki-67 LI was lower (average, 8.7%) compared with the previously reported data(from 41.8% to 47.1%)[35-38]. A low Ki-67 LI (13%) in the submucosal invasive area has also been reported by Endoh *et al*[11], and this finding reflects the slow growth and reduced aggressiveness of EWDA of the stomach.

The reported prevalences of abnormal expression of p53 and c-erbB-2 protein have been shown to range from 47% to 60%[39-42] and from 5.7% to 33.0%[43-46], respectively. It has been reported that the over-expression of p53[37-39] and c-erbB-2 protein[43] is a marker of poor prognosis in gastric carcinoma. Fortunately, none of the lesions of EWDA showed over-expression of p53 or c-erbB-2 in our study. It seems reasonable to regard these lesions as having a low-grade malignancy, but an ability to invade downward into the gastric wall, a finding which is supported by the data of low Ki-67 LI and no over-expression of p53 or c-erbB-2. In addition, these immunoreactivities of p53 and c-erbB-2 seemed to be useless for the diagnosis of EWDA.

In the practical diagnosis of a stomach biopsy, it is important to bear in mind the existence of extremely well-differentiated adenocarcinoma (EWDA) of both intestinal and gastric types.

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Anticancer and cytotoxic properties of the latex of *Calotropis procera* in a transgenic mouse model of hepatocellular carcinoma

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Abstract

AIM: To evaluate the anticancer property of the dried latex (DL) of *Calotropis procera*, a tropical medicinal plant, in the X15-*myc* transgenic mouse model of hepatocellular carcinoma and to elucidate its mechanism of action in cell culture.

METHODS: The young transgenic mice were orally fed with the aqueous suspension of DL (400 mg/kg for 5 d/wk) for 15 wk and their liver was examined for histopathological changes at 20 wk. Serum levels of vascular endothelial growth factor (VEGF) were also measured in these animals. To characterize the active fraction, DL was extracted with petroleum ether followed by methanol. The methanolic extract was sub-fractionated on a silica gel G column using a combination of non-polar and polar solvents and eleven fractions were obtained. Each fraction was analysed for cytotoxic effect on hepatoma (Huh7) and non-hepatoma (COS-1) cell lines and non-transformed hepatocytes (AML12) using tetrazolium (MTT) assay. Finally, the mechanism of cell death was investigated by measuring the levels of Bcl2, caspase 3 and DNA fragmentation.

RESULTS: DL treatment of mice showed a complete protection against hepatocarcinogenesis. No adverse effect was observed in these animals. The serum VEGF level was significantly lowered in the treated mice as compared to control animals. Cell culture studies revealed that the methanolic extract of DL as well as its fraction 8 induced extensive cell death in both Huh-7 and COS-1 cells while AML12 cells were spared. This was accompanied by extensive fragmentation of DNA in Huh-7 and COS-1 cells. No change in the levels of canonical markers of apoptosis such as Bcl2 and caspase 3 was observed.

CONCLUSION: DL of *C. procera* has the potential for anti-cancer therapy due to its differentiable targets and non-interference with regular pathway of apoptosis.

Key words: *Calotropis procera*; Transgenic mice; Hepatocellular carcinoma; Cytotoxicity; Anticancer agent; Differential killing

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INTRODUCTION

The incidence of cancer is increasing worldwide and it is the single most common cause of deaths in both developed and developing countries[1,2]. Irrespective of the cause, the localized malignant tumors are best managed by surgical removal[3,4] while the treatment options for advanced and metastasized tumors include chemotherapy and radiotherapy[5-7]. However, in view of the side effects of drugs used in the chemotherapy of different cancers, traditional herbal medicine and complementary and alternative medicine (CAM) are becoming increasingly popular among cancer patients in the developed countries[8,9]. In the traditional Indian medicinal system, the Ak plant or *Calotropis procera* (Ait.) R. Br. (*Asclepiadaceae*) has been used for a variety of disease conditions that includes its use in the treatment of leprosy, ulcers, piles and tumors[10]. The root extract of *C. procera* has been found to produce a strong cytotoxic effect on COLO 320 tumor cells[11]. Recently, a hemi synthetic derivative of a cardenolide isolated from the root barks of *C. procera* shows a strong cytotoxic effect on several human cancer lines, a high *in vivo* tolerance to tumor growth and prolonged survival in the human xenograft models of nude mice[12]. The chloroform-soluble

fraction of its roots, ethanolic extract of its flowers and aqueous and organic extracts of its dried latex (DL) also exhibit a strong anti-inflammatory activity in animal models of acute and chronic inflammation[13-15]. Further, recent epidemiological studies have shown that daily intake of non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and ibuprofen results in a significant exponential decline in the risk of different clinical cancers[16]. In view of this, we have evaluated the cytotoxic and anti-tumor activities of the latex of *C. procera* respectively on hepatoma and non-hepatoma cell lines, and in a transgenic mouse model of hepatocellular carcinoma (HCC).

MATERIALS AND METHODS

Collection of latex and its fractionation

C. procera growing in wild was identified by the Raw Materials, Herbarium and Museum Division, National Institute of Science Communication, New Delhi, where a voucher specimen is preserved (Voucher No. PID1739). The latex was collected from the aerial parts of the plant and dried under shade (DL). DL was extracted with petroleum ether (B.P. 40-60 ℃) and methanol in a sequential order. The methanol extract (ME) was subjected to silica gel G (mesh 60-120) step column chromatography using a combination of non-polar and polar solvents and eleven fractions were collected[17]. The fractions were evaporated to dryness and tested for cytotoxic properties.

Chemicals and Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and Caspase 3 assay kit were procured from Invitrogen (California, USA). Dimethylsulfoxide (DMSO) and (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (Missouri, USA). Silica gel G, petroleum ether (PE) and other chemicals were bought from Merck, India.

Treatment of oncomouse

The X15-*myc* transgenic mice expressing HBx and c-*myc* genes in the liver and thereby resulting in hepatocellular carcinoma[18], were used for this study following approval of the Institutional Animal Ethics Committee. Animals of matched age (5 wk old)) of either sex were used in this study. The overnight fasted animals were fed with bread soaked in aqueous suspension of the DL at a dose of 400 mg/kg for 15 wk (5 d/wk, $n = 6$). The control animals were given bread alone ($n = 6$). At 20 wk, the animals of both groups were sacrificed and their liver was collected in buffered formalin (100 mL/L). Paraffin blocks of samples were made and the tissue sections were stained with hematoxylin and eosin to observe histopathological changes.

Estimation of vascular endothelial growth factor (VEGF)

The serum level of VEGF was measured in the mice using a mouse specific ELISA kit (Oncogene Research Products, Germany).

Cell culture and cytotoxicity assay

The human hepatoma Huh-7 cell line[19] was a kind gift from Dr. A. Siddiqui (University of Colorado, Denver). COS-1 (African green monkey kidney cell line, CRL-1 650) and AML-12 cells (mouse hepatocyte, CRL-2 254) were purchased from the American Type Culture Collection (Virginia, USA). All cultures were grown in DMEM without phenol red ($DMEM^{-PR}$) supplemented with fetal bovine serum (100 mL/L), L-glutamine, penicillin and streptomycin and maintained at 37℃ in a CO_2 incubator. The cytotoxic effect of ME and each DL fraction was evaluated in cell cultures where the cells were seeded at a density of 4×10^5 cell/60 mm dish and allowed to settle for 12 h. Then these cells were incubated with different fractions of DL at concentrations ranging from 0.1-10 mg/L for either 24h or 48h. Cell viability was analyzed by MTT colorimetric assay[20]. Briefly, after treatment with the DL fractions, the cells were washed with culture medium and incubated with MTT solution (1 mg/mL in $DMEM^{-PR}$) for 1h at 37 ℃. The medium was decanted, the formazan product was dissolved in 1mL DMSO and the absorbance was read at 560 nm.

DNA fragmentation assay

Extract of the cells treated with DL fraction 8 was prepared in TET buffer (10 mmol/L Tris-HCl, pH 7.4, 5 mmol/L EDTA, 5 mL/L Triton X100) and incubated with proteinase K (40 mg/L) at 37 ℃ for 1h. After extraction with an equal volume of phenol, followed by phenol and chloroform, DNA was precipitated using ethanol. The DNA pellet was resuspended in H_2O and incubated with RNase A (40 mg/L) at 37 ℃ for 1h before electrophoretic separation in a 15 g/L TBE agarose gel[21].

Bcl-2 and caspase assay

The intracellular level of Bcl-2 was measured by immunoprecipitation using anti-Bcl-2 antibody (Santa Cruz Biotechnology, California, USA). The relative caspase activity was measured using the caspase 3 assay kit (Invitrogen, USA). The cell extract was prepared as per supplier's protocol and the caspase activity was measured in 50 μL aliquots by incubating with the substrate for 2 h at 37 ℃ and the absorbance was read at 405 nm.

Statistical analysis

The values are expressed as mean ± SE ($n = 6$) and the statistical analysis was performed by Student's t test. $P < 0.05$ was considered significant.

RESULTS

DL exhibits in vivo chemopreventive effect

The chemopreventive effect of orally administered DL was studied in the X15-*myc* transgenic mice. Histological examination of the liver of untreated mice at 20 wk showed a marked nuclear atypia, hyperchromatia, necrosis and loss in sinusoidal architecture (Figure 1A). Treatment of mice with DL (400 mg/kg) for a period of 15 wk protected these mice from malignant changes occurring in the liver while sinusoidal architecture and cellular integrity was slightly disrupted as compared to normal and hydropic changes were observed (Figures 1B and 1C). We further

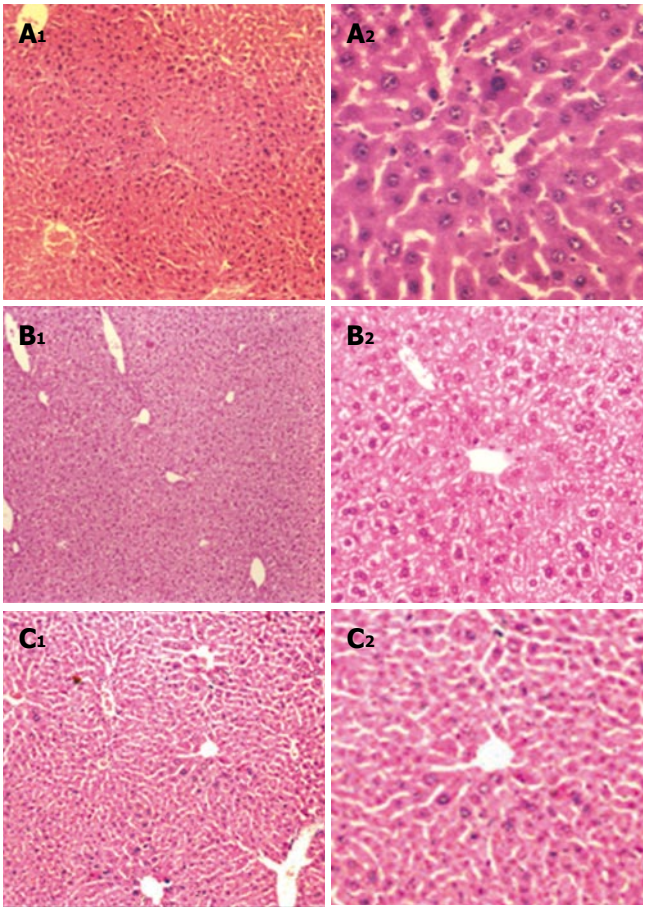

Figure 1 Histological analysis of mouse liver. **A**: X15-myc control; **B**: X15-myc mouse treated with DL suspension; **C**: Normal mouse liver. (**A**$_1$, **B**$_1$, **C**$_1$ = x100 and **A**$_2$, **B**$_2$, **C**$_2$ = x400).

measured the levels of VEGF, a marker of angiogenesis, in these mice. Treatment with DL produced a significant decrease in the serum VEGF levels of X15-*myc* mice from 12.18 ± 0.64 μg/L to 9.75 ± 0.27 μg/L (P = 0.002) while the level of VEGF in normal mice was 7.00 ± 0.55 μg/L.

DL is cytotoxic to cancer cells

Since the aqueous extract of DL showed a strong *in vivo* chemopreventive effect, the molecular basis of its action was investigated in cell culture. The ME of DL was evaluated for cytotoxicity using MTT assay on two different cell lines, viz., Huh-7 and COS-1 cells (Figure 2). ME induced cell death in both cell lines in a concentration dependent manner. Even at low concentration (0.1 mg/L), ME could induce cell death in both the cell lines. The effect was, however, more pronounced (-90% cell death) at higher doses of ME (1 and 10 mg/L).

A polar fraction of DL contributes to its cytotoxic effect

To identify the active component of DL, ME was subjected to silica gel G column chromatography and eleven fractions were collected[17]. Each fraction was evaluated for its cytotoxic activity on Huh-7 and COS-1 cells at 10 mg/L concentration and cell viability was determined by MTT assay (Figure 3). Out of 11 fractions, only fraction 8 exhibited a potent cytotoxic effect on both Huh-7 and COS-1 cells (~90% cell death) and the effect was comparable to ME (input). A marginal inhibitory effect was also seen with fraction 1 on Huh-7 cells (-24% cell death).

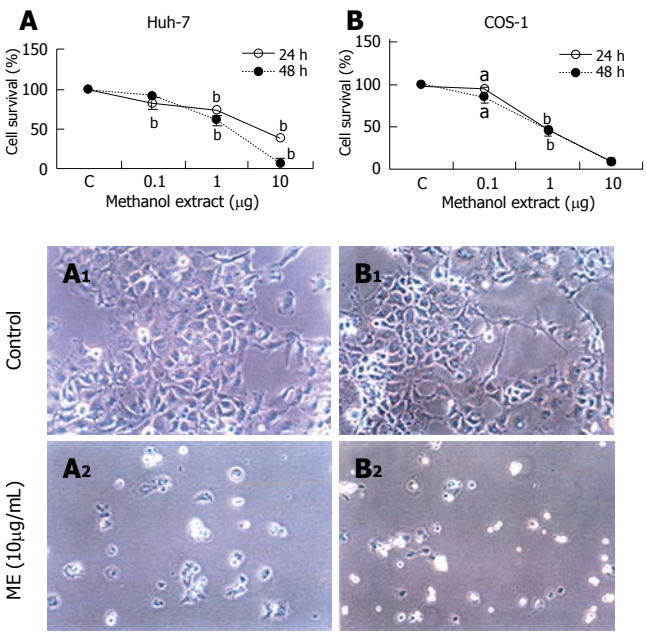

Figure 2 Cytotoxic effect of methanolic extract of DL on cancer cell lines. Huh-7 (**A**) and COS-1 cells (**B**) were incubated with different concentrations of ME and analyzed for cell viability at 24h or 48h (n = 6), mean ± SE. $^aP < 0.001$; $^bP < 0.01$; **A**$_1$ and **B**$_1$ are control Huh-7 and COS-1 cells; **A**$_2$ and **B**$_2$ are ME-treated Huh-7 and COS-1 cells (x200).

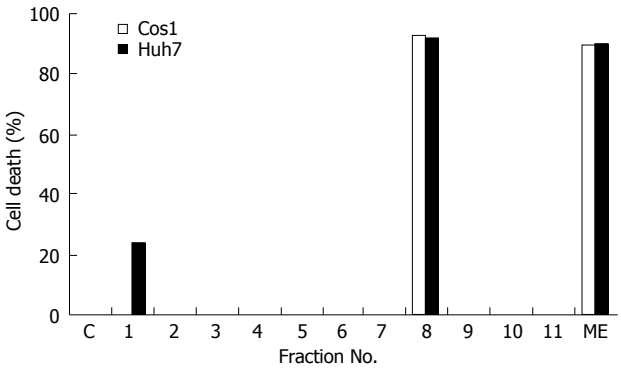

Figure 3 Cytotoxic effect of different fractions of the methanolic extract of DL on cancer cell lines. Huh-7 and COS-1 cells were incubated with either ME or its eleven fractions (all at 10 mg/L). Cell viability was measured at 48 h and results are expressed as % cell death.

We further investigated the relationship between the dose-response and cytotoxic effect of DL fraction 8 on Huh-7, COS-1 and non-transformed AML12 cells. At concentration ranging from 0.1 to 10 mg/L, it produced a dose-dependent decrease in the survival of Huh-7 and COS-1 cells leaving behind only 20%-30% living cells at 10 mg/L concentration. However, unlike the two cancer cell lines, AML12 cells showed a much better survival (-80%) in the presence of fraction 8 (Figure 4 A-C).

Fraction 8 of DL induces DNA fragmentation

To understand the mechanism of DL-induced cell death, the canonical markers of apoptosis like Bcl-2 and caspase 3 were measured in Huh-7 cells after treatment with fraction

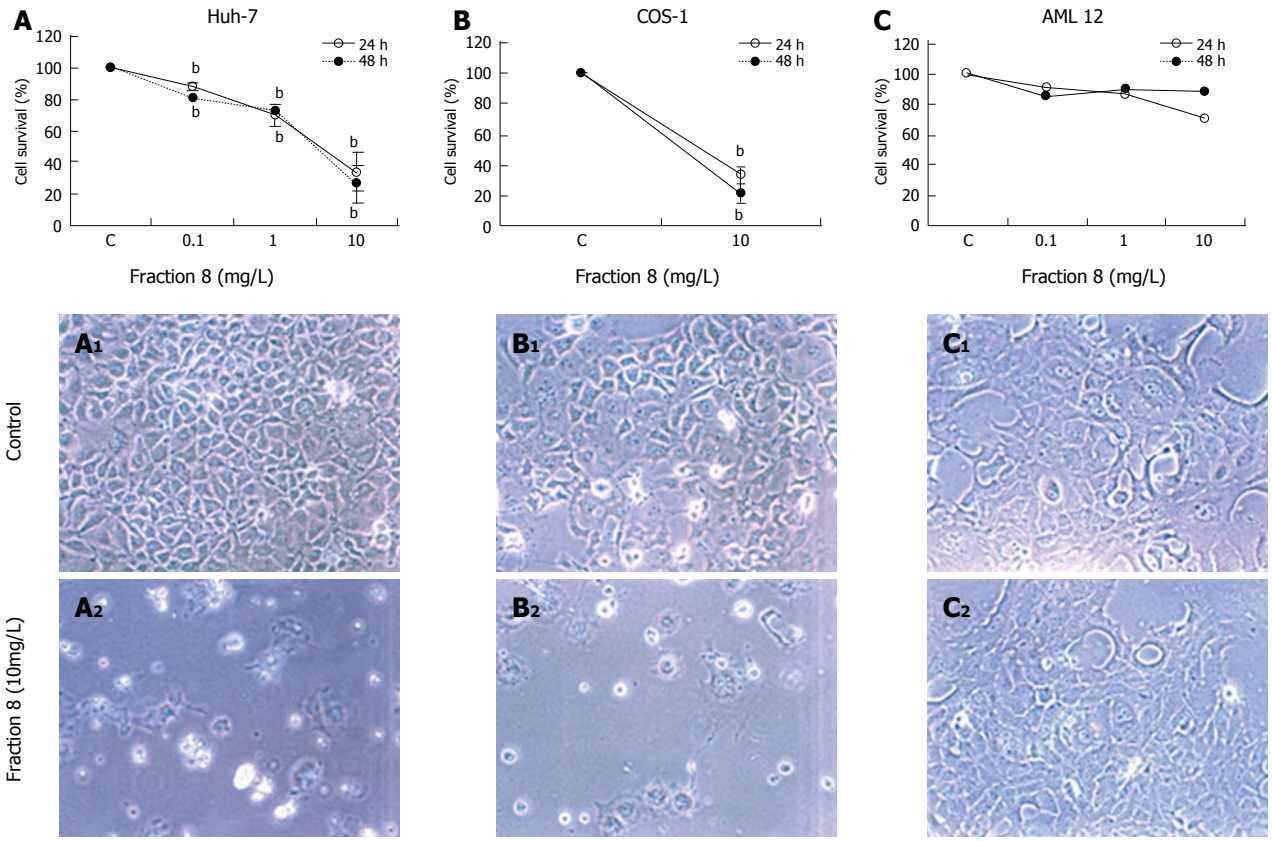

Figure 4 Cytotoxic effect of fraction 8 of the DL methanolic extract on different cell lines. Huh-7 (**A**), COS-1 (**B**) and AML12 cells (**C**) were incubated with different concentrations of fraction 8 and cell viability was measured at 24 and 48 h (mean ± SE, $^{b}P < 0.01$). **A**$_1$, **B**$_1$ and **C**$_1$ are control Huh-7, COS1 and AML12 cells; **A**$_2$, **B**$_2$ and **C**$_2$ are fraction 8 treated Huh-7, COS-1 and AML12 cells (x200).

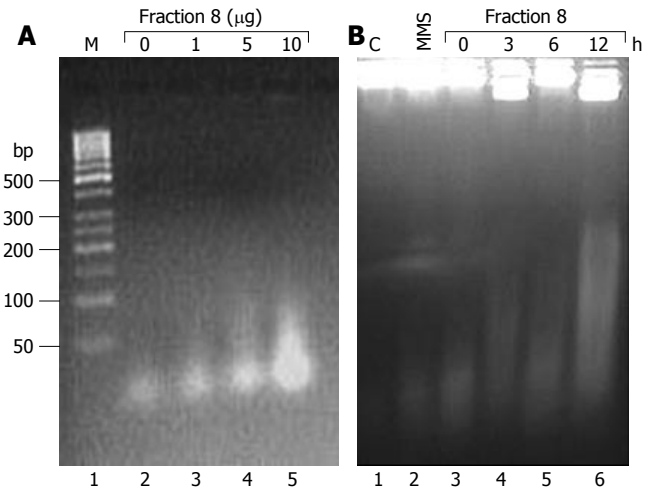

Figure 5 Effect of fraction 8 on DNA fragmentation in Huh-7 cells. Huh-7 cells were treated either with different concentrations (1, 5 and 10 mg/L) of fraction 8 for 3 h (**A**) or for 3, 6 and 12 h with 10 mg/L of fraction 8 (**B**). Total DNA was extracted after proteinase K and RNase A treatment and resolved by agarose gel electrophoresis. C: DNA from control cells; M: 100 base pair (bp) ladder; MMS, DNA from cells treated with methyl methanesulfonate (2 g/L) for 15 min.

8. Surprisingly, no change in the levels of both markers was observed (data not shown). Nonetheless, there was a marked increase in the fragmentation of cellular DNA that was both concentration as well as time dependent thereby suggesting a rise in the cellular nuclease activity (Figure 5).

DISCUSSION

The latex of *C. procera* has been shown to possess potent anti-inflammatory and antioxidant properties. In this study we have tested the latex for its chemopreventive and *in vitro* cytotoxic properties. The study was carried out in an mouse model of HCC developed by the integration of a chimeric transgene having hepatitis B virus X and murine c-*myc* genes exhibiting liver-specific neoplastic changes that are associated with inflammation and angiogenesis[18]. Five days a week oral administration of DL to the X15-myc mouse produced a marked chemopreventive effect as revealed by histological analysis. The mitotic changes produced by transgene expression were inhibited at the dose studied though some hydropic changes were observed. This was accompanied by inhibition of cellular infiltration. The DL might be producing chemopreventive effects through inhibition of different mediators of inflammation[15]. The pro-inflammatory cytokines and reactive oxygen and nitrogen species are known to activate signaling molecules involved in inflammation and carcinogenesis. These include nuclear transcription factor NF-κB, inducible nitric oxide synthetase (iNOS) and cyclooxygenase-2 (COX-2)[22]. The elevated levels of tumor COX-2 have been reported to correlate with invasiveness and elevated VEGF levels that brings about neovascularization and progression of HCC[23,24]. In fact, a recent study has revealed that COX-2 expression correlates with VEGF expression and microvessel density in HCC caused by hepatitis B virus[25]. It is interesting to note that DL not only inhibited

the inflammatory changes, but also produced a significant decrease in VEGF levels as observed with other anticancer drugs[26-28].

We further evaluated the cytotoxic effects of DL on hepatoma (Huh-7), non-hepatoma (COS-1) and non-cancerous (AML12) cell lines and observed that the cytotoxic activity was associated with one of the polar fractions of DL, i.e., fraction 8. Like total methanolic extract, fraction 8 also showed a strong cytotoxic effect on both transformed cell lines used here. However, a marginal effect on the killing of AML12 cells suggested a high degree of selectivity for transformed cells. Such differential killing of cancerous cells could relate to their altered metabolic status and/or membrane properties. In fact selective killing of cancerous cells by chemotherapeutic drugs like methotrexate and polyunsaturated fatty acids has been reported earlier as well[29,30]. Thus, it would be interesting to investigate the mechanism of such target selectivity by DL. The cytotoxic effect of DL was accompanied by intracellular fragmentation of target cell DNA. We observed a dramatic increase in the fragmentation of Huh-7 cells upon incubation with DL fraction 8. Though the action was rapid, it was not accompanied by expression of the common markers of intrinsic pathway of apoptosis such as Bcl-2 or caspase 3. Since Bcl-2 and caspases are markers of mitochondria-mediated apoptotic death, the genomic DNA can still undergo fragmentation by cellular DNase activity independent of mitochondrial pathway[31]. Hence a possible role of hypoxia and free radical-dependent activation of DNase activity or direct DNA damage and consequent degradation of cellular DNA[32] as evident here, cannot be ruled out. Thus, our study suggests that the latex of *C. procera* possesses an activity that has a chemopreventive action *in vivo* and cytotoxic action on cancer cell lines. Further study would be necessary to demonstrate its efficacy in HCC treatment.

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Usefulness of liver infiltrating CD86-positive mononuclear cells for diagnosis of autoimmune hepatitis

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Abstract

AIM: Although the pathogenic mechanism underlying autoimmune hepatitis (AIH) remains unclear, the immune system is thought to be critical for the progression of the disease. Cellular immune responses may be linked to the hepatocellular damage in AIH. Recently, much attention has been focused on the critical functions of costimulatory molecules expressed on mononuclear cells in the generation of effective T cell-mediated immune responses. Analysis of costimulatory molecule expressed on mononuclear cells from the patients with AIH may give us insight into the pathogenic mechanism of hepatocellular damage in AIH.

METHODS: Peripheral blood mononuclear cells (PBMC) were taken from the patients with AIH (34 cases) and healthy controls (25 cases). Liver infiltrating mononuclear cells (LIMCs) were taken from the patients with AIH (18 cases), the patient with chronic hepatitis C (CH-C) (13 cases) and the patients with fatty liver (2 cases). Using flow cytometry, the cells were analyzed for the expression of costimulatory molecules, such as CD80, CD86, and CD152 (CTLA-4). The results were compared with clinical data such as the level of gammaglobulin, histological grade, presence or absence of corticosteroids administration and the response to corticosteroids.

RESULTS: The levels of CD80+, CD86+ and CD152+ PBMC were significantly reduced in the patients with AIH as compared with healthy controls. By contrast, those cells were significantly higher in LIMC than in PBMC of the patients with AIH. Especially, the level of CD86+ LIMC showed a marked increase irrespective of the degree of disease activity in the patients with AIH, although CD86+ cells were rarely present in PBMC. The levels of CD86+ cells were present in significantly higher frequency in patients with AIH than in the patients with CH-C. Furthermore, the patients with AIH with high levels of CD86+ LIMC showed good responses to corticosteroids, whereas 2 cases of AIH with low levels of CD86+ LIMC did not respond well.

CONCLUSION: These results suggest that LIMC over-expressing costimulatory molecules such as CD80 and CD86 appears to play a role in the pathogenesis of AIH. Especially, CD86 molecule expressed on the LIMC may be useful for the diagnosis of AIH and for the prediction of the therapeutic effects of corticosteroids on AIH.

Key words: Autoimmune hepatitis; Costimulatory molecule; CD86 molecule; Peripheral blood mononuclear cells; Liver infiltrating mononuclear cells; Flow cytometry

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INTRODUCTION

In autoimmune liver diseases including autoimmune hepatitis (AIH) and primary biliary cirrhosis (PBC), autoimmune mechanisms are thought to affect the disease progression and manifestation. Although the mechanism of hepatocellular damage in AIH and PBC is still unclear, it is speculated that the immunological disorder is linked to host immunological targeting of hepatocytes in the liver[1-3]. It has been recently reported that polymorphism of cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and the susceptibility to AIH and PBC were associated[4]. Among T cell-mediated immune responses, especially those mediated by CD4+ or CD8+ T cells, are likely to be associated with the hepatocellular damage in AIH[5,6]. We have previously reported that the expression of bcl-2, an anti-apoptotic molecule, in CD4+ Th1 cells was increased in peripheral blood and in the liver of the patients with

AIH[7]. Moreover, it was reported that T cells bearing certain T cell receptor clonotypes were expanded in the liver of the patients with AIH[8]. Although extensive efforts have been made to identify the target antigens expressed on the hepatocyes in AIH, the real target antigen has not been identified yet.

For the generation of effective T cell-mediated immune responses, much attention has been paid on the critical functions of a series of costimulatory molecules expressed on the cell surface of T or B cells. The T cell receives signals from antigen-presenting cells (APC) through the interaction of the T cell receptor (TCR) and major histocompatibility complex (MHC) in an antigen-dependent manner. However, these signals alone are insufficient to generate effective T cell-mediated immune responses and often lead to T cell anergy. The aberrant expression of costimulatory molecules was reported in some liver diseases other than AIH[9-14]. Although it is clear that the costimulatory molecules play a crucial role in T cell activation, few reports have addressed the role of these costimulatory molecules in AIH. In the present study, to elucidate the role of costimulatory molecules on mononuclear cells in AIH, some of the costimulatory molecules expressed on PBMC and LIMC were analyzed using flow cytometry and the results were evaluated in terms of the clinical status of the patients with AIH.

MATERIALS AND METHODS

Patients

Thirty-four patients with AIH serologically and histologically diagnosed at Kagawa University Hospital were enrolled in the present study. All patients had anti-nuclear antibody (ANA) in their sera and 19 of 34 (55.88%) patients showed hyper-gammaglobulinemia (> 2.0 g/dL). Based on the criteria proposed by International Autoimmune Hepatitis Group, all patients satisfied the score over probable AIH. PBMCs were analyzed in 34 patients with AIH. LIMCs were also analyzed in 18 of 34 patients at the liver biopsy for diagnosis. As a disease control, PBMC and LIMC of thirteen patients with chronic hepatitis C (CH-C) were analyzed. Twenty-five healthy individuals without any symptoms of liver injury were selected as normal controls for the analysis of PBMC. All of these studies were conducted with informed consent at the time of the enrollment for this study in all patients.

Flow cytometric analysis of PBMC

PBMCs from patients and healthy individuals were separated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation (Histopaque; Sigma Chemical Co., St Lois, MO). Immediately after separating PBMC, cells were washed twice in ice-cold phosphate-buffered saline (PBS), and adjusted to 4×10^5 cells per well of a 96-well U-bottom cell culture plate in flow microfluorometry medium [FMF medium: Hanks' balanced salt solution (without Ca, Mg and phenol red) containing 2 g/L bovine serum albumin, and 1 g/L sodium azide]. After centrifugation at 1200 r/min for 5 min, the supernatant was removed and cells were incubated for 30 min with FITC-conjugated or phycoerythrin (PE)-conjugated antibodies. The combinations of antibodies used were anti-CD80-FITC (Pharmingen, San Diego, USA)/anti-CD152-PE (Pharmingen), anti-CD86-FITC (Pharmingen). FITC-conjugated IgG (Dainippon Pharmaceutical Co., Ltd. Osaka, Japan) and PE-conjugated IgG (Dako A/G, Denmark) were used as the negative controls. After being washed twice, the pellets were resuspended with FMF medium, and the fluorescence detections were performed with a flow cytometer, COULTER EPICS XL. Prior to the analysis, FL1, FL2 and color compensation were adjusted so that no CD4CD8 double-positive cells were detected. The gate was set for accumulation of PBMC and ten thousand events were acquired for each analysis. Flow cytometric analysis was done immediately after isolating PBMC.

Flow cytometric analysis of LIMC

Liver specimens from the 18 patients with AIH, 10 patients with CH-C and 2 patients with fatty liver were obtained using a 16 G biopsy needle for diagnosis. Most of the patients with AIH underwent laparoscopy to obtain the liver specimen and observe the change of the liver surface for diagnosis. After incubating the liver biopsy specimen in RPMI-1640 containing 1 g/L collagenase for 2 h to destroy the connective tissue, LIMCs were separated by Ficoll-Hypaque density gradient centrifugation at 3000 r/min for 10 min. Isolated LIMCs were stained with FITC- or PE-conjugated antibodies and analyzed using flow cytometry. The cells positive for CD69, a marker for activated T cells, were also analyzed in LIMC. An anti-CD69-PE (Dako Japan A/G) was used. Three thousand events were acquired for each analysis.

Clinical markers

Levels of alanine aminotransferase (ALT) (IU/L) and gammaglobulin (g/dL) and titer of ANA were monitored. These laboratory data were compared with the level of LIMC positive for costimulatory molecules obtained in the present study. Inflammation and fibrosis in histological analysis was graded according to the classification documented by Knodell *et al*[15]. Histological staging and grading were classified based on the classification documented by Desmet *et al*[16].

Statistical analysis

Statistical analysis was performed using Macintosh software, Statview II (version 4.2) and a Mann-Whitney *U* test (non-parametric analysis). $P < 0.05$ was considered statistically significant.

RESULTS

Flow cytometric analysis of PBMC

For the analysis of costimulatory molecules, we focused on several costimulatory molecules critical for the induction of effective T and B cell-mediated immune responses. Initially, we examined the expression of those molecules on PBMC in patients with AIH. The level of positive cells for each surface molecule on PBMC was compared between the patients and healthy controls (Table 1). The results revealed that the patients with AIH had significantly

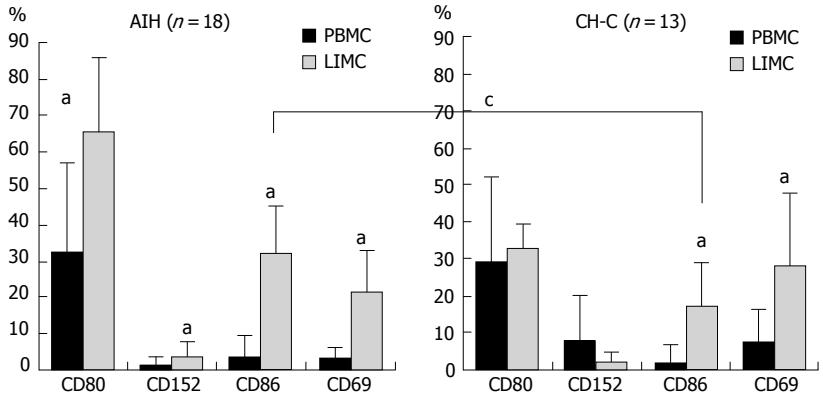

Figure 1 Isolation of PBMCs and LIMCs from peripheral blood and liver biopsy specimens in the patients with AIH and CH-C by using density gradient centrifugation. The cells were stained with anti-CD80, anti-CD86, anti-CD152 and anti-CD95 antibodies and analyzed using flow cytometry. The percentage of cells positive for those costimulatory molecules is shown. CD80+, CD86+ and CD152+ cells were significantly higher in LIMCs compared to PBMC in patients with AIH. The difference of CD86+ cells between LIMCs and PBMCs was more striking than that of CD80+ cells between LIMCs and PBMCs. (a) represents the significant difference ($P < 0.05$) from the data of PBMCs and (c) shows the significant difference ($P < 0.05$) of LIMCs between AIH and CH-C.

Table 1 Analysis of costimulatory molecules expressed on PBMCs (mean ± SD)

	Control n = 25	AIH Patients n = 34	P value
CD80	41.3 % ± 7.04 %	32.7 % ± 23.5 %	0.008
CD86	1.15 % ± 1.19 %	0.78 % ± 0.75 %	0.04
CD152 CTLA-4	13.8 % ± 10.9 %	2.32 % ± 2.80 %	0.01

The P values calculated by a Mann-Whitney's U-test are shown. Compared to the healthy controls, CD80+, CD86+ and CD152+ PBMC of the patients with AIH are shown to be significantly fewer.

fewer CD80+, CD86+ and CD152+ (CTLA-4+) cells in PBMC as compared with the healthy controls. Among the cells analyzed, CD86+ cells were present in low frequency in PBMC of both the patients and healthy controls.

Flow cytometric analysis of LIMCs

CD80 and CD86 are expressed on professional APC and activated lymphocytes, and have unique expression pattern[17]. In the patients with AIH, the predominant location of inflammation and damage is seen in the liver. Therefore, it might be reasonable to think that those decreased cells in the blood, such as CD80+, CD86+ or CD152+ cells, accumulate in the liver and are likely to be important to analyze the expression of costimulatory molecules on LIMCs as well as on PBMCs. Although it may be necessary to compare the expression of costimulatory molecules on LIMCs between the patients and healthy controls, it is ethically difficult to take mononuclear cells from the liver of healthy controls. Therefore, the frequency of LIMC positive for each costimulatory molecule was compared between PBMC and LIMC in patients with AIH and CH-C in the present study (Figure 1). In both AIH and CH-C, the ratios of CD69+ cells in LIMCs were significantly higher than that in PBMCs, suggesting infiltration of many activated T cells into the liver in both AIH and CH-C. In AIH, the ratios of CD80+ and CD86+ cells were significantly higher in LIMCs compared to PBMCs. The difference of CD86+ cells between LIMCs and PBMCs was more striking than that of CD80+ cells between LIMCs and PBMCs. Although the ratio of CD152+ cells was significantly higher in LIMCs than that in PBMCs, both the ratios were very low. In CH-C, the ratio of CD86+ cells was significantly higher in LIMCs than that in PBMC, but the ratios of CD80+ and CD152+ cells in LIMC were not significantly different from those in PBMC. Although the ratios of CD86+ cells were significantly higher both in AIH and in CH-C, the ratio of CD86+ cells in LIMCs was significantly higher in AIH than that in CH-C. Taken collectively, the most dramatic and apparent difference between PBMCs and LIMCs was the marked increase of CD86+ cells in LIMCs of AIH. By contrast, the levels of CD86+ cells in LIMCs of patients with fatty liver were low (Table 2).

Relationship between the level of CD86+ LIMCs and clinical markers

Since the most dramatic change of frequency between PBMCs and LIMCs was the increase of CD86+ cells in patients with AIH, relationship between the level of CD86+ LIMC and the clinical parameters was examined in patients with AIH (Table 2). In most of the patients with AIH tested, the levels of CD86+ LIMC were elevated by more than 20%. Three patients (No. 1-3 in Table 2) were already being treated with corticosteroids when the biopsy was performed. Even in these 3 patients, the aberrant expression of CD86 molecule on LIMCs was observed. The level of CD80+ or CD86+ LIMC did not show any significant correlation with that of ALT [correlation coefficient (r): CD80 *vs* ALT = -0.160; CD86 *vs* ALT = -0.166]. Furthermore, no significant correlation of the level of CD86+ LIMC with the level of serum gammaglobulin, ANA titer and HAI score was observed. These results suggested that LIMCs in patients with AIH are over-expressing CD80 or CD86 molecule irrespective of the degree of hepatocellular damage or disease activity. Among 18 patients analyzed for the expression of costimulatory molecule on LIMCs, clinical course after the administration of corticosteroids could be followed up in 10 patients. Administration of corticosteroids was effective in decreasing the level of transaminase in 8 of 10 patients. All of these 8 patients showed the high levels of CD86+ LIMC (> 20%). By contrast, 2 patients who did not respond satisfactorily to corticosteroids showed low levels of CD86+ LIMC (11.1% and 5.9%, respectively).

Representative AIH cases reactive and non-reactive to corticosteroids

Clinical course, laparoscopic appearance of the liver surface and liver histology of two AIH cases reactive and non-reactive to corticosteroids are shown in Figures 2 and 3. The

Table 2 Analysis of CD86+ cells in liver infiltrating mononuclear cells

	γ -globulin (g/dL)	ALT (U/L)	ANA	Histology	HAI	CD86 (%)	CS	Reactivity to CS
No. 1	2.1	34	×40	CH (A0, F2)	9	44.0	On	Reactive
No. 2	1.1	55	×8	CH (A0, F1)	3	43.5	On	Reactive
No. 3	1.6	44	×20	CH (A2, F3)	13	27.5	On	NT
No. 4	2.0	151	×160	CH (A3, F3)	16	42.2	Off	Reactive
No. 5	2.0	99	×40	CH (A0, F1)	3	43.7	Off	NT
No. 6	2.0	105	×40	CH (A2, F2)	3	33.8	Off	NT
No. 7	2.3	71	×640	CH (A1, F1)	5	41.2	Off	NT
No. 8	2.4	18	×320	CH (A1, F2)	2	34.0	Off	NT
No. 9	1.6	50	×320	LC (A1, F4)	18	19.1	Off	NT
No. 10	2.7	84	×1280	CH (A2, F2)	14	11.1	Off	Non-reactive
No. 11	1.3	131	×5120	CH (A2, F2)	14	28.9	Off	Reactive
No. 12	2.4	93	×640	CH (A2, F2)	12	40.0	Off	Reactive
No. 13	1.7	363	×2560	CH (A2, F2)	15	27.3	Off	Reactive
No. 14	1.4	123	×40	CH (A1, F1)	2	5.9	Off	Non-reactive
No. 15	1.4	50	×20	LC (A3, F4)	18	25.7	Off	NT
No. 16	2.1	34	×20	CH (A2, F2)	12	29.4	Off	NT
No. 17	2.3	65	×40	LC (A2, F4)	20	24.1	Off	Reactive
No. 18	1.9	17	×1280	CH (A2, F3)	12	23.0	Off	Reactive
No. 19	1.2	45	(-)	Fatty liver	NT	11.8	Off	NT
No. 20	1.3	47	(-)	Fatty liver	NT	10.7	Off	NT

Comparison of the level of CD86+ LIMC and clinical data in patients with AIH (18 cases) and fatty liver (2 cases). LIMCs taken from the liver biopsy specimen of patients with AIH (18 cases) and 2 patients with fatty liver were stained with antibody against CD86 and analyzed by using flow cytometry. Three thousand events were acquired for each analysis. Patients with AIH showed high levels of CD86+ LIMC irrespective of the levels of γ-globulin, ALT, histological activity, and the presence or absence of the administration of corticosteroids (CS). Response to CS in patients with AIH was shown to be associated with the level of CD86+ LIMC. NT: not tested.

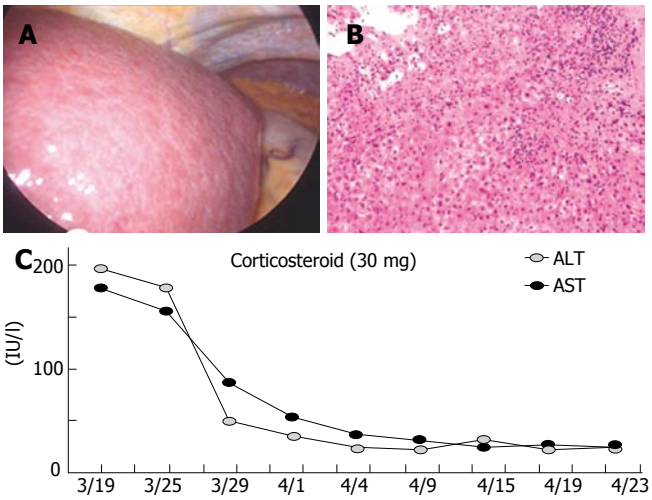

Figure 2 Laparoscopic findings, liver biopsy and clinical course after the administration of corticosteroids of a patient with AIH reactive to corticosteroids. **A:** Laparoscopic findings showing many reddish markings on the liver surface, indicating that the ongoing hepatitis was active; **B:** microscopic observation of the liver showing formation of rosette and bridging necrosis, infiltration of a large number of lymphocytes and plasma cells; and **C:** time-course of the levels of ALT and AST after the administration of corticosteroids. The levels of ALT and AST were rapidly decreased to normal ones after administration of corticosteroids.

former case was a 58-year-old female (No. 11 in Table 2) who showed a good response to the treatment of corticosteroids (Figure 2). At the time of diagnosis, she had ALT 131 IU/dL, AST 139 IU/dL, T-Bil 1.1 mg/dL, ALB 2.9 g/dL, IgG 2730 mg/dL and ANA ×5120. Laparoscopic findings of the liver showed many reddish markings on the surface of the liver, indicating that the ongoing hepatitis was active (Figure 2A). Histological findings were compatible to AIH showing the formation of rosette and bridging necrosis, infiltration of a large number of lymphocytes and many plasma cells (Figure 2B). The level of CD86+ LIMC was high, 28.9 %. The levels of ALT and AST were rapidly decreased to normal ones after the administration of corticosteroids (Figure 2C). By contrast, the latter case was a 59-year-old female (No. 10 in Table 2) who was not reactive to the treatment of corticosteroids (Figure 3). At the time of diagnosis, she had ALT 50 IU/dL, AST 29 IU/dL, T-Bil 1.9 mg/dL, ALB 2.9 g/dL, IgG 2880 mg/dL, and ANA ×1280. Laparoscopic findings of the liver showed many reddish markings and small lymph cysts on the surface of the liver (Figure 3A). Histological findings revealed the bridging necrosis, infiltration of a large number of lymphocytes and plasma cells (Figure 3B). The level of CD86+ LIMC was relatively low, 11%. Although the levels of ALT and AST were slightly improved after the administration of corticosteroids, those were elevated again despite the treatment.

As aforementioned, these 2 cases showed similar aspects of laboratory data, laparoscopic findings and histological findings. Nevertheless, one with a high level of CD86+ LIMC was responsive to corticosteroids, and the other with a low level of CD86+ LIMC was not. In these 2 cases, the differences were seen in the response to corticosteroids and in the level of CD86+ LIMC, but not in other clinical markers.

DISCUSSION

Although the mechanism of hepatocellular damage in AIH has not been well understood, many lines of evidence have demonstrated the presence of immunological disorders in AIH[5]. For example, wide ranges of circulating auto-antibodies are observed in the sera of patients with AIH[18-21]. Moreover, it has been shown that

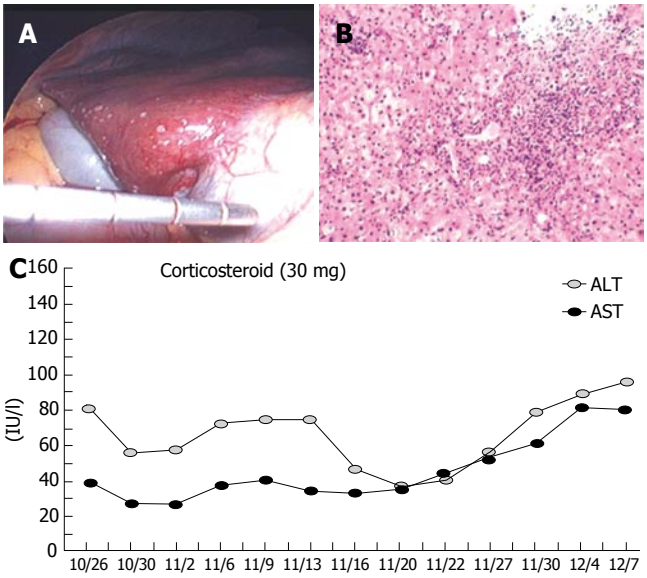

Figure 3 Laparoscopic findings, liver biopsy and clinical course after the administration of corticosteroids of a AIH patient non-reactive to corticosteroids. **A:** Laparoscopic view showing many reddish markings on the surface of the liver and small lymph cysts; **B:** microscopic findings of the liver showing bridging necrosis, infiltration of a large number of lymphocytes and plasma cells; and **C:** the time-course of the levels of ALT and AST after the administration of corticosteroids. The levels of ALT and AST were not improved to normal level after administration of corticosteroids.

the susceptibility to AIH was associated with HLA class II, DR3 and DR4 alleles[3,22,23]. Some reports have also suggested the association of cytotoxic T lymphocytes (CTL) with the hepatocellular injury in AIH using animal models[24-26].

Helper T cells (Th) receive a TCR signal from APC via HLA class II molecules. Helper T cells are divided into two subtypes, Th1 and Th2, according to their cytokine secretion profile[27]. Both Th1 and Th2 differentiate from Th0 cells. In the treatment of the patients with AIH, corticosteroids are effective in decreasing hepatocellular damage. This effect is thought to be due to the action of corticosteroids in inhibiting Th0 activation, leading to inhibition of CTL responses and antibody production[28,29]. CTL and Th receive signals from APC via the TCR-MHC complex[30]. However, this signal alone is not enough to activate T cells, and the expression of costimulatory molecules plays a crucial role in full T cell activation. Nevertheless, few reports have analyzed the expression of costimulatory molecules on mononuclear cells in AIH. In the present study, flow cytometry was used to analyze the expression of costimulatory molecules on mononuclear cells, because it is a sensitive method for detecting positive cells. PBMCs expressing CD80+, CD152+ (CTLA-4+) and CD86+ were shown to be significantly lower in the patients with AIH as compared with the healthy controls. However, in the patients with AIH, these cells existed more frequently in LIMCs than that in PBMCs. Interestingly, the most dramatic difference in frequency between PBMCs and LIMCs was detected in the ratios of cells positive for CD86 molecule, formerly designated as B7-2. Although CD86+ cells were barely detected in PBMCs obtained from the patients and healthy controls, they were considerably high in LIMCs obtained from the patients with AIH.

CD80 and CD86 molecules have unique expression patterns on professional APC and activated lymphocytes[31]. CD80 is constitutively expressed at low levels on lymphoid cells, whereas CD86 expression is rapidly increased upon activation[32]. Our results regarding the high level of CD86+ LIMC in the patients with AIH suggested that activated APC or lymphocytes might be enriched in the liver of the patients with AIH. Furthermore, the aberrant expression of CD86 was observed irrespective of the presence of hepatocellular damage and the treatment with corticosteroids, and the enhanced expression of CD86 was not observed in other liver diseases. These results suggest that the costimulatory molecules, such as CD80 and CD86, are continuously expressed on mononuclear cells in the liver even when the hepatocellular damage is not present. In addition, we often observe the relapse of liver dysfunction during the time course of tapering the dose of corticosteroids. Therefore, these lead us to speculate that excessive antigen presentation by APC to T cells, via the interaction between the CD80/86 and CD28 molecules, might contribute to the hepatocellular damage in AIH and administration of corticosteroids may block the signal transduction between APC and T cells. Indeed, it is important to identify which subtypes of PBMC or LIMC really express CD86 molecule. However, we have not identified it yet. Because the expression of CD86 on PBMCs was extremely low and the amount of LIMCs purified from a tiny liver specimen was quite a few, it was technically difficult to identity a specific subtype of the cells. The more detail analysis of this subtype and the function CD86+ cells in AIH awaits further elucidation in the next study.

Recently, extensive efforts have been made to elucidate the critical function of costimulatory molecules in various diseases. The aberrant expression of costimulatory molecules on mononuclear cells has been reported in a variety of autoimmune diseases, such as lupus, multiple sclerosis (MS), and experimental autoimmune encep halomyelitis[33-35]. The enhanced expression of CD80 and CD86 has also been reported in liver diseases, such as fulminant hepatic failure, primary biliary cirrhosis (PBC), primary sclerosing cholangitis, hepatitis C and hepatocellular carcinoma[9-14]. However, all of these reports focused on the CD80 or CD86 molecules expressed on either hepatocytes or bile ducts. There are few reports expressing the role of costimulatory molecules in AIH. The costimulatory molecules, such as CD80, CD86 and CTLA-4 (CD152), are essentially expressed on mononuclear cells. To our best of knowledge, this is the first descriptive report showing the aberrant expression of CD86 molecule on LIMCs of patients with AIH. Recently, anti-CD86 antibody or soluble CD152 (CTLA-4) was used as blocking agents for CD86 function in the treatment of autoimmune diseases[36-38]. Similarly, our data suggest that anti-CD86 antibody might be used to stabilize liver function for the treatment of AIH.

In PBC, presence of anti-mitochondria M2 antibody is a useful diagnostic marker[39,40]. In AIH, presence of ANA and the scoring system proposed by the International

Autoimmune Hepatitis Group are currently being used for the diagnosis of AIH[41]. However, some cases are still difficult to classify as definite or probable AIH. Actually, the cases with low levels of gammaglobulin (< 2.0 g/dL) were present in 8 of 18 cases whose LIMCs were examined in the present study. In contrast, all cases except No. 14 in Table 2 showed high levels of CD86+ LIMC. Furthermore, 2 patients who did not respond satisfactory to corticosteroids showed low levels of CD86+ LIMC. It is well known that non-response to the treatments of corticosteroids is uncommon in AIH. Therefore, these 2 cases might not be a real AIH although they were clarified as AIH by the scoring system. Thus, the detection of CD86+ LIMC may be useful for the diagnosis of AIH and this may lead to be helpful for the prediction of therapeutic effects of corticosteroids on AIH in the future.

In summary, our results showed lower proportions of CD80+, CD86+ and CD152+ (CTLA-4+) PBMC in the patients with AIH as compared with the healthy controls. These cells are present in greater frequency in LIMCs, suggesting that the aberrant expression of these costimulatory molecules on LIMCs might be associated with pathogenic mechanism of AIH. Especially, the level of CD86+ LIMC is likely to be helpful for the diagnosis of AIH and for the prediction of therapeutic effects of corticosteroids on AIH.

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VIRAL HEPATITIS

Antibody to E1 peptide of hepatitis C virus genotype 4 inhibits virus binding and entry to HepG2 cells *in vitro*

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Abstract

AIM: To analyze the neutralizing activity of antibodies against E1 region of hepatitis C virus (HCV). Specific polyclonal antibody was raised via immunization of New Zealand rabbits with a synthetic peptide that had been derived from the E1 region of HCV and was shown to be highly conserved among HCV published genotypes.

METHODS: Hyper-immune HCV E1 antibodies were incubated over night at 4℃ with serum samples positive for HCV RNA, with viral loads ranging from 615 to 3.2 million IU/ mL. Treated sera were incubated with HepG2 cells for 90 min. Blocking of viral binding and entry into cells by anti E1 antibody were tested by means of RT-PCR and flow cytometry.

RESULTS: Direct immunostaining using FITC conjugated E1 antibody followed by Flow cytometric analysis showed reduced mean fluorescence intensity in samples pre-incubated with E1 antibody compared with untreated samples. Furthermore, 13 out of 18 positive sera (72%) showed complete inhibition of infectivity as detected by RT-PCR.

CONCLUSION: In house produced E1 antibody, blocks binding and entry of HCV virion infection to target cells suggesting the involvement of this epitope in virus binding and entry. Isolation of these antibodies that block virus attachment to human cells are useful as therapeutic reagents.

Key words: Flow cytometry; Hepatitis C virus; E1 envelope; Therapeutic antibodies; Direct immuno-fluorescence; HepG2 cells

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INTRODUCTION

Hepatitis C virus (HCV) is the major etiology of non-A, non-B hepatitis that infects 170 million people worldwide. Approximately 70% to 80% of HCV patients develop chronic hepatitis, 20% to 30% of which progress to liver cirrhosis[1]. At present, there is no vaccine available to prevent HCV infection, and current therapies are not optimal. The initial steps of HCV infection (binding and entry) that are critical for tissue tropism, and hence pathogenesis, are poorly understood. Studies to elucidate this process have been hampered by the lack of robust cell culture systems or convenient small animal models that can support HCV infection. HCV is an enveloped, positive-stranded RNA virus that belongs to the *Flaviviridae* family. Based on the sequence heterogeneity of the genome, HCV is classified into six major genotypes and 100 subtypes[1]. The viral genome (9.6 kb) is translated into a single poly-protein of 3000 amino acids (aa). A combination of host and viral proteases are involved in poly-protein processing to give at least nine different proteins[2]. Like other enveloped viruses, E1 and E2 proteins most likely play a pivotal role in the assembly of infectious particle and in the initiation of viral infection by binding to its cellular receptor(s). It has been suggested that the humoral and cellular immune responses to the E1 envelope protein are largely impaired in patients with chronic active hepatitis C, and that such responses may be important for clearance of HCV[3]. Leroux-Roels *et al*,[4] have previously reported that cellular immune responses to the E1 envelope protein are almost absent in patients with chronic active hepatitis C, while long-term responders to IFN- therapy, on average, show higher levels of E1 antibodies[5]. Depraetere *et al*,[6] suggesting that E1 antibodies contribute, at least

partially, in viral elimination. Baumert *et al*[7] confirmed the presence of such higher antibody levels directed at the HCV envelope in sustained viral responders to IFN-based therapy. Maertens *et al*[8] have been able to show that therapeutic vaccination of chronically infected chimpanzees with the HCV E1 protein induces the appearance of T-helper immune responses and antibodies which are very rarely seen in patients[6,7] or chimpanzees[9] with chronic active hepatitis C. The use of a viral envelope protein has the advantage of potentially inducing not only T-cell responses, but also neutralizing antibodies and complement activation. The E1 protein was chosen as vaccine rather than the E2 protein not only because E2 has the disadvantage of displaying a very high strain-to-strain variation in the hypervariable region I (HVRI), but also because of the higher degree of inter-genotype cross-reactivity of E1 as compared to E2. The E2 hypervariable region is immunodominant and neutralizable[10]. However, strong anti-E2 vaccine responses directed against the HVR I do not cross-neutralize with the infecting strain[11,12]. Although the E1 antigen is also variable between genotypes, it shows a relatively high degree of conservation within the subtypes, such as subtype 1b[13], the most widespread genotype worldwide. In the present study, we aimed to examine the neutralizing -related activity of an in house made antibody against the most conserved region of HCV E1 protein, for blocking the entry of HCV virion to HepG2 cells.

MATERIALS AND METHODS

Infected Serum samples

We selected 28 serum samples which tested positive for HCV RNA at different viral loads (ranging from 615 to 3.2 million IU/ mL) for infection experiments. The presence of HCV RNA was determined by nested RT-PCR and genotyped using Innolipa system (Bayer, Germany). Viral loads were determined by branched DNA method (Bayer, Germany).

Design of E1 conserved synthetic peptides

Sequence analysis of HCV quasi-species in local patients (Data not shown), revealed several conserved regions within the core and the E1 proteins. We designed 4 core and one E1-specific peptides and analyzed their ability to detect circulating antibodies in infected patients. The results of these studies showed that only one core-peptide (C1) had reasonable sensitivity and specificity. However the rest of peptides including E1 peptide had poor reactivity with circulating antibodies[14]. In the present study, we raised HCV specific polyclonal antibodies against the 4 core and an E1 peptide as follows:

(C1) DVKFPGGGQIVGGVYLLPRR, (C2) PRLGVRATRKTSERSQPRG,
(C3) IPKARRPEGRTWAQPGY, (C4) IPKDRRSTGKSWGKPGY,
(E1) GHRMAWDMM

Production of polyclonal antibodies against core and Envelope regions of HCV

New Zealand rabbits were immunized independently (two rabbits per each peptide) with purified synthetic peptides coupled with KLH protein. Equal volume of diluted core and E1 synthetic peptides and Freund's complete adjuvant were emulsified and injected subcutaneously into the rabbits in three different sites. On d 15 and 28, the rabbits were immunized again with the same protein emulsified with Incomplete Freund`s adjuvant. On d 32 the rabbits were sacrificed and sera were separated and stored at -20 ℃. For direct immuno-fluorescence, immunized polyclonal antibodies were digested with pepsin A (porcine 1:60 000 grade (sigma P-7012) ST. Louis, Mo, USA) at acidic pH and the $F(ab)_2$ portion was labeled with fluorescence isothiocyanate-FITC according to Hudson and Hay[15].

Flow cytometric analysis of E1 binding to HepG2 cells

The interaction of E1 glycoprotein with cells was quantified using a fluorescence activated cell sorting (FACS) based assay. Surface labeling was performed by direct immuno-fluorescence. Twenty eight serum samples that tested positive for HCV RNA with broad range of viral loads (617-3.2×10^6 IU / mL) were incubated with anti E1 antibody (diluted 1:250 in cultured media) overnight at 4 ℃. The pretreated sera with E1 antibody were incubated with HepG2 cells for 90 min at 37 ℃ in CO_2 incubator. Cells were centrifugated and supernatants were removed. Cell pellets were washed 4 times with PBS and incubated with FITC labeled $F(ab)_2$ portion of HCV E1 antibody (at 1:1500 dilution) for 30 min at 4 ℃. Cells were washed 3 times with PBS containing 1% normal goat serum. Cells were suspended in 500 μL PBS and analyzed with flow cytometry (FACS Calibure, BD). The mean fluorescence intensity were determined using cell Quest software (Becton Dickinson)

Isolation and extraction of RNA from serum and HepG2 cells

RNA was isolated from serum samples and HepG2 cells as reported by Lohr *et al*[16]. Briefly, cells were precipitated and washed in the same buffer to remove adherent viral particles before lysis in 4 mol/L guanidinium isothiocyanate containing 25 mmol/L sodium citrate, 0.5% sarcosyl and 0.1 mol/L β-mercaptoethanol. Cellular RNA was extracted using the single-step method described originally by Chomczynski and Sacchi[17].

PCR of genomic RNA strands of HCV

Reverse transcription-nested PCR was carried out according to Lohr *et al*.[16] with few modifications. Retro-transcription was performed in 25 μL reaction mixture containing 20 units of AMV reverse transcriptase (Clonetech, USA) with either 400 ng of total PBMCs RNA or 3 μL of purified RNA from serum samples (equivalent to 30 μL serum) as template, 40 units of RNAsin (Clontech, USA), a final concentration of 0.2 mmol/L from each dNTP (Promega, Madison, WI, USA) and 50 pmol of the reverse primer P1 (for plus strand) or 50 pmol of the forward primer P2 (for minus strand). The reaction was incubated at 42 ℃ for 60 min. and denatured at 98 ℃ for 10 min. Amplification of the highly conserved 5'-UTR sequences was done using two rounds of PCR with 2 pairs of nested primers. First round amplification was done in 50 μL reaction con-

Table 1 Neutralizing activity of E1 antibody on infectivity of HCV to HepG2 cells

n	Viral Load	Genotype	Pre-incubation	
			PBS	Anti E1 Ab
1	<615	4c/4d	positive	positive
2	1481570	4	positive	positive
3	28469	4c/4d	Cells not infected[1]	Cells not infected[1]
4	4030	4e	Cells not infected[1]	Cells not infected[1]
5	42154	4	positive	positive
6	114598	4	positive	negative
7	<615	4c/4d	positive	positive
8	-	1b	Cells not infected[1]	Cells not infected[1]
9	2835590	4a	positive	negative
10	110933	4c/4d	positive	negative
11	1052310	4c/4d	positive	negative
12	806482	4e	positive	negative
13	4721	4c/4d	positive	negative
14	817331	4	Cells not infected[1]	Cells not infected[1]
15	2077	4a	Positive	negative
16	177021	4	Positive	negative
17	147226	4c/4d	Cells not infected[1]	Cells not infected[1]
18	3284580	4c/4d	Positive	negative
19	1136230	4	Positive	negative
20	506773	4c/4d	Cells not infected[1]	Cells not infected[1]
21	380695	4	Cells not infected[1]	Cells not infected[1]
22	159725	4	Positive	negative
23	1216640	4e	Positive	negative
24	<615	4a	Cells not infected[1]	Cells not infected[1]
25	<615	4c/4d	Positive	positive
26	284962	4	Cells not infected[1]	Cells not infected[1]
27	336705	4a	Positive	negative
28	<615	4	Cells not infected[1]	Cells not infected[1]

The presence of HCV RNA was determined by nested RT-PCR and genotyped using Innolipa system (Bayer, Germany). Viral loads were determined by branched DNA method (Bayer, Germany).

PBS: phosphate buffer saline

[1]Cells not infected: HepG2 cells did not infected by positive HCV serum.

taining 50 pmol from each of P2 forward primer and P3 reverse primer, 0.2 mmol/L from each dNTP, 10 μL from RT reaction mixture as template and 2 units of Taq DNA polymerase (Promega, USA) in 1X buffer supplied with the enzyme. The thermal cycling protocol was as follows: 1 min. at 94℃, 1 min at 55℃ and 1 min at 72℃ for 30 cycles. The second round amplification was done similar to the first round, except for use of the nested reverse primer P4 and forward primer P5 at 50 pmol each. A fragment of 172 bp was identified in positive samples. Primer sequences were as follows: P1: 5' ggtgcacggtctacgagacctc 3' P2 : 5' aactactgtcttcacgcagaa 3', P3: 5' tgctcatggtgcacggtcta 3', P4: 5' actcggctagcagtctcgcg 3', P5: 5' gtgcagcctccaggaccc 3'. To control for false detection of negative-strand HCV RNA and known variations in PCR efficiency[18,19], specific control assays and rigorous standardization of the reaction were employed as previously described[20]. These specific control assays were: (1) cDNA synthesis without RNA templates to exclude product contamination, (2) cDNA synthesis without RTase to exclude Taq polymerase RTase activity, (3) cDNA synthesis and PCR step done with only the reverse or forward primer to confirm no contamination from mixed primers. These controls were consistently negative. In addition, cDNA synthesis was carried out using only one primer followed by heat inactivation of RTase activity at 95℃ for 1 h, in an attempt to diminish false detection of negative-strand prior to the addition of the second primer.

Infection of HepG2 cells with HCV

Cells were grown for 48h to semi-confluence in complete DMEM medium, washed twice with FCS -free medium then inoculated with serum samples (500 μLl plus 500 μL FCS-free DMEM/3×10^6 cells) obtained from HCV infected patients (RT-PCR and antibody positives). The viral load in the used sera was quantitated by bDNA technology and the average copy number was 615-3.2×10^6 IU/mL. After 90 min, DMEM containing FCS was added to make the overall serum contents 10% in a final volume 8 mL including the volume of human serum used for infection and cells were maintained overnight at 37℃ in 50 mL/L CO_2. Next day, adherent cells were washed three times with culture medium to get rid of the remaining infectious serum and incubation was continued in complete medium containing 10% FCS with regular medium changes. An inhibition assay of viral absorption or attachment to presumed susceptible cells has been developed for assessing the neutralizing related capacity of antibodies[21]. To analyze the neutralizing-related activity of antibodies against E1 region of HCV as compared with antibodies against core peptides, serial dilutions of serum samples were incubated with equal volumes of different amounts of the studied specific polyclonal antibodies in PBS at 4℃ overnight. One hundred microliters of pretreated serum samples were incubated with 1 mL of the cell suspension of HepG2 cells containing 0.5 million cells. Appropriate controls included HCV RNA positive sera that have neither been treated with E1 nor with core antibodies as positive controls, HepG2 cells not infected with HCV RNA positive sera as negative controls. Retrotranscription-PCR was performed on intracellular HCV RNA of HepG2 cells under the above described circumstances.

RESULTS

Selection of highly conserved peptide sequence among various HCV genotypes

The most conserved 10 amino acid stretch within the N-terminal region of E1 protein derived from several reported HCV isolates is shown in figure 1. When this 10mer peptide (GHRMAWDMM) was synthesized and used for immunization of New Zealand rabbits, the reactivity of hyper-immune E1 antibody was confirmed by enzyme-linked immunosorbent assay (ELISA) and western blot for detection of E1 protein in infected sera and infected HepG2 cells (Data not shown).

Selection of HCV positive sera and infection of HepG2 cells

Twenty eight HCV RNA positive sera with different viral loads and various subtypes of genotype 4 were used for infection experiments (Table 1). Only 18 samples were able to infect HepG2 cells, thus producing 18 different cell lines of HCV infected HepG2 cells.

```
AB67036[H77]      GHITGHRMAWDMMMNWSPTAALVVAQLLRIPQAIMDMIAGAHWGVLAGIAYFSMVGNWAK 160
BAD73999[1b]      GHVSGHRMAWDMMMNWSPTTALVVSQLLRIPQAVVDIVVGAHWGVLAGLAYYSMVGNWAK 180
BAB08107[2b]      GHITGQRMAWDMMLNWSPTLTMILAYAARVPELVLEIVFGGHWGVVFGLAYFSMQGAWAK 180
CAA72338[4a]      GHITGHRMAWDMMMNWSPTTTLVLAQVMRIPTTLVDLLSGGHWGVLVGVAYFSMQANWAK 170
CAC16101[4d]      GHITGHRMAWDMMMNWSPTATLVLAQLMRIPGAMVDLLAGGHWGILVGIAYFSMQANWAK 179
CAC16106[4f]      GHITGHRMAWDMMMNWSPTTTLVLAQIMRVPAALVDMLAGGHWGVLAGMAFFSMQANWAK 179
CAA73640[5a]      GHITGHRMAWDMMMKWSPTTALLMAQLLRIPQVVIDIIAGGHWGVLLAAAYFASTANWAK 180
AAW56714[6a]      GHVTGHRMAWDMMMSWSPTTTLVLSSILRVPEICASVIFGGHWGILLAVAYFGMAGNWLK 180
                  **:::*:********:.**** :::::   *:*   .:: *.***:: . *::.  . * *
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Figure 1 Sequence alignment of amino acids no 311 to 370 (numbering starts from initiating methionin in the core protein of genotype 4a) of HCV E1 among representative genotypes. Eight sequences of different subtypes were aligned using ClustalW software, the output diagram is shown with the legend on the left having the accession number and the subtypes. Sequences shown in bold represent the highly conserved amino acid stretch (GHRMAWDMM) used for production of polyclonal antibody.

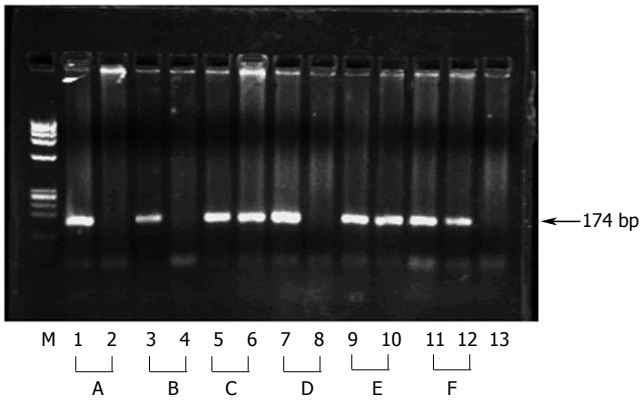

Figure 2 Inhibition of HCV entry into HepG2 cells by anti E1 Ab. Sera from 6 HCV infected patients (A to F) were used for infection of HepG2 cells before (Lanes 1,3,6,7,9 and 11) and after pre-incubation with anti E1 Ab (Lanes 2,4,6,8,10 and 12). RNA was detected in HepG2 lysates by nested HCV RT-PCR and the products were resolved on 2% agarose gel as described in materials and methods. The presence of a 174 bp band indicates presence of viral RNA while absence of the band indicates successful blocking of viral entry into cells. Lane M shows φx Hae III digest as a molecular weight marker, lane 13 shows RT-PCR of the PBS used for the last wash step of the cells.

Inhibition of HCV entry into HepG2 cells by specific E1 antibodies

Although an in vitro culture for HCV is not available, an inhibition assay of viral absorption or attachment to presumed susceptible cells has been developed by another laboratory for assessing the neutralizing related capacity of antibodies[21]. In the present study we used the HepG2 cell line to examine the biological function of E1 antibody. All 18 serum samples were positive for HCV RNA and have the ability to infect HepG2 cells as determined by PCR (Figure 2). After incubation of serum samples with E1 antibodies overnight at 4 ℃, only 5 out of 18 samples remained infectious to HepG2 cells while the remaining 13 samples did not infect HepG2 cells with an inhibition rate of 72% (Figure 2)

Inhibition of HCV binding to HepG2 cells by specific E1 antibodies

The mean fluorescence intensity of bound HCV particle was determined by flow cytometric analysis of HepG2 cells incubated with FITC labeled $F(ab)_2$ portion of HCV E1 antibody after subtraction of the nonspecific fluorescence value. Figure 3 showed a 6 fold (from 12% to 2%) reduction of mean fluorescence intensity in HepG2 cells treated with serum samples pre-incubated with specific anti E1 antibodies compared with cells incubated with untreated positive sera.

DISCUSSION

Although a detailed analysis of the viral genomic organization has led to the identification of various genetic elements[2] and the establishment of subgenomic replicons[22] in transfection experiments, the study of whole viral entry and infection is still hampered by the inability to propagate the virus efficiently in cultured cells and the limited animal tropism of the virus. The chimpanzee is the only nonhuman host serving as a model for HCV infection[23]. Binding of individually expressed recombinant glycoprotein E2 to human cell lines has been used as a surrogate model for binding of virus to host cells, allowing the study of antibody mediated neutralization of binding[24]. Using this surrogate assay, Pileri *et al*[25] have demonstrated that envelope glycoprotein E2 interacts with the large extracellular loop of cellular membrane protein CD81, a member of the tetraspanin family[26]. CD81 has been suggested as a candidate receptor for HCV[27]. Recently Brazzoli *et al*[28] suggested that productive folding of the major HCV spike protein E2 is assisted by E1. In the present study ,we developed, in house, a monospecific polyclonal antibody for an E1 peptide. The observed great homology within the N-terminal region of E1 suggests that this domain plays a major role in E1/E2 interaction and proper folding of envelop glycoproteins[29,30]. Therefore, a monospecific antibody against the amino terminal domain of E1 protein was expected to interfere with virus binding to membrane receptor. The immunogenicity of this anti E1 Ab was demonstrated by our laboratory in immunoassay techniques such as flow cytometry for detection of E1 glycoprotein in infected cells[31]. In vitro infection experiments, rather than the artificial replicon assays, was reported by others to mimic the intracellular events occurring *in vivo*[32,33]. Besides, study of the E1 Ab activity in blocking infection of cells by several infected sera is easier to accomplish via direct infection than the use of the laborious cloning to produce replicon (s) from each sample. However direct infection experiments does not facilitate 100% efficiency of HCV infection into HepG2 cells in all studied cases. In the current study, only 18 out

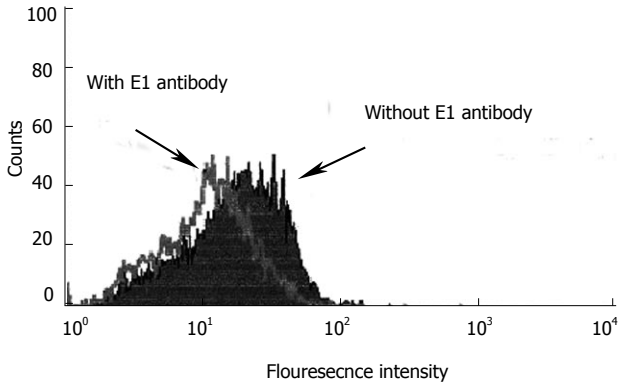

Figure 3 Neutralization of HCV infection into HepG2 cells by anti E1 antibody. The pretreated sera with anti E1 Ab or with PBS were incubated with HepG2 cells for 90 min at 37 °C in CO_2 incubator. Cell pellets were washed with PBS, incubated with FITC labeled $F(ab)_2$ portion of anti E1 Ab and analyzed with flow cytometry as described in Materials and Methods The mean fluorescence intensities decreased from 12.5% in cells incubated with PBS to 2% after treatment with anti E1 Ab using cell Quest software (Becton Dickinson).

of 28 positive samples (64%) had the ability to infect HepG2 cells as determined by intracellular detection of HCV RNA by RT-PCR. The reasons for the inability of the other ten serum samples to infect HepG2 cells is not clear. The quasispecies pool in each sample seems to play a significant role in determining viral entry in each case[34,35], Moreover, the competitive binding of viral particles and low density lipoproteins (LDL) toward the limited number of LDL receptors on cell membrane contributes in the sample to sample variation probably due to individual variations in LDL levels. Enjoji *et al*[36] reported that LDL competitively inhibit the infection of hepatocytes with HCV. On the other hand, our results showed that variations in viral counts appear not to be involved in determining the efficiency of HCV entry into HepG2 cells, a finding that agrees with earlier reports[37]**.** Anti E1 Ab could completely inhibit entry of viral particles into cells in 13 out of 18 (72%) samples. The reasons why the remaining 5 cases (28%) escaped the inhibitory effect of anti E1 Ab may be related to the relative protection of circulating viral particles by exosomes against neutralizing antibodies[38]. Alternatively, the concentration of E1 antibody may be not sufficient for complete inhibition of binding in all tested samples due to variations in the levels of circulating free E1 antigen.

The results of RT-PCR in HepG2 cells were confirmed by the results of flow cytometry. The direct immunostaining of E1 antibody conjugated with FITC and flow cytometric analysis showed reduced mean fluorescence intensity in the samples pre-incubated with E1 Ab compared with samples without E1 Ab. Shimizu *et al*[39] and Farci *et al*[11] demonstrated that a rabbit hyper-immune serum prepared against a peptide representing the 21 C-terminal amino acids of the HVR1 H77 of HCV 1a could neutralize the homologous virus *in vitro* and *in vivo*. These studies provided the first identification of a neutralization epitope on the surface of HCV. They also demonstrated that the neutralization was highly strain-specific and that minor variants of HCV bearing divergent sequences in the HVR1 were not neutralized and emerged in the cell culture and the chimpanzee as neutralization escape mutants. The development of a vaccine against HCV, based on stimulating neutralizing antibody to the HVR1, appeared to be a daunting task[40]. In the present study we provide alternative approach which may bear new hope for developing HCV vaccine based on conserved N-terminal region of E1 protein. Recently, Leroux-Roels *et al*[41] suggested that immunization of healthy individuals against HCV with the E1 protein as a prophylactic vaccine may not only raise useful (potentially neutralizing) anti-E1 antibodies but could also induce a strong T-cell response that might contribute to the prevention of chronic evolution in cases of acute hepatitis C.

In conclusion, in house produced anti E1 Ab that was raised in rabbits against the most conserved region among reported viral strains, blocks HCV infection to target cells suggesting the involvement of this epitope in virus binding and entry. Isolation of similar humanized antibodies that block virus binding and entry will be useful in providing potential therapeutic reagents and for vaccine development.

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BASIC RESEARCH

A comparison of gene expression in mouse liver and kidney in obstructive cholestasis utilizing high-density oligonucleotide microarray technology

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Abstract

AIM: To assess the effects of obstructive cholestasis on a wider range of gene expression using microarray technology.

METHODS: Male C57BL/6J mice underwent common bile duct ligation (BDL) and were matched with pair-fed sham-operated controls. After 7 d, the animals were sacrificed and total RNA was isolated from livers and kidneys. Equal amounts of RNA from each tissue were pooled for each group and hybridized to Affymetrix GeneChip®MG-U74Av2 containing a total of 12488 probe sets. Data analysis was performed using GeneSpring® 6.0 software. Northern analysis and immunofluorescence were used for validation.

RESULTS: In sham-operated and BDL mice, 44 and 50% of 12488 genes were expressed in livers, whereas 49 and 51% were expressed in kidneys, respectively. Seven days after BDL, 265 liver and 112 kidney genes with GeneOntology annotation were up-regulated and 113 liver and 36 kidney genes were down-regulated in comparison with sham-operated controls. Many genes were commonly regulated in both tissues and metabolism-related genes represented the largest functional group.

CONCLUSION: Following BDL, microarray analysis reveals a broad range of gene alterations in both liver and kidney.

Key words: Bile duct ligation; Cholestasis; Kidney; Liver; Microarray

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INTRODUCTION

Cholestasis, defined as impairment of bile secretion, is a feature of many hepatic disorders and systemic diseases. The recent cloning and functional characterization of different transport proteins for bile acids, organic anions and cations in hepatocytes and cholangiocytes have provided new insights into the molecular biology and physiology of bile formation and have increased understanding of the pathophysiology of cholestatic disorders[1]. Thus it is now established that a number of transport proteins in the basolateral and canalicular hepatocyte membrane undergo adaptive regulation in response to cholestatic liver injury to minimize the hepatic accumulation of toxic substances, such as hydrophobic bile acids[2-4]. Previous studies have indicated that in addition to the liver, adaptive regulation of these transporters in cholestasis also occurs in extrahepatic tissues, including the kidney[5] and the intestine[6]. Other alterations in cholestasis affect hepatic signal transduction[7,8], vesicular transport[7], apoptosis[9,10], metabolism[11], and the structure of the extracellular matrix[12,13].

Given the wide range of signaling, regulatory, and metabolic pathways, structural elements, and transport proteins which may be affected in cholestasis, much

further research will be necessary to more fully understand the extent of these adaptations. High-density DNA microarrays containing thousands of DNA fragments and oligonucleotides are a potentially promising approach to identify additional genes of interest that play a role in this pathophysiologic process. Based on their ability to monitor large numbers of genes at a time, high-density DNA microarrays are a sensitive, time-saving, and efficient tool in determining gene expression and finding regulatory pathways[14].

In the present study, we, therefore, have utilized high-density oligonucleotide microarray technology to screen for gene alterations in the liver and kidney following bile duct ligation (BDL) in mice, an established model of obstructive cholestasis. This study has allowed a comprehensive gene expression profile to be obtained in cholestatic mouse liver and kidney as well as it has highlighted a number of genes whose expression is particularly altered by this process.

MATERIALS AND METHODS

Animals and animal treatment

Male C57BL/6J mice (8-12-wk-old) purchased from Jackson Lab (Bar Harbor, ME) underwent BDL or sham-surgery as previously described[15]. The common bile duct was identified, ligated twice close to the liver hilum immediately below the cystic duct, and then divided between the ligatures. Control mice underwent sham-surgery in which the common bile duct was exposed but not ligated. Since sham-operated mice tend to consume more food than BDL mice and the expression of some genes may be affected by caloric intake, food intake of BDL mice was monitored daily and sham-operated mice were pair-fed so as to receive the same amount of food as BDL mice. Animals were sacrificed 7 d after surgery and livers and kidneys were harvested. The protocol was approved by the Yale Animal Care and Use Committee, and the animals received humane care as outlined in the "Guide for the Care and Use of Laboratory Animals" (NIH publication 86-23, revised 1985).

Isolation of total RNA

Blood-free livers and kidneys were homogenized in GTC solution containing 4 mol/L guanidinium thiocyanate, 25 mmol/L Na-citrate, and 5 g/L N-lauroylsarcosine and subjected to CsCl gradient centrifugation. The recovered total RNA was further purified by phenol/chloroform extraction and ethanol precipitation. The RNA concentration was determined spectrophotometrically and the RNA quality was confirmed by formaldehyde-agarose gel electrophoresis. Equal amounts of liver and kidney, respectively, total RNA from each of four BDL and four sham-operated mice were pooled to minimize inter-animal variations and used for biotin-labeling.

DNA microarray hybridization and analysis

The biotin-labeled RNA from the different groups was hybridized with two replicates for each condition to individual high-density oligonucleotide microarray chips (GeneChip®MG-U74Av2) from Affymetrix (Santa Clara, CA) containing a total of 12488 probe sets. Microarray expression data were generated with Affymetrix Microarray Suite 5.0 software and further analysis was carried out with GeneSpring®6.0 (Silicon Genetics, Redwood City, CA). Raw intensity values from each chip were normalized to the 50th percentile of the measurements taken from that chip to reduce chip-wide variations in intensity. Each gene was normalized to the average measurement of that gene in the respective paired controls to enable comparison of relative changes in gene expression levels between different conditions. Cross-gene error model was active based on the replicates. Comparisons of gene expression data were made between BDL and sham-operated mice. Signal and detection flag from Microarray Suite 5.0 were used as quality controls. Only genes with a minimum signal intensity of 600, a detection flag present in both replicates in at least one of the comparison conditions, and a two-fold and above change in gene expression were used for further analysis. For identification of differentially expressed genes in the different groups, a one-sample *t*-test with a *P* value cutoff of 0.05 was performed to determine if the average log of the ratio of the replicates was significantly different from 1.0, which was the value of the control samples after normalization. Finally, genes were categorized into GeneOntology (GO) and annotated using NetAffx®, an analysis web interface from Affymetrix.

Northern analysis

To validate alterations in gene expression on the microarray, changes in the expression of selected genes were confirmed in aliquots of the same RNA samples used for the microarray by Northern analysis as previously described[16]. The following primers were used for the generation of specific probes: cytochrome P450 7b1 (GenBank accession number U36993): 5'-GAATCTC AGCTTAGAGAGTAAGAG-3' (sense), 5'-TTTGTA CCTAAAGGAGACGGCAG-3' (antisense); organic cation transporter 1 (Oct1) (GenBank accession number U38652): 5'-GCAGCCTGCCTCCTCATGATC-3' (sense), 5'-GGTAAATCGTGTTTTCTTTGGCC-3' (antisense); similar to putative integral membrane transport protein (GenBank accession number AI647632): 5'-TGATTACA AGAAATGTCAAGCAGG-3' (sense), 5'-CCTCTTCCTG ACTCCATCCATG-3' (antisense).

Immunofluorescence

Indirect immunofluorescence with a polyclonal antibody against Oct1[17,18] (dilution 1:100; kindly provided by Prof. Dr. H. Koepsell, Würzburg, Germany) was conducted on liver specimens from sham-operated and BDL mice as previously described[19].

RESULTS

Gene expression profile in mouse liver in obstructive cholestasis

Of the total of 12488 genes on the microarray chip, 44 and 50% were expressed in the livers of sham-operated and BDL mice, respectively. After 7 d of obstructive cholestasis 265 genes with GO annotation were up-regulated and 113 were down-regulated in livers of BDL

mice by a factor of two or more in comparison with sham-operated pair-fed controls. Metabolism-related genes represented the largest functional group among the altered genes after BDL in liver (Table 1). It should be noted that the grouping of the altered genes was primarily done to achieve a clearer arrangement for the reader. Since a considerable number of the encoded proteins have multiple, little characterized or even unknown functions, we want to point out that the classification provided is subject to the personal opinions and emphasis of the authors (Table 1). Upon request, a complete list of the altered genes including genes without GO annotation that are not mentioned here can be obtained from the authors. Alternatively, the complete list of altered genes can be accessed via http://livercenter.yale.edu/datalist.html.

Gene expression profile in mouse kidney in obstructive cholestasis

In the kidneys of sham-operated and BDL mice, 49 and 51% of the 12 488 genes on the microarray chip were expressed, respectively. Seven days after surgery, 112 genes with GO annotation were up-regulated and 36 were down-regulated in the kidneys of BDL mice at least two-fold when compared with the sham-operated pair-fed controls. Thus the number of altered genes in kidney seven days after BDL was considerably smaller than that in liver (148 *vs* 378). Of the 112 GO genes up-regulated in kidney after BDL, 53 were also up-regulated in cholestatic liver. In contrast, of the 36 genes down-regulated in kidney, 7 were also down-regulated in liver (Table 1). What was particularly striking is that many of the most highly up-regulated genes in liver were also the same genes that were most highly up-regulated in kidney, irrespective of their functional class (Table 1). This suggests that both the liver and the kidney may be responding to similar transcriptional signaling molecules in this cholestatic model. For example, the acute phase gene, *serum amyloid A3*, was up-regulated 10.0-fold in liver and 36.1-fold in kidney, the gene encoding *chemokine (C-X-C motif) ligand 1* was increased 9.6-fold in liver and 4.2-fold in kidney, and the gene encoding the transport molecule *lipocalin 2* was up-regulated 13.1-fold in liver and 66.5-fold in kidney. In addition, a number of cell adhesion and extracellular matrix genes were similarly up-regulated in both liver and kidney. However, only one membrane transporter gene was up-regulated in both tissues, the gene encoding the *β1 subunit of the voltage-gated sodium channel* (Table 1). Interestingly, several genes for nucleic acid binding proteins were also highly up-regulated in both liver and kidney including the genes encoding the transcription factors *FBJ osteosarcoma oncogene* (alias *c-Fos*), *CCAAT/enhancer binding protein (C/EBP), delta*, and *activating transcription factor 3*.

In contrast, only seven genes were commonly down-regulated in both liver and kidney. These included the *RIKEN cDNA 1700013L23 gene* and the genes encoding *similar to putative integral membrane transport protein, major urinary protein 2*, *transthyretin*, *cytochrome P450 7b1* (GenBank accession numbers AV141027 and U36993), and *thioether S-methyltransferase*.

Northern analysis of selected genes

Gene expression results from the microarray were confirmed by Northern analysis for selected genes that included *cytochrome P450 7b1*, *organic cation transporter 1 (Oct1; solute carrier family 22, member 1)* and *similar to putative integral membrane transport protein* from aliquots of the RNA samples utilized for the microarray (Figure 1).

Tissue immunofluorescence of Oct1 in liver

Indirect immunofluorescence was performed to illustrate the decreased expression of the organic cation transporter Oct1 in BDL mouse liver. Figures 2A and B demonstrate that the findings are consistent with the microarray and the Northern blot results and corroborate that obstructive cholestasis leads to a down-regulation of Oct1 in mouse liver similarly as demonstrated previously in rat liver following BDL[19,20].

DISCUSSION

Ligation of the common bile duct in rodents is a well-established model of obstructive cholestasis. While most previous studies have been limited to investigations of small numbers of genes and their encoded proteins, we have been able to simultaneously monitor the responses of large numbers of genes in this cholestatic model by using high-density oligonucleotide microarray technology. In contrast to a recent study which investigated gene expression in obstructive cholestasis only in the livers of BDL mice[21], we additionally monitored alterations of gene expression in the kidneys because the kidney is functionally closely linked to the liver and provides an alternative excretory route for cholephilic substances in cholestasis[5]. One of the interesting conclusions from this analysis is the finding that many of the most highly up-regulated genes were shared in both liver and kidney, possibly due to a common response to similar transcriptional signaling molecules in both tissues. The interpretation and discussion of our data is based on the assumption that changes in gene expression lead to changes in protein expression although it is known that changes at the mRNA level do not always result in changes in protein expression in certain time periods[22]. As others have done, we first evaluated the observed changes in gene expression in terms of what is already known about the effects of cholestasis. We then attempted to identify novel regulatory processes that have not yet been investigated[22].

For example, our microarray data largely confirm previous results obtained by conventional determination of transcription in obstructive cholestasis, such as the up-regulation of the canalicular cation transporter *multidrug resistance P-glycoprotein 1a* (*Mdr1a*, Abcb1a)[23] or the down-regulation of the basolateral *sodium-taurocholate cotransporting polypeptide* (*Ntcp*, Slc10a1)[24]. In addition, our gene expression profile obtained from cholestatic liver also closely matched the gene expression profile recently generated by Campbell *et al*[21], although there are a substantial number of additional gene alterations in our data set. This difference can be explained since Campbell

Table 1 Fold increase/decrease in liver and kidney, GenBank accession number, and classification of altered genes in mice 7 d after bile duct ligation in comparison with pair-fed sham-operated controls

Liver	Kidney	Accession number	Description
Cell death			
3.6		AF011428	CD5 antigen-like
3.2		AW046181	Serum/glucocorticoid regulated kinase
2.6		AV373612	Bcl2-associated athanogene 3
-2.6		X65128	Growth arrest specific 1
-2.7		AA770736	Induced in fatty liver dystrophy 2
	2.8	M61737	Fat-specific gene 27
	2.7	AV003873	Clusterin
	2.3	D14077	Clusterin
	-7.0	AJ000062	Deoxyribonuclease I
Stress response			
10.0	36.1	X03505	Serum amyloid A 3
5.0		M13521	Serum amyloid A 2
2.8		M12566	Orosomucoid 2
2.5		J04633	Heat shock protein 1, alpha
2.5		X60676	Serine (or cysteine) proteinase inhibitor, clade H, member 1
	7.4	M96827	Haptoglobin
	-2.2	Z36774	Serine (or cysteine) proteinase inhibitor, clade F, member 2
Immune and inflammatory response			
31.9	5.0	M94584	Chitinase 3-like 3
17.3		M19681	Chemokine (C-C motif) ligand 2
10.7		X53798	Chemokine (C-X-C motif) ligand 2
9.6	4.2	J04596	Chemokine (C-X-C motif) ligand 1
9.4		AW120786	Chemokine (C-X-C motif) ligand 14
9.2		U18424	Macrophage receptor with collagenous structure
8.4		AV370035	Chemokine (C-C motif) receptor 5
7.1		U56819	Chemokine (C-C) receptor 2
6.9	5.5	AF002719	Secretory leukocyte protease inhibitor
6.1		M18237	Immunoglobulin kappa chain variable 8 (V8)
5.9		U34277	Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)
5.4		M83218	S100 calcium binding protein A8 (calgranulin A)
3.6	3.7	X04673	Adipsin
3.6		AI844520	Interferon gamma inducible protein 30
3.6		AF081789	Complement component 1, q subcomponent, receptor 1
3.5		X12905	Properdin factor, complement
3.3		L32838	Interleukin 1 receptor antagonist
3.2		U96752	Histocompatibility 2, Q region locus 1
3.2	3.4	M22531	Complement component 1, q subcomponent, beta polypeptide
3.2		X15591	Cytotoxic T lymphocyte-associated protein 2 alpha
3.1		X63782	Lymphocyte antigen 6 complex, locus D
2.9		M58004	Chemokine (C-C motif) ligand 6
2.9		M21932	Histocompatibility 2, class II antigen A, beta 1
2.9		U16985	Lymphotoxin B
2.9		M14639	Interleukin 1 alpha
2.9	4.2	X58861	Complement component 1, q subcomponent, alpha polypeptide
2.8		U77461	Complement component 3a receptor 1
2.8		M31314	Fc receptor, IgG, high affinity I
2.8		X52643	Histocompatibility 2, class II antigen A, alpha
2.7		AB007599	Lymphocyte antigen 86
2.6		X15592	Cytotoxic T lymphocyte-associated protein 2 beta
2.6		AF013715	Periplakin
2.6	2.1	X66295	Complement component 1, q subcomponent, gamma polypeptide
2.5	3.1	L38444	T-cell specific GTPase
2.5		AA596710	Leukotriene B4 12-hydroxydehydrogenase
2.5		AB019505	Interleukin 18 binding protein
2.4		M34815	Chemokine (C-X-C motif) ligand 9
2.4	2.0	X00496	Ia-associated invariant chain
2.4	2.8	AJ007970	Guanylate nucleotide binding protein 2
2.3		D86382	Allograft inflammatory factor 1
2.3		L22181	Formyl peptide receptor 1
2.2		AF038149	Paired-Ig-like receptor B
2.1		AW060457	Immunoglobulin superfamily, member 7
2.1		U03003	Defensin related cryptdin 6
2.0		M29855	Colony stimulating factor 2 receptor, beta 2, low-affinity (granulocyte-macrophage)
2.0		AF003525	Defensin beta 1
-2.8		M29007	Complement component factor h

Liver	Kidney	Accession number	Description
-4.6		L22977	X-linked lymphocyte-regulated 3b
	13.6	U47810	Complement component factor i
	6.5	K02782	Complement component 3
	6.0	X06454	Complement component 4 (within H-2S)
	4.2	AI563854	Tumor-associated calcium signal transducer 2
	3.8	AA986114	T-cell immunoglobulin and mucin domain containing 2
	3.5	U49513	Chemokine (C-C motif) ligand 9
	3.0	Y08830	Tumor-associated calcium signal transducer 2
	2.4	AA270365	Cytokine receptor-like factor 1
	2.2	AI152789	Sema domain, immunoglobulin domain (Ig), and GPI membrane anchor, (semaphorin) 7A
Signal transduction			
12.9		U88328	Suppressor of cytokine signaling 3
5.4		Z48043	Coagulation factor II (thrombin) receptor-like 1
5.0	2.7	M14044	Annexin A2
4.0	2.2	AJ001633	Annexin A3
3.6		AI641895	Shroom
3.6		U90715	Coxsackievirus and adenovirus receptor
3.6		AI317205	Mitogen activated protein kinase kinase kinase 1
3.4		J03023	Hemopoietic cell kinase
3.1		AW209098	IQ motif containing GTPase activating protein 1
3.0		AW049806	RIKEN cDNA 1700093E07 gene
3.0		X84797	Hematopoietic cell specific Lyn substrate 1
2.9	3.0	AB015978	Oncostatin M receptor
2.6		X93328	EGF-like module containing, mucin-like, hormone receptor-like sequence 1
2.3		D63423	Annexin A5
2.3	2.1	M69260	Annexin A1
2.2		M68902	Hemopoietic cell phosphatase
2.2		AF020313	Amyloid beta (A4) precursor protein-binding, family B, member 1 interacting protein
2.1	3.6	AV374868	Suppressor of cytokine signaling 3
2.1		AA608387	Interleukin 13 receptor, alpha 1
-2.0		AC002397	Gene rich cluster, C9 gene
-2.0		AW125649	Guanine nucleotide binding protein, alpha 12
-2.4		AI839138	Thioredoxin interacting protein
-2.6		AV321519	Sorting nexin 17
-2.7		AA691492	RIKEN cDNA D530020C15 gene
-5.6		D17444	Leukemia inhibitory factor receptor
-11.7		AV349152	Regulator of G-protein signaling 16
-15.3		U94828	Regulator of G-protein signaling 16
	2.3	AF084466	Ras-related associated with diabetes
	2.1	AF009246	RAS, dexamethasone-induced 1
	-2.1	AF054623	Frizzled homolog 1 (Drosophila)
	-2.2	D85605	Cholecystokinin A receptor
	-2.2	AI834895	Membrane progestin receptor alpha
	-2.3	AW046638	PDZ domain containing 1
Cell growth and maintenance			
8.7		M33960	Serine (or cysteine) proteinase inhibitor, clade E, member 1
6.9		X98471	Epithelial membrane protein 1
5.8	4.9	X66449	S100 calcium binding protein A6 (calcyclin)
5.4		AF055638	Growth arrest and DNA-damage-inducible 45 gamma
5.1		M17298	Nerve growth factor, beta
3.6		AI849928	Cyclin D1
3.5		X59846	Growth arrest specific 6
3.2		M64292	B-cell translocation gene 2, anti-proliferative
3.2		AW048937	Cyclin-dependent kinase inhibitor 1A (P21)
3.1		AF009366	Neural precursor cell expressed, developmentally down-regulated gene 9
2.7		M21019	Harvey rat sarcoma oncogene, subgroup R
2.7		X06368	Colony-stimulating factor 1 receptor
2.2		X81579	Insulin-like growth factor binding protein 1
2.1		AI851454	Cysteine rich protein 2
2.0		AA529583	Mortality factor 4 like 2
-2.1		X95280	G0/G1 switch gene 2
-2.2		M31680	Growth hormone receptor
-2.5		U15012	Growth hormone receptor
	3.4	AI852641	Nuclear protein 1
	2.8	M34094	Midkine
	2.8	AF058798	Stratifin
	2.1	X81580	Insulin-like growth factor binding protein 2
Protein biosynthesis			
2.3		Y11460	Integrin beta 4 binding protein

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2.1		NM_011690	Valyl-tRNA synthetase 2
-2.0		AV055186	Ribosomal protein, large, P1
Proteolysis and protein degradation			
7.6	2.4	X61232	Carboxypeptidase E
6.1		AW060527	Ubiquitin-conjugating enzyme E2 variant 2
4.0		AJ000990	Legumain
4.0	5.0	AJ223208	Cathepsin S
3.7		AL078630	Ubiquitin D
2.0		U35833	Ubiquitin-like 1 (sentrin) activating enzyme E1B
-2.2		AI844932	F-box only protein 8
-2.4		L21221	Proprotein convertase subtilisin/kexin type 4
-2.6		AV359471	Ubiquitin specific protease 15
	-2.2	J04946	Angiotensin converting enzyme
	-2.5	L15193	Meprin 1 beta
Protein amino acid phosphorylation and dephosphorylation			
3.2		D89728	Serine/threonine kinase 10
3.2		M97590	Protein tyrosine phosphatase, non-receptor type 1
2.6		D37801	Protein tyrosine phosphatase, non-receptor type 21
2.0		X61940	Dual specificity phosphatase 1
-2.1		L31783	Uridine monophosphate kinase
Cell adhesion and extracellular matrix			
24.7		L36244	Matrix metalloproteinase 7
20.6		U43525	Proteinase 3
10.7		M82831	Matrix metalloproteinase 12
10.4	2.3	D00613	Matrix gamma-carboxyglutamate (gla) protein
9.0	3.1	X16834	Lectin, galactose binding, soluble 3
8.9		L02918	Procollagen, type V, alpha 2
8.1		M31039	Integrin beta 2
7.2		M62470	Thrombospondin 1
6.2		X13986	Secreted phosphoprotein 1
5.9	2.4	U03419	Procollagen, type I, alpha 1
5.8		D14010	Regenerating islet-derived 1
4.9	2.1	X52046	Procollagen, type III, alpha 1
4.7	2.5	M90551	Intercellular adhesion molecule
4.2		X58251	Procollagen, type I, alpha 2
4.0		L57509	Discoidin domain receptor family, member 1
3.4	4.3	U12884	Vascular cell adhesion molecule 1
3.2	3.3	M84487	Vascular cell adhesion molecule 1
3.2		L29454	Fibrillin 1
3.0		Z22532	Syndecan 1
2.9		M23552	Serum amyloid P-component
2.8		X04017	Secreted acidic cysteine rich glycoprotein
2.7		M38337	Milk fat globule-EGF factor 8 protein
2.7		AA763466	Procollagen, type I, alpha 1
2.5		AA919594	Elastin
2.5		M70642	Connective tissue growth factor
2.5		D88577	C-type (calcium dependent, carbohydrate recognition domain) lectin, superfamily member 13
2.3		M15832	Procollagen, type IV, alpha 1
2.2		X59990	Catenin alpha 1
2.2		U82624	Amyloid beta (A4) precursor protein
2.1		X53928	Biglycan
2.1		U89915	F11 receptor
2.0		X04647	Procollagen, type IV, alpha 2
2.0	2.2	V00755	Tissue inhibitor of metalloproteinase 1
2.0		X91144	Selectin, platelet (p-selectin) ligand
-2.1		AF101164	CEA-related cell adhesion molecule 2
-2.2		AI840501	Camello-like 1
	2.1	L19932	Transforming growth factor, beta induced
Cytoskeleton and structural elements			
5.0	7.3	M36120	Keratin complex 1, acidic, gene 19
4.8		V00830	Keratin complex 1, acidic, gene 10
4.5		U38967	Thymosin, beta 4, X chromosome
3.6	2.3	AI852553	Thymosin, beta 10
3.6		U42471	Wiskott-Aldrich syndrome homolog (human)
3.4		U29539	Lysosomal-associated protein transmembrane 5
3.4		M22479	Tropomyosin 1, alpha
3.2	2.2	M28739	Tubulin, beta 2
3.2		AW215736	RIKEN cDNA 2310057H16 gene

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3.2	4.5	M22832	Keratin complex 1, acidic, gene 18
3.1		AI505453	Myosin heavy chain IX
3.0		X15662	Keratin complex 2, basic, gene 8
2.8		X60671	Villin 2
2.7		D49733	Lamin A
2.7		AW125446	Golgi phosphoprotein 2
2.6		AW050256	Tubulin, beta 3
2.6		AI839417	Moesin
2.4		AW125698	Myosin heavy chain IX
2.4		AW212775	Actin-related protein 2/3 complex, subunit 1B
2.4		AV356071	Lysosomal-associated protein transmembrane 5
2.2		M28727	Tubulin, alpha 2
2.2		AI835858	Tropomyosin 4
2.2		M12347	Actin, alpha 1, skeletal muscle
2.1		D88793	Cysteine and glycine-rich protein 1
2.1		AF020185	Dynein, cytoplasmic, light chain 1
2.1		AI837625	Cysteine and glycine-rich protein 1
2.1	3.2	X54511	Capping protein (actin filament), gelsolin-like
2.0	2.1	X04663	Tubulin, beta 5
2.0		AI841606	Actin-binding LIM protein 1
2.0		M21495	Actin, gamma, cytoplasmic
2.0		AI849152	Clathrin, light polypeptide (Lcb)
2.0		M60474	Myristoylated alanine rich protein kinase C substrate
-2.2		AW123904	Gamma-aminobutyric acid (GABA(A)) receptor-associated protein-like 1
	3.3	AB000713	Caudin 4
	2.6	AA755126	Keratin complex 2, basic, gene 7
	2.6	AF087825	Claudin 7
	2.3	AI195392	Actinin, alpha 1
Transport			
13.1	66.5	X81627	Lipocalin 2
12.1	2.0	L48687	Sodium channel, voltage-gated, type I, beta polypeptide
7.2		U04827	Fatty acid binding protein 7, brain
3.7		M24417	ATP-binding cassette, sub-family B (MDR/TAP), member 1A
3.4		AI842825	Glycolipid transfer protein
3.2		NM_033444	Chloride intracellular channel 1
3.2		X99347	Lipopolysaccharide binding protein
2.9		L13732	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1
2.8		U72680	FXYD domain-containing ion transport regulator 5
2.8		X60367	Retinol binding protein 1, cellular
2.5		U27315	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4
2.4		AI842065	Expressed sequence AW538430
2.3		AI849583	RIKEN cDNA 6330416G13 gene
2.3		AI852578	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2
2.1		D87661	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide
2.1		U28960	Phospholipid transfer protein
-2.0	-2.2	AA670737	RIKEN cDNA 1700013L23 gene
-2.1		M16360	Major urinary protein 5
-2.1		AF072757	Solute carrier family 27 (fatty acid transporter), member 2
-2.1		M16358	Major urinary protein 4
-2.1		L28836	ATP-binding cassette, sub-family D (ALD), member 3
-2.3		U38652	Solute carrier family 22 (organic cation transporter), member 1
-2.3		M16357	Major urinary protein 3
-2.3		U95131	Solute carrier family 10 (sodium/bile acid cotransporter family), member 1
-2.3	-2.3	AV355798	Major urinary protein 2
-2.3		AV104178	Serine (or cysteine) proteinase inhibitor, clade A, member 6
-2.4		U95132	Solute carrier family 10 (sodium/bile acid cotransporter family), member 1
-2.4		M16359	Major urinary protein 1
-2.4		AB028737	ATP-binding cassette, sub-family C (CFTR/MRP), member 6
-2.6	-2.0	D00073	Transthyretin
-2.9		AJ011080	Afamin
-3.8	-3.8	AI647632	Similar to putative integral membrane transport protein
-4.4		AI255271	Major urinary protein 2
-6.4		Y14660	Fatty acid binding protein 1, liver
-6.6		X70533	Serine (or cysteine) proteinase inhibitor, clade A, member 6
	4.0	M55413	Group-specific component
	2.7	AF047838	Chloride channel calcium activated 1
	2.7	AI849587	Protein distantly related to the gamma subunit family
	2.6	D00466	Apolipoprotein E
	2.4	AI661431	Aquaporin 2
	2.3	AI197481	Amiloride binding protein 1 (amine oxidase, copper-containing)
	-2.1	AI606956	Solute carrier family 2 (facilitated glucose transporter), member 5

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	-2.1	AW122706	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 8
	-2.2	AI120514	Solute carrier family 26 (sulfate transporter), member 1
	-2.3	AI837530	Solute carrier family 9 (sodium/hydrogen exchanger), member 8
Cell surface markers and membrane proteins			
12.9		X13333	CD14 antigen
6.7		X97227	CD53 antigen
6.4		M65027	Glycoprotein 49 A
5.9		D16432	CD63 antigen
5.3	2.3	AW209486	Prostate stem cell antigen
5.0	3.4	AF024637	TYRO protein tyrosine kinase binding protein
3.6		M58661	CD24a antigen
3.3		U37438	Deleted in malignant brain tumors 1
3.3		M55561	CD52 antigen
3.3		AI854863	RIKEN cDNA 1200015A22 gene
3.0		AF039663	Prominin 1
2.7		AI849180	Integral membrane protein 2C
2.6		AI787183	RIKEN cDNA 0610011I04 gene
2.5	2.5	X68273	CD68 antigen
2.2		AB031386	RIKEN cDNA 1810009M01 gene
2.0		L11332	CD38 antigen
-2.5		AI843959	RIKEN cDNA 5730403B10 gene
	3.3	AW261569	RIKEN cDNA D630035O19 gene
	2.0	AI847784	CD34 antigen
	-2.5	L23108	CD36 antigen
Transcription factors and nucleic acid binding proteins			
8.1	7.2	V00727	FBJ osteosarcoma oncogene
6.2	3.6	AW124113	Brain abundant, membrane attached signal protein 1
4.9	2.2	AW049031	Core promoter element binding protein
4.0		M90397	B-cell leukemia/lymphoma 3
3.8		M31885	Inhibitor of DNA binding 1
3.8	3.0	AA614971	Molecule possessing ankyrin-repeats induced by lipopolysaccharide
3.2	3.6	X61800	CCAAT/enhancer binding protein (C/EBP), delta
3.2		AF017258	Ribonuclease, RNase A family, 2
2.7		AB016424	RNA binding motif protein 3
2.6	2.4	U19118	Activating transcription factor 3
2.5		AF016294	E74-like factor 3
2.4		L03215	SFFV proviral integration 1
2.3		AI642098	RIKEN cDNA 4921515A04 gene
2.3		U20735	Jun-B oncogene
2.2		M60523	Inhibitor of DNA binding 3
2.2		D26089	Minichromosome maintenance deficient 4 homolog (S. cerevisiae)
2.1	2.2	U20344	Kruppel-like factor 4 (gut)
-2.0		U36799	Retinoblastoma-like 2
-2.0		AF038995	DEAD (Asp-Glu-Ala-Asp) box polypeptide 6
-2.1		L20450	Zinc finger protein 97
-2.1		X77602	Upstream transcription factor 2
-2.2		AF064088	TGFB inducible early growth response 1
-2.2		U95945	One cut domain, family member 1
-2.4		U62674	Histone 2, H2aa1
-2.4		AA002843	Nuclear factor I/X
-2.7		AI834950	Nuclear receptor subfamily 1, group D, member 1
-2.8		AW047343	D site albumin promoter binding protein
-3.4		X57638	Peroxisome proliferator activated receptor alpha
	4.7	AI840339	Ribonuclease, RNase A family 4
	2.7	M28845	Early growth response 1
	2.3	X16995	Nuclear receptor subfamily 4, group A, member 1
Metabolism			
8.5		M13018	Cysteine-rich protein 1 (intestinal)
6.1		AV327760	Stearoyl-Coenzyme A desaturase 2
6.0	37.5	X51547	P lysozyme structural
5.9		AW046124	Cytochrome b-245, alpha polypeptide
5.1	4.6	M21050	Lysozyme
4.9		X97047	Pyruvate kinase, muscle
4.2		AV368209	Pyruvate kinase, muscle
4.1		U43384	Cytochrome b-245, beta polypeptide
4.1		AA726364	Lipoprotein lipase
4.0		AI846517	Cytochrome b-561
4.0		AI854821	RIKEN cDNA 0610041P13 gene
3.8		U13705	Glutathione peroxidase 3
3.8		U12961	NAD(P)H dehydrogenase, quinone 1

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3.6	2.1	M26270	Stearoyl-Coenzyme A desaturase 2
3.6		M34141	Prostaglandin-endoperoxide synthase 1
3.6		X07888	3-hydroxy-3-methylglutaryl-Coenzyme A reductase
3.5		M31775	Cytochrome b-245, alpha polypeptide
3.5		AI847162	RIKEN cDNA 1300017C10 gene
3.4		U87147	Flavin containing monooxygenase 3
3.4		AA690863	ATPase, class VI, type 11A
3.4		J04696	Glutathione S-transferase, mu 2
3.3		X56824	Heme oxygenase (decycling) 1
3.0		AJ238894	Acyl-Coenzyme A thioesterase 3, mitochondrial
3.0		D42048	Squalene epoxidase
2.9		AW060927	Lanosterol synthase
2.8		J03953	Glutathione S-transferase, mu 3
2.4		U49350	Cytidine 5'-triphosphate synthase
2.4		AI594518	Chitinase, acidic
2.2		J02980	Alkaline phosphatase 2, liver
2.2		U27455	Serine palmitoyltransferase, long chain base subunit 2
2.2		AI327450	Phospholipase A2, group IB, pancreas
2.2		AF077527	Syndecan binding protein
2.2		AA710635	Colipase, pancreatic
2.1		M62766	3-hydroxy-3-methylglutaryl-Coenzyme A reductase
2.1		AW049778	Mevalonate (diphospho) decarboxylase
2.1		AF057368	7-dehydrocholesterol reductase
2.0		U49385	Cytidine 5'-triphosphate synthase 2
-2.0		AW123316	Methylcrotonoyl-Coenzyme A carboxylase 1 (alpha)
-2.0		AA824102	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7
-2.0		AF098009	Fatty acid amide hydrolase
-2.1		AV216468	Expressed in non-metastatic cells 1, protein
-2.1		L42996	Dihydrolipoamide branched chain transacylase E2
-2.1		AI846934	Lipin 1
-2.1		AV071102	Cytochrome c oxidase, subunit VIc
-2.1		AI839995	Sarcosine dehydrogenase
-2.1		X61397	Carbonic anhydrase 8
-2.1		AF022894	Sulfotransferase family 1B, member 1
-2.1		U24493	Tryptophan 2,3-dioxygenase
-2.2		AA675075	Proline dehydrogenase (oxidase) 2
-2.2		AV276715	Aldehyde dehydrogenase family 3, subfamily A2
-2.3		L11333	Esterase 31
-2.3		L11163	Acyl-Coenzyme A dehydrogenase, short chain
-2.3		AI840013	Peroxisomal delta3, delta2-enoyl-Coenzyme A isomerase
-2.4		M27347	Elastase 1, pancreatic
-2.4		U32684	Paraoxonase 1
-2.4		M77015	Hydroxysteroid dehydrogenase-3, delta<5>-3-beta
-2.4		AF030343	Enoyl coenzyme A hydratase 1, peroxisomal
-2.4		AF047542	Cytochrome P450, family 2, subfamily c, polypeptide 37
-2.4		AF047727	Cytochrome P450, family 2, subfamily c, polypeptide 40
-2.4		Z14050	Dodecenoyl-Coenzyme A delta isomerase (3,2 trans-enoyl-Coenyme A isomerase)
-2.5		D17674	Cytochrome P450, family 2, subfamily c, polypeptide 29
-2.5		AI844846	2,4-dienoyl CoA reductase 1, mitochondrial
-2.6		X83202	Hydroxysteroid 11-beta dehydrogenase 1
-2.6		U14390	Aldehyde dehydrogenase family 3, subfamily A2
-2.6		AW012588	3-ketoacyl-CoA thiolase B
-2.7		AI530403	Acetyl-Coenzyme A acyltransferase 1
-2.7		X51971	Carbonic anhydrase 5a, mitochondrial
-2.7		AF031170	Hydroxysteroid dehydrogenase-6, delta<5>-3-beta
-2.8		AI266885	RIKEN cDNA 1700124F02 gene
-2.9		AF030513	Retinol dehydrogenase 6
-3.0		U15977	Fatty acid Coenzyme A ligase, long chain 2
-3.0		X04283	Cytochrome P450, family 1, subfamily a, polypeptide 2
-3.4		X63349	Dopachrome tautomerase
-3.6		M15268	Aminolevulinic acid synthase 2, erythroid
-4.0		D63764	Pyruvate kinase liver and red blood cell
-4.1		AF026074	Sulfotransferase related gene X1
-4.1		Y14004	Cytosolic acyl-CoA thioesterase 1
-4.3	-3.8	AV141027	Cytochrome P450, family 7, subfamily b, polypeptide 1
-4.3		AJ132098	Vanin 1
-4.6		AW226939	Carboxylesterase 3
-5.1		U49861	Deiodinase, iodothyronine, type I
-6.1	-3.4	U36993	Cytochrome P450, family 7, subfamily b, polypeptide 1
-6.4		U12791	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2
-6.6	-2.8	M88694	Thioether S-methyltransferase
-6.9		AF090317	Cytochrome P450, family 8, subfamily b, polypeptide 1

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-14.0		AB018421	Cytochrome P450, family 4, subfamily a, polypeptide 10
-17.9		Y11638	Cytochrome P450, family 4, subfamily a, polypeptide 14
-28.0	2.5	AJ006474	Carbonic anhydrase 3
-37.8		M21855	Cytochrome P450, family 2, subfamily b, polypeptide 9
-93.8		L41519	Hydroxysteroid dehydrogenase-5, delta<5>-3-beta
	6.4	AB006034	Cytochrome P450, family 27, subfamily b, polypeptide 1
	4.8	U49430	Ceruloplasmin
	3.0	AF032466	Arginase type II
	2.9	J05277	Hexokinase 1
	2.6	Z19521	Low density lipoprotein receptor
	2.6	U04204	Aldo-keto reductase family 1, member B8
	2.6	AI848668	Sterol-C4-methyl oxidase-like
	2.6	U31966	Carbonyl reductase 1
	2.5	U49915	Adipocyte complement related protein
	2.4	AW124337	Microsomal glutathione S-transferase 1
	2.3	U18975	UDP-N-acetyl-alpha-D-galactosamine:(N-acetylneuraminyl)-galactosylglucosylceramide-beta-1, 4-N-acetylgalactosaminyltransferase
	2.2	L06047	Glutathione S-transferase, alpha 4
	2.1	AA718169	Resistin
	2.1	D88994	AMP deaminase 3
	2.0	AA710564	N-acetylneuraminate pyruvate lyase
	-2.0	U19265	Glucosaminyl (N-acetyl) transferase 1, core 2
	-2.0	AB005450	Carbonic anhydrase 14
	-2.1	M75886	Hydroxysteroid dehydrogenase-2, delta<5>-3-beta
	-2.1	AB020239	Adenylate kinase 4
	-2.2	U48896	UDP-glucuronosyltransferase 8
	-2.2	U89352	Lysophospholipase 1
	-2.2	M12330	Ornithine decarboxylase, structural
	-2.3	U90535	Flavin containing monooxygenase 5
	-2.3	AF009605	Phosphoenolpyruvate carboxykinase 1, cytosolic
	-2.3	AB015426	Fucosyltransferase 9
	-2.4	U89906	Alpha-methylacyl-CoA racemase
	-2.5	AA840463	Lysophospholipase 1
	-3.5	X06358	UDP-glucuronosyltransferase 2 family, member 5
Other			
18.6	8.2	U69488	G7e protein
10.9	2.4	X67644	Immediate early response 3
7.6		U78770	Trefoil factor 2 (spasmolytic protein 1)
2.9		AI117936	Mus musculus 11 days embryo head cDNA, RIKEN full-length enriched library, clone: 6230409N14 product:unknown EST, full insert sequence
2.7		AI852545	Transgelin 2
2.6	2.0	AW121336	RIKEN cDNA 1600023A02 gene
2.6		X58196	H19 fetal liver mRNA
2.5	2.2	U25844	Serine (or cysteine) proteinase inhibitor, clade B, member 6a
2.4		AA980164	SPARC related modular calcium binding 2
2.4		D38410	Trefoil factor 3, intestinal
2.2		U44426	Tumor protein D52
2.1		U22262	Apolipoprotein B editing complex 1
2.1	4.6	AW230891	Leucine-rich alpha-2-glycoprotein
-2.1		U32170	Regucalcin
-2.3		AI854813	Mus musculus 3 days neonate thymus cDNA, RIKEN full-length enriched library, clone: A630086H07 product:RAS GTPASE-ACTIVATING-LIKE PROTEIN IQGAP2 homolog [Homo sapiens], full insert sequence
-2.3		AW049373	RIKEN cDNA 2310016A09 gene
-2.8		AI326963	Angiopoietin-like 4
-3.0		AA797604	Angiopoietin-like 4
-3.4	10.7	AB011030	Protein related to DAN and cerberus
	9.8	AA986050	Fibrinogen, gamma polypeptide
	6.8	M64086	Serine (or cysteine) proteinase inhibitor, clade A, member 3N
	5.0	AA880891	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 10
	2.6	AI876446	Fibrinogen, alpha polypeptide
	2.1	X61597	Serine (or cysteine) proteinase inhibitor, clade A, member 3C
	2.0	X59520	Cholecystokinin
	2.0	D13003	Reticulocalbin
	-2.1	AI314227	RIKEN cDNA 0610006H10 gene
	-2.2	AW122036	Mus musculus transcribed sequence with strong similarity to protein ref:NP_005351.2 (H.sapiens) v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian); v-maf musculoaponeurotic fibrosarcoma (avian) oncogene homolog; Avian musculoaponeurotic fibrosarcoma (MAF) protooncogene [Homo sapiens]
	-3.4	M93264	Pregnancy zone protein

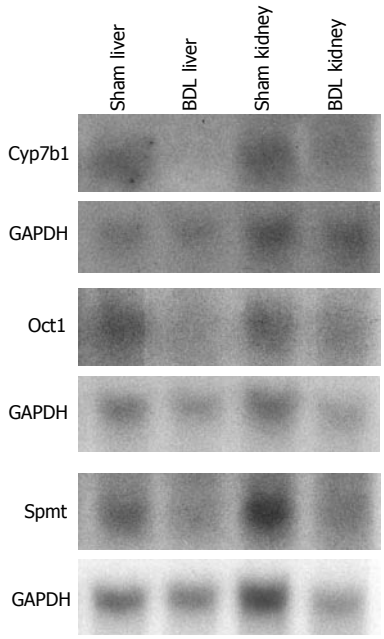

Figure 1 Confirmation of microarray data by Northern analysis of selected genes. Northern blots were performed with aliquots of pooled RNA from livers and kidneys from each 4 pair-fed sham-operated and 4 bile duct ligated mice, 7 d after surgery. Cyp7b1: Cytochrome P450 7b1; Oct1: Organic cation transporter 1; Spmt: Similar to putative integral membrane transport protein; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; Sham: Sham-surgery; BDL: Bile duct ligation.

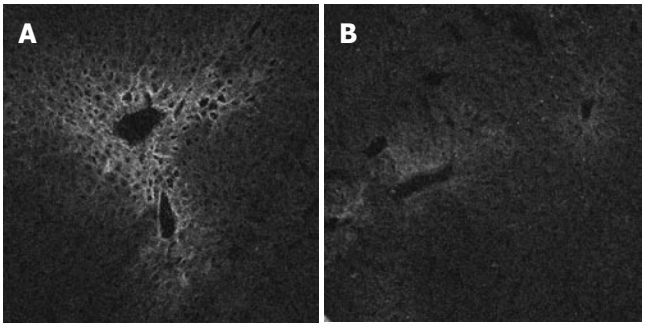

Figure 2 Indirect immunofluorescence of organic cation transporter 1 in murine liver sections. **A:** A low magnification view (x 20) shows antibody labeling at the basolateral membranes of hepatocytes of the pericentral zone of the liver lobule in the liver section of a sham-operated mouse, 7 d after surgery; **B:** In contrast, there is only a weak signal for organic cation transporter 1 after bile duct ligation.

et al[21] excluded genes with expression levels of less than 1 000, whereas we included genes with a mininum signal intensity of 600 and above. This approach led to the identification of a number of novel gene alterations of functional significance for the cholestatic phenotype. For instance, the decrease in expression of the gene encoding *Oct1* in BDL liver in the present microarray, an alteration not reported by Campbell *et al*[21] but previously reported by Ogawa *et al*[20] in the rat, led us to study this important basolateral cationic drug transporter in more detail. We were subsequently able to demonstrate that Oct1 is indeed down-regulated in rat liver, but not in kidney, in obstructive cholestasis at the mRNA as well as the protein levels and that this decrease results in reduced hepatic uptake of the Oct1 substrate tetraethylammonium[19]. Northern analysis and immunofluorescence microscopy of hepatic Oct1 performed in the present study indicated a similar pattern in mouse and confirmed the results of our microarray.

A number of other observations emerge from this analysis that deserve further study. For example, among the cell growth-related genes, the number of genes up-regulated in liver after BDL surpassed by far the number of down-regulated genes, a pattern which might reflect the extensive fibroproliferative process and tissue remodeling that takes place in this model of obstructive cholestasis. Similarly, a large number of genes related to cell adhesion, the extracellular matrix, and the cytoskeleton were found to be altered that have not been identified yet. We presume that many of these genes may play an important but as yet to be identified role in the fibrogenic response of the liver to bile duct obstruction. Alterations in the composition of the extracellular matrix are typical features of hepatic fibrosis[13], including substantial increases of collagens and non-collagenous components[25,26]. Accordingly, we observed a uniform up-regulation of genes encoding the *procollagen types* $I\alpha_1$, $I\alpha_2$, $III\alpha_1$, $IV\alpha_1$, $IV\alpha_2$, and $V\alpha_2$ in this mouse model of obstructive cholestasis. In addition, two members of the matrix metalloproteinase family, the *matrix metalloproteinases 7* and *12*, were up-regulated more than ten-fold following BDL when compared with the sham-operated controls. Matrix metalloproteinases represent a group of calcium-dependent enzymes involved in physiological and pathological degradation of extracellular matrix and tissue-remodeling[27]. *Matrix metalloproteinase 7 (matrilysin)*, an enzyme which is associated with poor prognosis in hepatocellular[28] and cholangiocellular carcinomas[29], has been closely related to the fibro-proliverative process in chronic hepatitis C[30] but not in cholestatic liver diseases. In contrast, *matrix metalloproteinase 12*, to our knowledge, has not been associated with liver fibrosis before and deserves future attention. Interestingly, the genes encoding *tissue inhibitor of metalloproteinase 1*, *vascular cell adhesion molecule 1* and *intercellular adhesion molecule* were up-regulated both in liver and kidney of BDL mice. Genes encoding the *procollagen types* $I\alpha_1$ *and* $III\alpha_1$ were also increased in the kidney of BDL mice although at lower levels than in the liver. The up-regulation of fibrosis-associated factors in kidney following BDL might be due to a paracrine action of fibrogenic mediators such as *connective tissue growth factor* whose hepatic expression is increased in cholestasis as previously described[31,32] and confirmed in our microarray. However, the functional relevance of the increased expression of these fibrotic genes in the kidney remains to be determined. Alternatively, the simultaneous up-regulation of important regulators of transcription following BDL such as *FBJ osteosarcoma oncogene*, *core promoter element binding protein*, and *activating transcription factor 3* in both liver and kidney supports the idea of coordinated gene regulation in different tissues as response to a specific stimulus. Another non-collagenous component of the extracellular matrix which was up-regulated in BDL liver is the gene for the matricellular protein *secreted acidic cysteine rich glycoprotein*. Matricellular proteins are a group of matrix-associated factors that mediate cell-matrix interactions but do not serve primarily as structural elements[13]. In particular, the expression of *secreted acidic cysteine rich glycoprotein* has been associated with cell proliferation, migration, and extracellular matrix remodeling in tissues, and *secreted acidic cysteine rich glycoprotein* has been found to be increased in different models of hepatic fibrosis[33].

The expression of a number of genes encoding

membrane proteins and transporters that were not previously known to be affected by cholestasis was also of interest. For example, the gene encoding the *β1 subunit of the voltage-gated sodium channel* which is important for the maturation and function of this channel[34] was up-regulated in liver as well as in kidney of BDL mice. In contrast, the expression of the gene encoding the ATP-binding cassette transporter *multidrug resistance-associated protein 6* (*Mrp6,* Abcc6) was reduced in cholestatic mouse liver as previously described for the rat[20]. Since mutations of human MRP6 are associated with pseudoxanthoma elasticum, a disorder characterized by calcification of the elastic fibres and abnormalities of the collagen fibrils[35], it is tempting to speculate that reduced hepatic Mrp6 expression in cholestasis might have functional implications for the development of liver fibrosis. Other genes up-regulated in cholestatic liver were the genes encoding the macrophage receptor markers *CD14 antigen* and *CD68 antigen*. Hepatic expression of both markers is increased in patients with biliary atresia[36], and expression of *CD68 antigen* may be an indicator of prognosis[37]. The functional significance of the concomitant *CD68 antigen* elevation in BDL kidney is unclear at the moment but illustrates again the close linkage between liver and kidney in this model of cholestasis and supports again a concept of coordinated gene regulation in different tissues.

In accordance with previous studies[38], obstructive cholestasis decreased the expression of a number of genes encoding *cytochrome P450* isoenzymes in liver. Since BDL results in an increase in liver concentrations of bile acids[15], the down-regulation of the *cytochrome P450 7b1 (oxysterol 7α-hydroxylase)* and *cytochrome P450 8B1 (sterol 12α-hydroxylase)* genes, that encode key enzymes in the conversion of cholesterol to bile acids[39], may represent adaptive responses to minimize the liver levels of cytotoxic bile salts. The increase of the gene encoding *cytochrome P450 27b1 (25-hydroxyvitamin D_3 1α-hydroxylase)* in BDL kidney is another interesting observation. *Cytochrome P450 27b1* catalyzes the conversion of 25-hydroxyvitamin D_3 to 1,25-dihydroxyvitamin D_3, the last step in vitamin D activation, which takes place in kidney[40]. Thus the increase in renal *cytochrome P450 27b1* expression may reflect an adaptive response to compensate for 25-hydroxyvitamin D deficiency in cholestasis. This may be a pathophysiologically important mechanism since patients with primary biliary cirrhosis often present with deficiencies of 25-hydroxyvitamin D but normal or even elevated levels of 1, 25-dihydroxyvitamin D[41].

In summary, the present study provides a comprehensive gene expression profile from mouse liver and kidney in obstructive cholestasis. Changes in gene expression were validated by Northern analysis, immunofluorescence, or comparison with the literature. The findings in this study provide new insights for generating novel hypotheses concerning the adaptive responses of gene expression in this mouse model of cholestasis.

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CLINICAL RESEARCH

Impact of lymph node micrometastasis in hilar bile duct carcinoma patients

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Abstract

AIM: To immunohistochemically examine micrometastasis and VEGF-C expression in hilar bile duct carcinoma (HBDC) and to evaluate the clinical significance of the results.

METHODS: A total of 361 regional lymph nodes from 25 patients with node-negative HBDC were immunostained with an antibody against cytokeratins 8 and 18 (CAM 5.2), and immunohistochemical staining of VEGF-C was performed in 34 primary resected tumors.

RESULTS: Lymph node micrometastasis was detected in 6 (24%) of the 25 patients and 10 (2.8%) of the 361 lymph nodes. Patients with micrometastasis showed significantly poorer survival rates than those without ($P = 0.025$). VEGF-C expression was positive in 17 (50%) of 34 HBDC, and significantly correlated with lymph node metastasis ($P = 0.042$) and microscopic venous invasion ($P = 0.035$).

CONCLUSIONS: It is suggested that immunohistochemically detected lymph node micrometastasis has an impact on the outcome of HBDC. VEGF-C expression is highly correlated with lymph node metastasis in HBDC and might therefore be a useful predictor.

Key words: Hilar bile duct carcinoma; Lymph node metastasis; Micrometastasis; Vascular endothelial growth factor-C

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INTRODUCTION

Hilar bile duct carcinomas (HBDC) are one of the most difficult to cure malignant gastroenterological tumors[1-4] and curative resection is essential for long-term survival. Because hilar bile duct tumors are in close proximity to vital structures in the hepatic hilum, such as the hepatic artery and portal vein, and since they tend to spread to the proximal biliary tract and perineural and perilymphatic spaces, hepatectomy with thorough systematic extended lymph node dissection is frequently required for curative resection. However, even with margin-negative resection, the prognosis after curative resection remains poor. One possible reason for the poor outcome is existence of occult lymph node metastasis that cannot be detected by conventional hematoxylin and eosin (HE) staining at the time of surgical resection. Immunohistochemical and molecular techniques have, however, made it possible to identify lymph node micrometastasis missed by traditional methods. Recently, immunohistochemical and/or genetic detection of lymph node micrometastases of various tumors, including carcinomas of the breast[5,6], lung[7,8], esophagus[9,10], stomach[11-14], colorectum[15,16] and gallbladder[17-19], has been reported. However, we were able to find only one report documenting this in HBDC[20].

Vascular endothelial growth factor C (VEGF-C) is a member of the highly glycosylated vascular endothelial growth factor (VEGF) family that regulates vasculogenesis, hematopoiesis, angiogenesis, lymphangiogenesis and vascular permeability, and has been implicated in many physiological and pathological processes[21,22]. Overexpression of VEGF-C cDNA in the skin of transgenic mice has been shown to selectively induce lymphatic endothelial cell proliferation and hyperplasia of the lymphatic vasculature[23]. It was also recently reported that a VEGF-C-transfected tumor cell line implanted into the stomach of nude mice gave rise to numerous lymph node metastases[24]. The most prominent VEGF-C expression has been detected in the human heart, placenta, muscle, ovary, and small intestine[25], and a positive correlation between expression and various clinicopathological factors, especially lymph node metastasis, has been reported in a number of tumors,

including carcinomas of the thyroid[26], breast[27], lung[28], esophagus[29], stomach[30,31], colorectum[32], prostate[33] and pancreas[34]. However, no investigations have been conducted with regard to VEGF-C expression in HBDC and possible clinicopathological associations. In this study, we examined lymph node micrometastasis and VEGF-C expression in patients with HBDC and evaluated the clinical significance of the results.

MATERIALS AND METHODS

Patients and specimens

From January 1981 to August 2000, 61 patients with HBDC underwent surgical resection plus systematic lymph node dissection in the First Department of Surgery, Mie University School of Medicine. Of these patients, 34 underwent macroscopic and microscopic margin-negative resection. Patients consisted of 21 males and 13 females with a mean age of 64.4±11.0 years (range: 37-89 years). The median follow-up period was 31.8 mo (minimum: 1.0 mon). No lymph node metastases were detected in 25 (73.5%) of the 34 patients by traditional pathologic examinations with HE staining.

Hepatectomy was performed in 29 (85.3%) of the 34 patients: extended right hepatectomy in 9 patients, left hepatectomy in 8, resection of segments 4a and 5 in 5, hilar resection in 4, extended left hepatectomy in 2, and caudate lobectomy only in 1. All 29 patients underwent combined resection of the caudate lobe. Two patients underwent combined resection of the portal vein, and 3 underwent pancreatoduodenectomy (PD) or pylorus-preserving pancreatoduodenectomy. Another 5 patients were treated with bile duct resection alone, including 2 patients who underwent combined PD.

A total of 361 lymph nodes dissected from 25 node-negative patients were examined immunohistochemically by staining with an antibody against cytokeratins 8 and 18, and all 34 primary tumors were immunohistochemically stained for VEGF-C. Tumor specimens and lymph nodes were collected from pathology files after obtaining informed consent from all patients in accordance with institutional guidelines.

Lymph node groups and resected margin status

Identification of the sites of lymph node metastasis were performed in accordance with the TNM Classification of Malignant Tumors proposed by the International Union Against Cancer (UICC)[35], which defines regional lymph nodes as the cystic duct, pericholedochal, hilar and peripancreatic (head only), periduodenal, periportal, celiac and superior mesenteric nodes, N0 as no regional lymph node metastasis and N1 as regional lymph node metastasis.

Evaluation of resected margin status was performed in accordance with the General Rules for Surgical and Pathological Studies on Cancer of the Biliary Tract (The 5th Edition) proposed by the Japanese Society of Biliary Surgery (JSBS)[36], which defines pEM0 as no tumor invasion within 5 mm of the resected margin, pEM1 as tumor invasion within 5 mm of the resected margin and pEM2 as distinct tumor invasion of the resected margin. pEM0 and pEM1 resections were defined as margin-negative in this study.

Immunohistochemical staining

Tissue samples were fixed in 10% formaldehyde with phosphate-buffered saline (PBS) and embedded in paraffin. Lymph node tissue was cut into six 5-μm thick sections, and primary tumor tissue was cut into a single 5-μm thick section. Briefly, the sections were deparaffinized with xylene and rehydrated through graded concentrations of ethanol. For antigen retrieval, sections were placed in 0.1*mol*/L citrate buffer (pH 6.0) and heated three times for 3 min each in a microwave oven (500 W). Lymph node sections were then incubated with a mouse monoclonal antibody (CAM 5.2; Becton Dickinson, San Jose, CA) specific for cytokeratins 8 and 18, and tumor sections were incubated with affinity-purified goat polyclonal antibodies (IBL, Fujioka, Japan) to VEGF-C at 1:30 dilution. Immunohistochemical detection of CAM 5.2 and VEGF-C was performed by a standard avidin-biotin method on an automated Ventana ES immunostainer (Ventana Medical Systems, Tucson, AZ) according to the manufacturer's instructions[37].

We examined 6 sections per lymph node and diagnosed micrometastasis when tumor cells were detected immunohistochemically, after being missed by routine histologic examinations with HE staining. VEGF-C immunoreactivity was mainly present in the cytoplasm of cancer cells and/or in the connective tissue around cancer cells. For evaluation of VEGF-C immunostaining, we examined at least 200 cancer cells per case. Cases in which at least 10% of the cancer cells were immunoreactive were defined as VEGF-C positive. All immunohistochemical evaluations were performed by an experienced histopathologist unaware of the clinicopathological features of the patients.

Statistical analysis

All statistical calculations were carried out using StatView-J 5.0 statistical software (SAS Institute, USA). Results are expressed as the means±SD. Statistical analysis for comparisons of VEGF-C expression and clinicopathological factors (age, gender, lymphatic vessel invasion, microscopic venous invasion, perineural invasion and lymph node metastasis) were performed using the chi-square test and Fisher's exact probability test. Analysis for comparisons of VEGF-C expression and other factors (pT classification and histopathological grading) was performed using the Mann-Whitney *U*-test. The Kaplan-Meier method was used to estimate postoperative survival rates, and the generalized log-rank test was used to compare differences in survival rates. All P values were two-sided and $P<0.05$ was considered statistically significant.

RESULTS

Patient outcomes

Of the 34 patients with margin-free resected HBDC, 4 died of other causes; three of multiple organ failure including 1 postoperative death (within 1 mo), and 1 of unknown causes. In addition, 15 (50.0%) of the remaining 30 patients died of disease. Recurrence sites were the

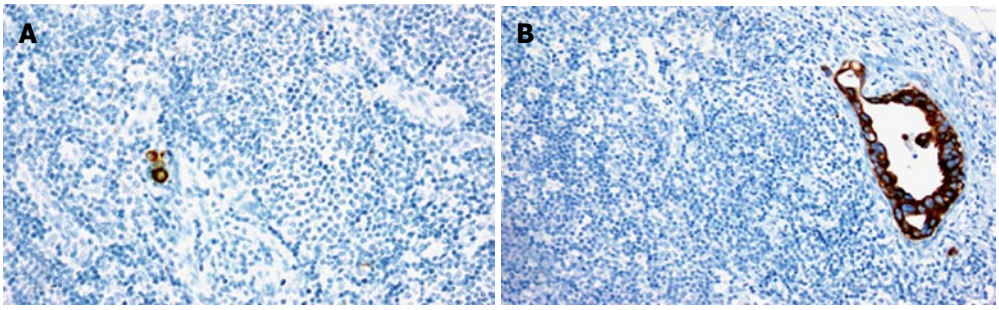

Figure 1 Immunohistochemical staining of lymph node micrometastasis with the monoclonal antibody CAM 5.2. **A:** Micrometastasis consisting of a single cell (original magnification, × 200). **B:** Micrometastasis consisting of a small cluster of tumor cells (original magnification, × 100).

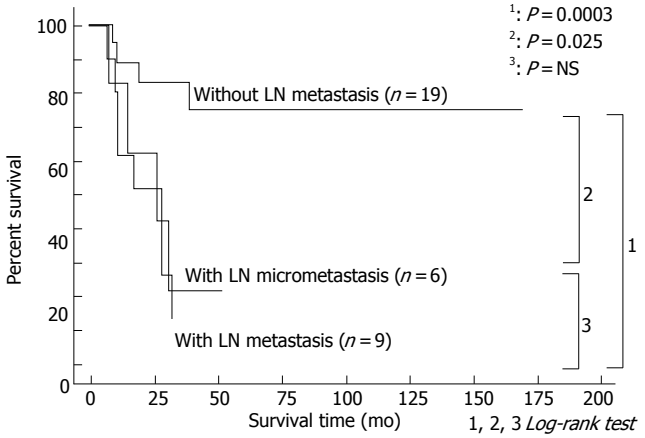

Figure 2 Survival curves after resection for hilar bile duct carcinoma according to the presence of lymph node metastasis, including micrometastasis.

liver in 2 patients including 1 patient with combined lung recurrence, the peritoneum in 1 patient, and local regions in 11 patients. Of these 11 patients, 3 showed combined recurrence in other sites; 1 showed combined liver metastasis, 1 showed combined lung metastasis, and 1 showed combined peritoneum recurrence.

Detection of lymph node micrometastasis

Micrometastasis was detected in 6 (24.0%) of the 25 node-negative patients and 10 (2.8%) of the 361 lymph nodes by immunohistochemical examination with CAM5.2. Lymph node micrometastasis was present in the form of a single-cell metastasis (Figure 1A) or a small cluster of tumor cells (Figure 1B). Of the 6 patients with lymph node micrometastasis, 5 had regional lymph node micrometastasis and 1 had regional lymph node with para-aortic lymph node micrometastases.

Impact of lymph node micrometastasis on survival

Cumulative survival rates were compared according to nodal status: the without lymph node metastasis group versus lymph node micrometastasis group versus HE diagnosed (overt) lymph node metastasis group (Figure 2). The 3- and 5-year survival rates of the 19 patients without lymph node metastasis were 81.6 and 72.5%, respectively, as opposed to 20.8 and 20.8%, respectively, in the 6 patients with micrometastasis and 29.6% and 0.0%, respectively, in the 9 patients with overt lymph node metastasis. Patients with lymph node micrometastasis showed significantly worse survival rates than those without ($P = 0.025$), and moreover, patients with overt lymph node metastasis showed worse survival rates than those without ($P = 0.0003$). There were no statistical differences between patients with lymph node micrometastasis and those with overt lymph node metastasis ($P = 0.469$). Five patients died of disease without overt lymph node or micrometastasis. Of these, 4 died of local recurrence, including 1 patient with combined liver metastasis. The remaining patient died of liver and lung metastasis. Follow-up revealed that 3 patients with lymph node micrometastasis survived with no evidence of disease for 11.7 and 36.7 and 60.3 mon after surgical resection, respectively.

To further evaluate the impact of lymph node micrometastasis on survival, survival rates were compared according to two groups: patients without lymph node metastasis versus those with overt lymph node and micrometastasis (Figure 3), and patients without lymph node metastasis and those with lymph node micrometastasis versus those with overt lymph node metastasis (Figure 4). The 3- and 5-year survival rates of the 19 patients without lymph node metastasis were 81.6 and 72.5%, respectively, as opposed to 25.5 and 8.5%, respectively, in the 15 patients with overt lymph node and micro metastasis ($P = 0.0004$). On the other hand, the 3- and 5- year survival rates of the 25 patients without lymph node metastasis and those with lymph node micrometastasis were 66.9 and 60.2%, respectively, as opposed to 14.8 and 0.0%, respectively, in the 9 patients with overt lymph node metastasis ($P = 0.0015$).

VEGF-C expression and clinicopathological factors

VEGF-C expression was observed in 17 (50.0%) of the 34 primary tumors (Figure 5). The correlations between VEGF-C expression and clinicopathological factors are shown in Table 1. Microscopic venous invasion ($P = 0.035$) and lymph node metastasis ($P = 0.042$) were significantly correlated with VEGF-C expression.

Prognostic factors for hilar bile duct carcinoma

To identify useful prognostic factors, we performed univariate analysis of the following possible independent prognostic factors: age (above 60 years versus 60 years or less), gender, operative procedure (hepatectomy versus bile duct resection), histopathological grading (well differentiated versus moderately or poorly differentiated), lymphatic vessel invasion, microscopic venous invasion, perineural invasion, microscopic resection margin (em0 versus em1), VEGF-C expression, lymph node metastasis (including micrometastasis) and lymph node metastasis (excluding micrometastasis) (Table 2). Ultimately, 4 independent variables (microscopic resection margin ($P = 0.040$), VEGF-C

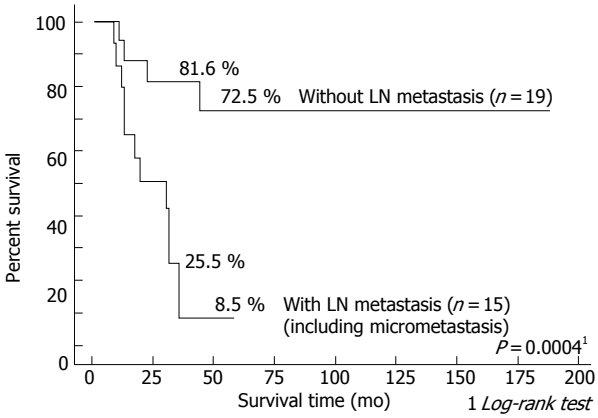

Figure 3 Survival curves after resection for hilar bile duct carcinoma according to the presence of lymph node metastasis: patients without lymph node metastasis versus those with overt lymph node and micro metastasis.

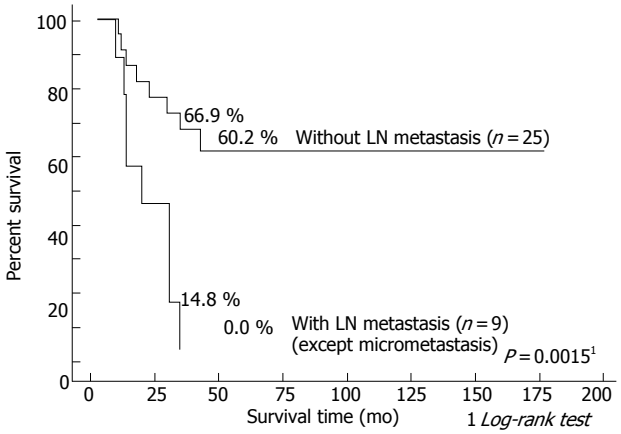

Figure 4 Survival curves after resection for hilar bile duct carcinoma according to the presence of lymph node metastasis: patients without lymph node metastasis and those with lymph node micrometastasis versus those with overt lymph node metastasis.

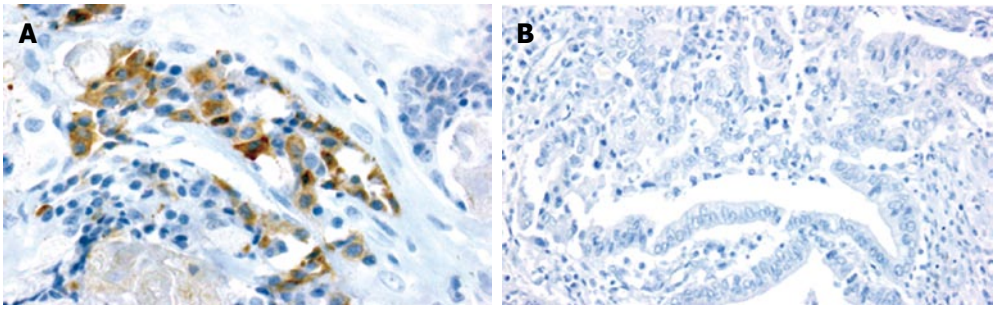

Figure 5 Immunohistochemical staining of primary tumors with VEGF-C polyclonal antibody. **A:** VEGF-C positive (original magnification, ×400). **B:** VEGF-C-negative (original magnification, ×200).

Table 1 Clinicopathological factors and VEGF-C expression

		VEGF-C expression		
		Positive (n = 16)	Negative (n = 18)	P Value
Age		64.7±11.6	64.1±10.8	0.870
Gender (M / F)		10 / 6	11 / 17	0.999
pT classification[1]	pT1	1	4	0.56
	pT2	8	11	
	pT3	7	3	
Histopathological Grading[1]	G1	10	15	0.225
	G2	6	2	
	G3	0	1	
Lymphatic vessel invasion	(presence)	14 (87.5 %)	13 (72.2 %)	0.405
Venous invasion	(presence)	10 (62.5 %)	4 (22.2 %)	0.035
Perineural invasion	(presence)	14 (87.5 %)	10 (55.6 %)	0.063
Lymph nodes metastasis	Metastasis (-)	6	13	0.042
	Metastasis (+) (including micrometastasis)	10	5	

[1]According to the TNM staging system. pT classification: pT1: Tumor confined the bile duct; pT2: Tumor invades beyond the wall of the bile duct; pT3: Tumor invades the liver, gallbladder, pancreas, and/or unilateral tributaries of the portal vein (right or left) or hepatic artery (right or left); pT4: Tumor invades any of follwing: main portalvein or its tributaries bilatellary, common hepatic artery, or other adjacent structures, e.g., colon, stomach, duodenum, abdominal wall. Histopathological Grading: G1: Well differentiated; G2: Moderately differentiated; G3: Poorly differentiated.

expression (P = 0.036), lymph node metastasis (including micrometastasis) (P = 0.0004) and lymph node metastasis (excluding micrometastasis) (P = 0.0017)) were identified as statistically significant predictors of survival.

DISCUSSION

Lymph node metastasis is a well known important predictor of prognosis with a wide variety of malignant tumors, and some studies have reported a significant relationship

Table 2 Univariate analysis of survival

Variable		5-yr survival (%)	*P* Value
Age	<60 *vs* ⩾60	40.0 *vs* 44.9	0.912
Gender	male *vs* female	42.3 *vs* 45.5	0.872
Operative procedure	hepatectomy *vs* bile duct resection	42.2 *vs* 60.0	0.430
Histopathologic Grading[1]	G2, G3 *vs* G1	37.5 *vs* 46.0	0.393
Lymphatic vessel invasion	present *vs* absent	37.4 *vs* 75.0	0.076
Venous invasion	present *vs* absent	36.4 *vs* 49.7	0.185
Perineural invasion	present *vs* absent	36.3 *vs* 68.6	0.064
Microscopic resection margin[2]	em1 *vs* em0	31.7 *vs* 83.3	0.040
VEGF-C expression	positive *vs* negative	23.3 *vs* 70.5	0.036
Lymph node metastasis (Including micrometastasis)	positive *vs* negative	8.5 *vs* 72.5	0.0004
Lymph node metastasis (except micrometastasis)	positive *vs* negative	0.0 *vs* 60.2	0.0017

[1] According to the TNM staging system. Histopathological Grading: G1 Well differentiated, G2 Moderately differentiated, G3 Poorly differentiated.

[2] According to the Japanese Society of Biliary Surgery. General Rules for Surgical and Pathological Studies on Cancer of the Biliary Tract. em0: no tumor invades within 5 mm from resected margin; em1: tumor invades within 5mm from resected margin.

between lymph node metastasis and prognosis of HBDC patients[38-40]. However, patients with early stage carcinoma and no apparent lymph node metastasis sometimes die of metastasis after surgery despite complete resection of the primary lesion. One of the possible reason for the poor outcome in these patients is occult lymph node metastasis not identified by conventional HE staining at the time of surgical resection.

Numerous studies on the incidence and significance of lymph node micrometastasis in cancer patients have been conducted in recent years. A number of investigators have proposed the prognostic significance of lymph node micrometastasis for various tumors including lesions of the lung, esophagus, stomach and colon, while others have suggested that lymph node micrometastasis is not significant for patient outcome. Thus, there is no consensus on the clinical significance of lymph node micrometastasis. However, we were able to find only one report documenting this in HBDC. Tojima *et al*[20] investigated 954 nodes from 45 patients with pN0 hilar cholangiocarcinoma after curative resection, and found micrometastasis in 13 (1.4%) nodes from 11 (24.4%) patients. Their data yielded similar survival curves for patients with and without lymph node micrometastasis (5-year survival rates: 43.6% *vs* 42.1%, respectively).

In this study, we demonstrated significant differences between outcomes of HBDC patients with and without lymph node micrometastases. Interestingly, a stronger correlation was recognized when patients with lymph node micrometastasis were treated as lymph node metastasis positive, compared to when they were treated as lymph node metastasis negative ($P=0.0004$ versus $P=0.0017$) (Figures 3 and 4). This might suggest the need to consider lymph node micrometastasis as overt lymph node metastasis.

One possible reason for the above-mentioned differing results is the number of sections examined. The number of sections immunohistochemically stained is considered an important factor in the diagnosis of lymph node micrometastasis. Many investigators examine lymph node micrometastasis using various sections of different thickness for immunohistochemical staining; however, the total thickness examined tends to range from 3 to 30 μm[6,8,10,13-20]. Sasaki *et al* examined the correlation between the number of CAM 5.2 sections and cumulative positive rate of lymph node metastasis[41]. They found that positive metastasis detection reached a plateau when over 9 sections (total thickness 27 μm) were examined. In this study, to identify lymph node micrometastasis, we examined six 5-μm sections (total thickness 30 μm) per lymph node by immunohistochemical staining. When we examined only one to four sections per lymph node, we found fewer lymph node micrometastases (data not shown).

Another possible reason for the differing results is the criteria of lymph node micrometastasis. In many studies, including ours, micrometastasis is defined as tumor cells detected only by immunohistochemical staining. However, some authors set size criteria for micrometastasis, such as deposits less than 2[42] or 0.5 mm in diameter[20,43]. Recent progress in molecular biological techniques has led to the development of genetic methods for detecting micrometastasis, including RT-PCR. RT-PCR is capable of detecting more micrometastasis foci than immunohistochemical staining[44]. Five patients without overt lymph node or micro metastasis died of disease recurrence in this study. If we use RT-PCR to detect lymph node micrometastasis, we will be able to evaluate lymph node micrometastasis in more detail, and the significance of lymph node micrometastasis will potentially increase. Therefore, further examinations using RT-PCR appear necessary.

VEGF-C is a specific ligand of VEGFR-3 and VEGFR-2, and has been shown to stimulate lymphangiogenesis and angiogenesis both *in vitro* and *in vivo*. Nakashima *et al*[45] investigated VEGF-C expression in 52 patients with gallbladder carcinoma and found that expression was significantly stronger ($P<0.001$) in patients with lymph node metastasis than those without, and that the VEGF-C-positive group showed poorer outcomes than the negative group ($P<0.001$).

Our study revealed a significant correlation between VEGF-C expression and both the presence of lymph node metastasis (HE detected and micrometastasis) and outcome of HBDC. These results suggest that VEGF-C expression might play an important role in causing lymph node metastasis in HBDC, consistent with the findings of previous studies regarding other malignant tumors.

In conclusion, our findings suggest that immunohistochemical detection of lymph node micrometastasis provides very useful information of survival rates after surgery for HBDC. However, considering that 1 patient with lymph node micrometastasis survived for more than 5-years with no evidence of tumor recurrence, long-term survival is thus possible for some patients with lymph node micrometastasis; therefore, extended lymph node dissection is necessary in HBDC patients. Although further study is needed, VEGF-C seems to be a useful predictor of overt and micro lymph node metastasis.

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CLINICAL RESEARCH

Evaluation of thermal water in patients with functional dyspepsia and irritable bowel syndrome accompanying constipation

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Abstract

AIM: To evaluate the efficacy of water supplementation treatment in patients with functional dyspepsia or irritable bowe syndrome (IBS) accompanying predominant constipation.

METHODS: A total of 3872 patients with functional dyspepsia and 3609 patients with irritable bowel syndrome were enrolled in the study by 18 Italina thermal centres. Patients underwent a first cycle of thermal therapy for 21 d. A year later patients were re-evaluated at the same centre and received another cycle of thermal therapy. A questionnare to collect personal data on social and occupational status, family and pathological case history, life style, clinical records, utilisation of welfare and health structure and devices was administered to each patient at basal time and one year after each thermal treatment. Sixty patients with functional dyspepsia and 20 with IBS and 80 healthy controls received an evaluation of gastric output and oro-cecal transit time by breath test analysis. Breath test was performed at basal time and after water supplementaton therapies. Gastrointestinal symptoms were evaluated at the same time points. Breath samples were analyzed with a mass spectometer and a gascromatograph. Results were expressed as $T_{1/2}$ and T-lag for octanoic acid breath test and as oro-cecal transit time for lactulose breath test.

RESULTS: A significant reduction of prevalence of symptoms was observed at the end of the first and second cycles of thermal therapy in dyspeptic and IBS patients. The analysis of variance showed a real and persistant improvement of symptoms in all patients. After water supplementation for 3 wk a reduction of gastric output was observed in 49 (87.5%) of 56 dyspeptic patients. Both $T_{1/2}$ and T-lag were significantly reduced after the therapy compared to basal values [91 ± 12 ($T_{1/2}$) and 53 ± 11 (T-lag), Tables 1 and 2] with results of octanoic acid breath test similar to healthy subjects. After water supplementation for 3 wk oro-cecal transit time was shorter than that at the beginning of the study.

CONCLUSION: Mineral water supplementation treatment for functional dyspepsia or conspipation accompanying IBS can improve gastric acid output and intestinal transit time.

Key words: Mineral water; Constipation; Dispepsia; Thermal therapy

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INTRODUCTION

Functional dyspepsia and irritable bowel syndrome accompanying predominant constipation (IBSc) are two of the most prevalent diseases in the industrialized world. These disturbances are among the first diseases for a gastroenterologic examination with high social and economical costs. In particular, dyspepsia is the first cause of specialized blood and invasive examination in Europe and USA[1]. Dyspepsia may be organic when associated with specific gastrointestinal or liver diseases. To diagnose functional dyspepsia gastroenterologists need specific criteria known as "Roma criteria"[2]. Prevalence of dyspepsia in general population is variable in different studies between 14% and 41%[3-5]. About 65% of dyspeptic patients result from functional dyspepsia[6]. About 35%

of patients with functional dyspepsia present a delayed gastric output which is related to clinical symptoms[7,8] or other disturbances of gastrointestinal motility[9,10]. On the contrary, the link between gastric *H pylori* infection and functional dyspepsia has not been clarified yet[6] because the data on the effect of *H pylori* eradication on dyspeptic symptoms are discordant[11,12].

Treatment of dyspepsia is based on drugs that inhibit the gastric acid secretion (such as proton pump inhibitors or H2 blocker agents) or drugs that stimulate gastrointestinal motility (prokinetics agents). However, the efficacy of such therapies is often unsatisfactory in particular for the short duration of the improvement of symptoms. It should be underlined that a prolonged treatment with these drugs is related to a high incidence of side effects. Although there is no scientific evidence, several dietetic regimens have been used in combination with drugs by dyspeptic patients.

A high enrichment of fibers in diet is the first therapeutic steps for constipation[13]. In fact, a high fiber intake is related to an increased fecal mass in healthy subjects (with high interindividual variability)[14,15]. The efficacy of this treatment seems to be time-related, probably for an intestinal adaptation to the high intake of fibers[16]. IBSc is a chronic functional disorder associated with psychological, environmental, emotional, social factors (drugs, stress, lifestyle)[17]. A reduction of water intake may play an important role in the pathogenesis of constipation and water supplementation is often suggested by a general practitioner in clinical practice to IBSc patients[18,19]. Recently, it has been described that an increased water intake up to 1.5 liters of mineral water is able to increase the effect of a diet with fiber enrichment in patients with constipation[20]. In Europe, especially in Italy, Germany and France, a large number of thermal centers stress on the real role of a treatment with thermal mineral water in functional gastrointestinal diseases. For this reason we need international studies or controlled trial to evaluate the effect of mineral water on functional dyspepsia or IBSc. These studies may allow a medical prescription by physicians and evaluate the socio-economical impact on public health systems of these treatments compared to pharmacological therapies. The efficacy of thermal treatment with mineral water on gastrointestinal diseases[21] remains to be further clarified.

This study was to evaluate the economic and health indicators for the assessment of the efficacy of thermal therapies in reducing the health costs. Moreover, the effect of water supplementation therapy on functional dyspepsia and IBSc was also evaluated.

MATERIALS AND METHODS

Patients

A total of 3872 patients with functional dysepsia and 3609 patients with IBSc were enrolled in the study from 1999 to 2000 by the medical staff of 18 thermal centres distributed throughout Italy (Bagni di Lucca, Chianciano, Comano, Franciacorta, Pejo e Rabbi, Recoaro, Sangemini, Sant'Andrea Bagni, San Carlo, S. Elena, SanPellegrino, Sarnano, DI Stabia, Vallio, Vulpacchio, Montecatini, Angolo, Boario). Patients with a history of gastrointestinal, liver, pancreatic, gall bladder, neurological, muscular, rheumatological, autoimmunitary and immunological diseses were excluded from the study. Moreover, patients with severe high blood pressure (diastolic>110, systolic>180), cancer, recent surgical resection, and pregnant women were also excluded from the study. All enrolled patients underwent abdominal ultrasonography and fecal occult blood test. Only patients negative in both tests were enrolled in the study and underwent a first cycle of thermal therapy for a standard period (2000 mL of mineral water for 21 d). Compliance was evaluated by the percentage of empty bottles returned by patients at the end of the cycle. Before the first cycle of thermal therapy was started, an informed consent was obtained from all enrolled subjects. The anamnestic and clinical data were collected by submitting a questionnaire. The questionnaire included approximately 1400 closed-answer questions (Table 1) for identifying the exposure variables and risk indicator, with special reference to the social-demographic and clinical variables including different sections (personal data, social and occupational status, family and pathological case history, life style, clinical records and social data, also including indicators of the standard living, utilisation of welfare and health structure and devices). According to the first study plan, the patients were re-examined at the same thermal centre one year after the first therapeutic cycle and received a second cycle of thermal treatment. A second follow-up was made after another year. During the follow-up the effect of the treatment was evaluated by assigning a score on side effects, personal opinion of the treatment and overall tolerability of therapy (Table 1). Moreover a retrospective assessment of the clinical follow-up, drug intake and utilisation of welfare and health service between the first and second cycles was carried out. Statistical analysis was performed using Bowker's symmetry test and ANOVA to compare uncontinuos variables.

Table 1 Main items included in the questionnaire

Anagraphic data
Blood pressur, heart rate
Occupation
Physical activity[1]
Disease duration
Main symptoms[1]
Admission to hospital (d)[1]
Missed work days[1]
Clinical relapse[1]
Drugs comsumption[12]
Igienic-dietary habit[1]
Personal opinions on therapy[2]
Reasons of Good opinions on therapy[2]
Side effects[2]

[1]During past 12 mo; [2]Evaluation after treatment.

Methods

Sixty patients (30 females, 30 males, mean age 44±6 years) with functional dyspepsia and 60 healthy controls (30 males, 30 females, the mean age of 41±5 years) were enrolled. All patients underwent upper digestive endoscopy and abdominal ultrasonography to exclude peptic ulcer

disease, gastroesophageal reflux disease, liver, pancreatic and gall bladder diseases. Diagnosis of functional dyspepsia was made based on the Roma II criteria. All patients underwent ^{13}C octanoic acid breath test (OBT) and filled in a questionnaire for evaluation of gastrointestinal symptoms (post-prandial fullness, epigastric pain, bloating, heartburn, nausea, vomiting).

After OBT, all patients started a diet supplemented with mineral water (2000 mL of mineral water/d for 3 wk). During the mineral water treatment, patients were controlled with a standardized diet (similar caloric and fiber intake)[20]. Compliance to thermal therapy was evaluated by the number of empty bottles returned at the end of the study (20% or more of full bottles returned were considered as the indicator of in adequate compliance). After 3 wk of mineral water supplementation treatment, the OBT was repeated and a new questionnaire for evaluation of gastrointestinal symptoms was administered. Patients after an overnight fasting had 91 mg of 13-C octanoic acid dissolved in an egg with a standardized meal (50 g ham, 150 mL fruit juice, 100 g white bread and 100 mL water) in 10 min. Breath samples were collected in a test tube before and every 15 min for 4 h after ingestion of the labeled substrate.

Analysis of ^{13}C in the breath was performed using a mass spectrometer (Breath Mat; FinniganMat; Bremen, Germany). $0T_{1/2}$ and T-lag values were used to express the results after a regression analysis of exhaled air curves. Results were expressed as $T_{1/2}$ and Tlag. T-test for coupled or uncoupled data was used to compare the difference between groups. Difference in symptoms was evaluated by χ^2 test or Fisher's exact test. $P<0.05$ was considered statistically significant.

Twenty patients (10 females, 10 males, mean age 41 ± 5 years) with IBSc and 20 sex and age matched healthy controls (10 females, 10 males, mean age 40 ± 7 years) were enrolled. Diagnosis of IBSc was made based on the Roma II criteria. All patients underwent H_2-lactulose breath test (LBT) to evaluate the oro-cecal transit time. Then, all patients started a controlled diet with standard fiber and caloric intake supplemented with 2000 mL of mineral water. After 3 wk the diet was stopped and LBT was performed. All enrolled patients filled in a questionnaire for evaluation of gastrointestinal symptoms (bloating, hard stools, number of evacuations in a week, incomplete evacuation) before and after mineral water supplementation. Compliance to thermal therapy was evaluated by the number of empty bottles returned at the end of the study (20% or more of full bottles returned were considered as the indicator of in adequate compliance).

LBT was performed after 20 g of lactulose dissolved in 100 mL of water was administered. Breath samples were collected at basal time and every 15 min for 4 h in a specific test tube after the assumption of lactulose. The presence of hydrogen in the breath samples was evaluated by gascchromatography (Quintron Milwaukee, Wisconsin USA). The oro-cecal transit time was evaluated by curves of hydrogen exhaled during the test.

The Student t test for coupled or uncoupled data was used to compare the difference between groups. Difference in symptom prevalence was evaluated by χ^2 test or Fisher's exact test as appropriate. $P<0.05$ was considered statistically significant.

Table 2 Patients enrolled and re-evaluated after the first and second cycles of thermal therapy with mineral water

Water Type	Pathology	1st yr	2nd yr	3rd yr
	Dyspepsia			
Bicarbonate		1667	966	110
Salse		1282	979	30
Solfate		923	923	24
Total		3872	2868	164
	IBSc			
Bicarbonate		1471	861	59
Salse		1181	701	19
Solfate		957	949	30
Total		3609	2511	108

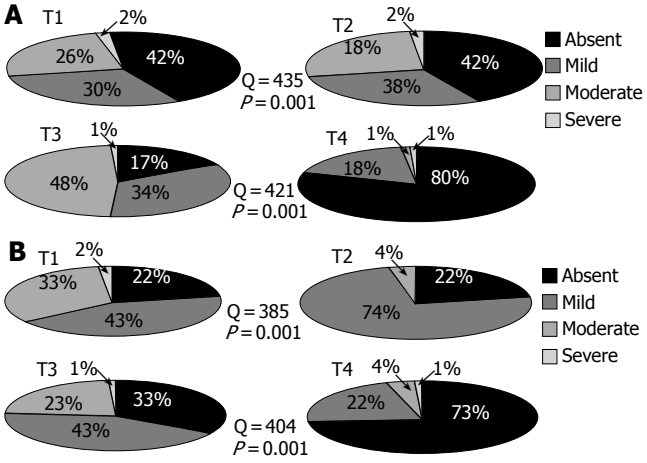

Figure 1 Prevalence of epigastric pain and heartbum (**A**) and postprandial gastric fullness (**B**) at the 4 time points of follow-up in dyspeptic patients.

RESULTS

A totae of 3872 patients with functional dyspepsia and 3069 patients with IBSc were enrolled in the first part of the study. After a year 74% and 69.5% came for follow up visit and to perform a second cycle of thermal therapy (Table 2). No difference among different mineral content of water supplemented was observed.

Figures 1A and 1B show the prevalence of dyspeptic symptoms (epigastric pain and post-prandial fullness) during the two cycles of thermal therapy. A significant reduction in the prevalence of dyspeptic symptoms was observed both at the end of the first and second cycles. In particular, 80% of the patients were symptom free after the first cycle of thermal therapy. Moreover, the score during the pretreatment period was similar between the two cycles. However, ANOVA showed reduction of symptoms when the whole follow-up period was considered. Similar results were observed for post-prandial fullness. The analysis of variance showed a real and persistant improvement of symptoms in all patients.

When the main symptoms of IBSc (bloating, hard stools, incomplete evacuation) were considered, significant improvement was observed with ANOVA (Figures

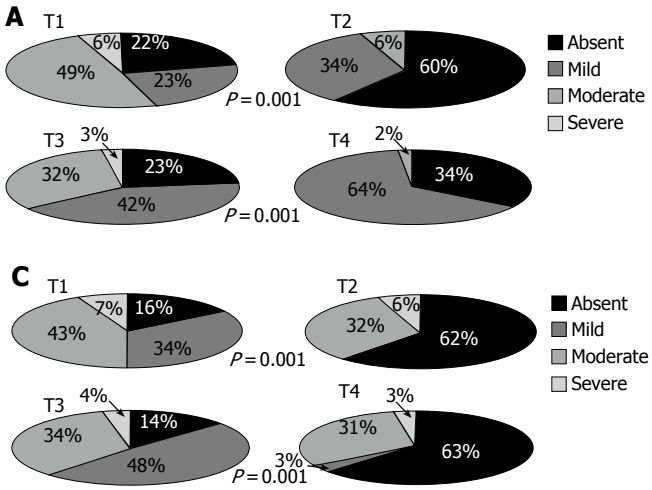

Figure 2 Prevalence of bloating (**A**), incomplete evacuation (**B**), and epigastric hard stool (**C**) at the 4 time points of follow-up in patients with IBS-c.

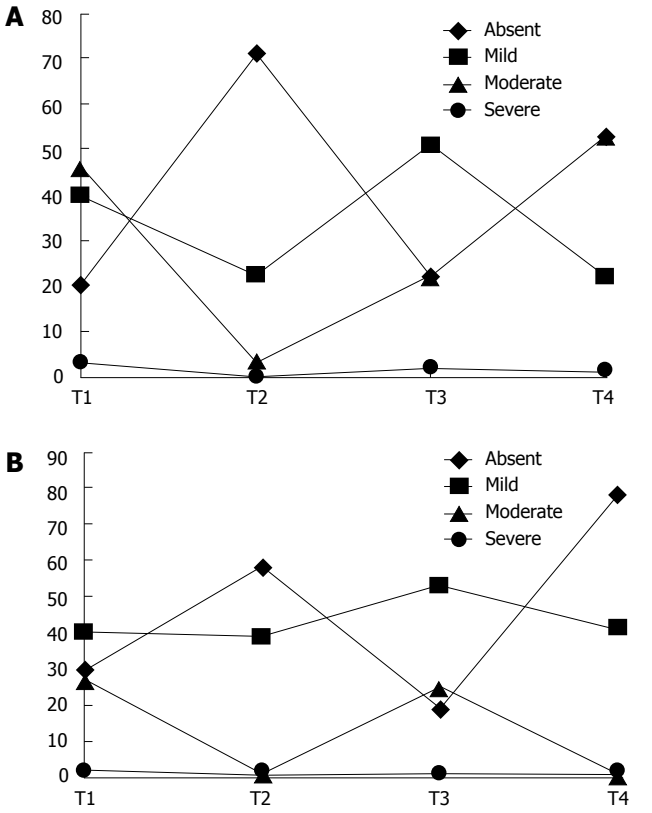

Figure 3 Mean score of symptoms in dyspeptic (**A**) and IBSc (**B**) patients at the 4 time points of follow-up.

2A-2C).

To better underline the temporal evolution of syptoms we reported the mean of scores for symptoms of dyspepsia and IBS at the 4 time points (Figures 3A and 3B).

The data on the number of hospitalization, days of absence from work and the clinical recrudescence before and after two cycles of thermal therapy were decreased by about 30% (Table 3). Moreover, data on overall tolerablity of treatment and reasons of acceptance of this treatment are shown in Table 4. About 80% of patients appreciated the treatment and about 95% gave a positive result of the treatment

Sixty patients and 60 healthy controls were enrolled in the study. Fifty-eight patients had a slow gastric output

Table 3 Dyspepsia-IBSc: relapse after two cycles of thermal therapy

	1 cycle (%)[2]	2 cycles (%)[2]
Admission to hospital[1]	5.6	2.0
Work lack[2]	3.4	2.2
Clinical relapse	29.6	21.9

[1] In previous 12 mo.
[2] On yearly working days.

Table 4 Subjective evaluation of dyspepsia and IBSc

Personal opinions on therapy (%)		Reasons of good opinion (%)	
Excellen	42	Care	95.6
Good	41.8	Relax	68
Lean	1.2	Stay	55.8
Null	6.3	Climate	45.3
No comment	8.4		

measured by OBT (mean $T_{1/2}$: 131 ± 18; Table 2). Patients with a normal gastric output were excluded. One patient and 2 controls refusing to give their consent were excluded. One patient was excluded for low compliance with the treatment (more than 20% of full bottles returned).

Dyspeptic patients presented an alterated gastric output and a significant difference at enollment compared with healthy controls when both $T_{1/2}$ (131 ± 18 *vs* 81 ± 7; $P < 0.01$) and T-lag (92 ± 11 *vs* 51±10; $P < 0.01$) were considered.

After mineral water supplementation for 3 weeks, a reduction of gastric output was observed in 49 (87.5%) of 56 dyspeptic patients. Both $T_{1/2}$ and T-lag were significantly reduced after the therapy compared to the basal values (91 ± 12 for $T_{1/2}$ and 53 ± 11 for T-lag, Table 5) with OBT similar to that in healthy controls. In controls and 7 patients, the gastric output did not change after mineral water supplementation treatment.

The prevalence of dyspeptic symtoms was significantly lower after the therapy than at enrolment. The prevalence of bloating and gastric fullness was significantly lower after mineral water supplementation treatment (Table 6). The

Table 5 Parameters of gastric emptyng rate (^{13}C octanoic acid breath test) in dyspeptic patients before and after mineral water supplementation therapy (mean ± SD)

	Pre-treatment	Post-treatment	*P*
$T_{1/2}$	131±18	91±12	<0.001
T-lag	92±11	53±11	<0.001

Table 6 Prevalence of gastrointestinal symptoms before and after mineral water supplementation therapy in dyspeptic patients

Symptomatology	T0 % (*n/n*)	T2 % (*n/n*)	*P*
Gastric fullness	86 (48/56)	25 (14/56)	<0.0001
Epigastric pyrosis	52 (29/56)	30 (17/56)	<0.05
Bloating	52 (29/56)	23 (13/56)	<0.005
Epigastric pain	24 (12/56)	11 (6/56)	NS
Nausea	5 (3/56)	0.2 (1/56)	NS
Vomiting	0.2 (1/56)	0 (0/56)	NS
Overall	100 (56/56)	68 (38/56)	<0.0001

Table 7 Oro-cecal transit time in IBC patients before and after mineral water based diet (mean ± SD)

	Pre treatament	Post treatament	*P*
Patients	1205±12	97±8	<0.001
Controls	85.5±14	81±8	NS
P	<0.001	<0.001	

Table 8 Gastrointestinal symptoms before and after mineral water supplementation therapy in patients with IBS

Symptoms	T0 % (*n/n*)	T21 % (*n/n*)	*P*
Bloating	90 (9/10)	20 (2/10)	<0.005
Abdominal Pain	40 (4/10)	20 (2/10)	NS
	T0 Media	T21 Media	
Evacuation/wk (*n*)	1.7	3.3	<0.001

T0: Basal time
T21: After mineral water supplementation therapy
NS: Not significant

overall prevalence of gastrointestinal symptoms was reduced after mineral water supplementation treatment too. At the basal point, a mean global score of symptoms was 15±4. After 30 d of mineral water supplementation treatment, the score was 7±3. A lower prevalence of abdominal pain, nausea, vomiting was also observed. No severe side effects were reported by patients. Only one control experienced mild diarrhea but treatment was not stopped. Five patients and 7 controls reported an increased number of evacuations during treatment. No effects of mineral water supplementation trealment on blood pressure, glycaemic control and heart rate were observed.

Oro-cecal transit time was longer in patients with IBSc than in controls (Table 7). All patients and 3 healthy controls had an abnormal oro-cecal transit time. After minere water supplementation treatment for 3 wk, the oro-cecal transit time was shorter than at the beginning of the study. A slight reduction in transit time was observed in healthy controls especially in those presenting a pathological transit time at the start of the treatment. The number of evacuations in a week was increased and bloating was reduced during mineral water supplementation treatment in patients with IBSc (Table 8). No side effects were reported by patients and controls.

DISCUSSION

Several diseases have been treated with thermal therapies for a long time in different countries. However, whether thermal therapy should be considered as pertinent to alternative medicines is a matter of debate and largely depends on the different cultural settings in which this practice is performed. In the Scandinavian, British and North American countries the therapeutic value and benefits of thermal (spa) treatment are seen with scepticism and looked at as an alternative and unorthodox practice. On the contrary, spa has been considered a credible medical treatment and supported by official undergraduate and postgraduate university teaching in most countries of the continent, Southern and Eastern Europe (France, Germany and Italy). It should also be mentioned that, differently from various forms of alternative medicine, spa therapy is firmly maintained in medical hands and undergoes orthodox medical control. As a consequence, thermal medicine appears as a supportive rather than alternative practice. Thus in the mentioned countries, spa therapy cannot be labelled as "alternative" medicine, but should be defined as "complementary" or "auxiliary" medicine. Nevertheless, also in these settings, an alignment to the Anglo-Saxon scepticism towards spa therapies has recently developed within the medical and academic community, although not all do so among patients and within the "civic" society.

Such scepticism is based on the scant number of studies published in medical journals of good reputation, apt to investigate with correct methodology and design the real benefits of spa therapies in various clinical conditions in term of efficacy and cost-effectiveness. Even in those countries in which "hydrology" is a recognized medical speciality with an academic background (as in Italy) most researcher work done in the past has been characterized by an approach mainly pathophysiologic and pharmacological, aimed at investigating the mechanism and biological effects of the mineral water rather than at assessing the related clinical and health economic effects with appropriate methods.

As in a number of European countries, variable kinds of financial support (public and private) have been provided for different forms of thermal treatment applied to various diseases. This issue is not a simple question of medical and academic relevance but has great implications from the socioeconomic point of view. In Italy, almost 340 thermal industries are crucial economics and social factor for many geographic areas. The yearly financial turnover related to the thermal activities amounts to 300 million dollars and 2000 million dollars as for health aspects and

linked activities, respectively, and is a key element of the national economy.

The evaluation of literature on the efficacy of spa therapies in the international bibliography can clearly show how wide the gap is in this context, if compared to traditional clinical domains and other nonconventional therapies. The limited impact of scientific research on the efficacy of spa therapies accounts the reservations of the scientific world to the actual efficacy of spa therapies, thus paving the way in Italy to discuss the public funding of these activities. On these bases in 1995, the Association of Thermal Industries and Curative Mineral Waters (Federterme", which officially represents all 340 Italian "medical" spa centres) have developed the epidemiological, health and cost-effectiveness aspects related to spa activities to assess the efficacy of thermal therapies in reducing the health costs.

The observational study has given very important results. The high number of patients who decided to come back for a second period of thermal treatment suggests a very good impact of the therapy on symptoms evaluted by ANOVA test. The data cannot be ascribed only to a placebo effect. We however, cannot conclude that thermal therapies are able to influence the natural history of studied diseases (dyspepsia and IBSc). It is possible that the beneficial effet of a single thermal treatment can influence the perception of symptoms as less severe even in a long follow-up period. This hypotesis was supported by reduction of day and number of hospitalization and day of absence by work during the follow-up period. It should be important to evaluate the economical impact of such results but it was not possible in this study. However, all cited parameters showed a reduction of about one third during the follow-up perion when compared to the former years. No reduction in drug use was observed (data not shown). It is possible to conclude that mineral water supplementation treatment for functional dyspepsia and IBSc can improve symptoms and reduce the medical cost as well as deserves further attentions. However, a best detailed analysis on cost/effectiveness should be performed.

Our data showed that mineral water supplementation therapy could reduce the gastric output of solid food and improve symptoms in patients with functional dyspepsia. The improvement of both studied parameters ($T_{1/2}$ and Tlag) suggests that mineral water can normalize both gastric output time and redistribution of alimentary bolus in the stomach. The pathophysiologic mechanism causing these effects are unknown. Our study demonstrated that mineral water supplementation therapy could improve symptoms and gastric acid output in functional dyspeptic patients evaluated by a questionnaire and OBT. The effects exerted by mineral water include stimulation of chemoreceptors and baroceptors in the gastric walls. The well known effect of water on intestinal motility (due to osmotic properties) with increased intestinal transit time induces an early duodenal transit of food with an earlier relaxation of pylorus and a faster transit of bolus from stomach to duodenum. Moreover, the presence of liquid in the stomach accelerates food disintegration and solubilization. The chemical content of particular water plays a role in the stimulation of specific gastric receptors that increases the gastric motility by secreting local hormones (gastrin, secretin, vasoactive intestinal peptide). The evidence that the effect of water is limited in patients suggests that hormones may be dysregulated in such patients. The presence of large amounts of calcium and magnesium in mineral water may directly stimulate the gastric smooth muscle to increase its motility and relax pylorus, thus producing its effect on gastric output and symptoms. The restoration of a correct gastric output reduces the time of exposition of bolus to intestinal bacteria, thus reducing the intensity of bloating due to intestinal bacteria overgrowth.

Constipation predominant irritable bowel sindrome is one of the frequent gastroenterological diseases in general population. Several pharmacological treatments have been proposed and used. Although therapies for constipation are efficacious, most of them are self-prescribed by patients with a high cost. Laxatives for example are widely used without medical control and may produce side effects. Moreover, the efficacy of laxative treatment is temporary and induces patients to increase dosage of drugs. A diet containing high fibers has been demonstrated to be a valid alternative to drugs for chronic constipation[20].

In our study, mineral water supplementation theray (2000 mL) for 3 wk accelerated oro-cecal transit time and improved symptoms in patients with IBSc. Compliance to therapy was excellent. The mechanisms of action of mineral water are not completely clear. Others iones present in mineral water may directly or indirectly (via neuroendocrine secretion of vasointestinal active peptides) stimulate smooth muscle to increase its motility. These actions reduce the transit time and increase the number of evacuations in a day with improvement of symptoms in IBSc patients. The decreased transit time improves bloating and reduces the time of contact between intestinal content and saprophytic flora with reduction in gas production.

In conclusion, mineral water supplementation theray can improve gastiric acid output, oro-cecal transit time and symptoms in patients with functional dyspepsia or IBSc. Mineral water supplementation therapy seems to be a simple, well-tolerated, cheap therapy for functional dyspepsia or IBSc and should be taken into account by physicians in the treatment of dyspepsia ad IBSc.

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RAPID COMMUNICATION

Interleukin-6 and its soluble receptor in patients with liver cirrhosis and hepatocellular carcinoma

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Abstract

AIM: To evaluate the immunohistochemical localization of interleukin-6 (IL-6) and IL-6 receptor (IL-6R) on tumor tissue specimens from patients with hepatocellular carcinoma (HCC) and the serum levels of IL-6 and sIL-6R in a group of patients with HCC as well as liver cirrhosis (LC) in a group of patients with LC alone and in a control group.

METHODS: Three groups of subjects were studied: group I ($n = 83$) suffering from HCC and LC, group II ($n = 72$) suffering from LC alone and group III ($n = 42$) as healthy controls. All patients had hepatitis C virus infection. Serum IL-6 and IL-6R levels were determined using a commercially available ELISA kit. Immunohistochemistry was performed using the streptavidin-biotin complex and rabbit polyclonal antibodies against IL-6 and IL-6R.

RESULTS: Immunohistochemistry analysis showed a medium to strong cytoplasmic and membrane reactivity for IL-6 and IL-6R respectively, in at least 40% of cases of HCC, whereas liver cirrhosis patients and controls were negative for IL-6 or showed a very mild and focal dot-like cytoplasmic reaction for IL-6R. Serum IL-6 levels in HCC group were significantly higher than those in LC and control groups ($P < 0.0001$). There was no significant difference in sIL-6R concentrations among 3 groups. When the patients with HCC were divided into groups according to Okuda's classification, a significant serum increase of IL-6 and sIL-6R level was observed from stage I to stage III ($P < 0.02$, $P < 0.0005$). When HCC and LC patients were divided into 3 classes of cirrhosis severity according to Child-Pugh, values in HCC patients were significantly higher than those in LC patients for each corresponding class ($P < 0.01$).

CONCLUSION: IL-6 serum levels in HCC patients are higher than those in LC patients and controls, suggesting an increased production of this cytokine by neoplastic cells. sIL-6R values are similar in all groups, increasing only in stage III HCC patients. These data suggest that they have a closer relationship with the neoplastic mass rather than with the residual functioning hepatic mass.

Key words: Interleukin-6; Cytokine; Chronic liver disease; Immunohistochemistry

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INTRODUCTION

Interleukin-6 (IL-6) is a proinflammatory cytokine which plays an important role in the host defence mechanism. Serum IL-6 levels are low in physiological conditions, but increase considerably in pathological conditions such as trauma, inflammation and neoplasia. In tumors, IL-6 may be involved in promoting the differentiation and growth of target cells. In fact, several neoplastic cell lines (such as esophageal cancer, renal cell carcinoma, multiple myeloma, prostate and ovarian cancer) have been shown to produce high *in vitro* levels of IL-6[1-5], and high concentrations of this cytokine are associated *in vivo* with a poor outcome of the disease in many types of tumours[6-12]. It has also been hypothesized that activation of the IL-6 gene is responsible for the derangement of some events which can lead to neoplastic degeneration[13].

IL-6 activity is mediated through the binding to its membrane receptor (IL-6R), which in turn promotes the

interaction with another receptor component, gp130, able to transduce IL-6 signalling at the intracellular level[14]. High concentrations of soluble IL-6R, like IL-6, are present in serum and other biological fluids in different pathological conditions, because it is released from cells expressing it on their surface[15].

Many works have reported high serum levels of IL-6 in various liver diseases, such as acute hepatitis[16], alcoholic cirrhosis[17], HBV-associated chronic hepatitis, primary biliary cirrhosis (PBC)[18], chronic hepatitis and HCV-correlated liver cirrhosis[19,20] and in hepatocellular carcinoma (HCC)[21-24].

Studies on animal models have shown that transgenic mice expressing high levels of IL-6 and sIL-6R develop hepatic nodular hyperplasia and signs of sustained hepatocyte proliferation, suggesting that IL-6 and sIL-6R could provide the primary stimulus to cell proliferation and are involved in development of HCC[25].

This study aimed to evaluate the immunohistochemical expression and localization of IL-6 and sIL-6R on tissue specimens from patients with HCC-associated liver cirrhosis and liver cirrhosis alone, and the serum levels of IL-6 and sIL-6R in patients with HCC- associated liver cirrhosis (LC) and to compare them in patients with LC alone and healthy controls.

MATERIALS AND METHODS

Patients

The study was performed in 207 subjects divided into three groups. Group I included 93 patients with HCC (61 males, 32 females, mean age 62.2 years, range 43-76 years). Diagnosis was made in 41 cases based on biopsy or cytological findings, diagnosis of the remaining cases was made on the basis of multiple, concordant imaging techniques (ultrasound, helicoidal computed tomography (CT), lipiodol-CT, selective angiography) and biochemical examination (AFP > 400 ng/mL). Some of the patients known as cirrhotics were enrolled in a prospective study for HCC screening, and others were referred to our center diagnosed as HCC. HCC was associated with the presence of serum HCV antibodies in all cases. The patients were then divided into the 3 stages of Okuda's classification[26] which as well as neoplasia size were also taken into account of serum values of bilirubin and albumin and the presence of ascites. The last three parameters were used to evaluate the hepatic functioning mass and the severity of the underlying cirrhosis. In brief, stage I was an initial stage in which the neoplasm (or the sum of the nodules) measured less than 50% of the whole liver section on CT scan. There was no ascites, albumin levels were over 3 g/dL and bilirubin levels were below 3 mg/dL. Stage II was moderately advanced, with two or more indices of advanced disease. Stage III was very advanced, with three or all indices of advanced disease. Group II included 72 patients (48 males, 24 females, mean age 56.5 years, range 36-75 years) suffering from liver cirrhosis, consecutively selected from out- or in-patients examined at our hospital. Diagnosis was made in 46 cases based on biopsy findings and diagnosis of the remaining cases was made on the basis of unequivocal clinical, biochemical and instrumental data. A post-study follow-up for at least 6 mo excluded the existence of neoplasia. The disease was associated with hepatitis C virus infection in all cases. The control group was composed of 42 healthy asymptomatic subjects (31 males, 11 females, mean age of 54.9 years, range 45-61 years recruited from donors at the blood bank of our hospital. Liver disease was excluded on the basis of anamnestic, biochemical and instrumental data. There were no cases of neoplastic disease, evaluated by a follow-up for at least six months. Daily alcohol consumption of above 30 g/d was found in none of the three groups.

Methods

Blood samples were taken after overnight fasting. After centrifugation part of the sera was used to assay the main parameters of liver function by routine methods. The remainder was frozen at - 40 ℃ for IL-6 and sIL-6R assay. Serological testing for anti-HCV was performed using the third generation of enzyme-linked immunosorbent assay (ELISA) (Orthodiagnostic System, Raritan, New Jersey, USA) in accordance with the manufacturer's instructions. Anti-HCV reacting samples were confirmed using the third generation of anti-HCV recombinant immunoblot assay (RIBA III, Chiron Corporation, Emeryville, CA, USA). Markers of HBV were tested using the Abbott RIA kit.

Liver biopsy samples were obtained for diagnostic purposes percutaneously according to the Menghini technique using 1.0-1.2 mm diameter needles (Surecut, Hospital Service, Rome, Italy). In some cases HCC was diagnosed with a thin needle (20 gauge, Surecut) guided by ultrascan using a Toshiba SSA 240 A apparatus with a 3.5 MHz probe.

Serum IL-6 and sIL-6R levels were determined using a commercially available ELISA kit (Quantikine, human IL-6 and sIL-6R, R & D Systems, Minneapolis, USA) in accordance with the manufacturer's instructions.

Immunohistochemistry analysis was performed on ten different HCC samples, five cirrhotic and two normal liver samples. Histologically normal liver tissues were obtained from patients during surgery for cholelithiasis. Written informed consent was obtained. The specimens were fixed in formalin and embedded in paraffin. Five- μm thick sections were cut and dewaxed, hydrated and incubated in 3% hydrogen peroxide for 20 min. Sections were then heated in microwave oven and non-specific binding was blocked by incubation with 3% rabbit normal serum for 20 min. Immunohistochemistry was performed using the streptavidin-biotin complex (StreptABC) with the following antibodies: rabbit polyclonal antibody against IL-6 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal antibody against IL6R (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:100 overnight in a 4 ℃ moist chamber and mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA, Dako, Santa Barbara, CA) at a dilution of 1:100 for 30 min at room temperature. Sections were then incubated for 30 min at room temperature with biotinylated anti-rabbit and anti-mouse immunoglobulin diluted in phosphate-buffered

Table 1 Median and range of serum values of IL-6 (pg/mL) and sIL-6R (ng/mL) in controls, liver cirrhotoc (LC) and hepatocellular carcinoma (HCC) patients and divided according to stage of disease

	Controls	LC	HCC	HCC Stage I	HCC Stage II	HCC Stage III	Z =	*P*
	(*n* = 42) median (range) (a)	(*n* = 72) median (range) (b)	(*n* = 93) median (range) (c)	(*n* = 28) median (range) (d)	(*n* = 46) median (range) (e)	(*n* = 19) median (range) (f)		
IL-6	3.6 (3-17)	6.4 (3-155)	14 (3-301)	6.5 (3-270)	12 (3-301)	32 (3-110)	b-c: z = 3.1	0.002
							a-c : z = 5.4	0.0001
							a-b : z = 2.3	0.03
							d-f : z = 2.8	0.005
							e-f : z = 2.7	0.01
							b-d : z = 1.9	0.05
sIL-6R	34.8 (7.2-80)	39.2 (4.8-80)	42.4 (17.6-80)	34.2 (17.6-65.6)	39.6 (18-80)	56 (30-80)	a-c : z = 2.1	0.04
							b-f : z = 2.7	0.01
							d-f : z = 3.2	0.002
							e-f : z = 2.9	0.004

Table 2 Median and range of serum values of IL-6 (pg/mL) and sIL-6R (ng/ml) in HCC and LC patients divided into groups according to Child-Pugh classes

	Class A		Class B		Class C	
	LC	HCC	LC	HCC	LC	HCC
	(*n* = 20) median (range)	(*n* = 44) median (range)	(*n* = 33) median (range)	(*n* = 30) median (range)	(*n* = 19) median (range)	(*n* = 19) median (range)
IL-6	3[a] (3-52)	6.5[a] (3-270)	8.5 (3-155)	14 (3-301)	8[c] (3-132)	28[c] (3-301)
sIL-6R	49.8[b] (21.2-80)	36.8[b] (17.6-67.2)	37.2 (4.8-72)	42.8 (18-72.8)	6.8[d] (24.4-57.2)	56.2[d] (24-80)

[a]$P < 0.04$ *vs* sIL-6R ; [b]$P < 0.04$, [d]$P < 0.03$ *vs* IL-6.

saline (PBS), with streptavidin-biotin complex for 50 min at room temperature and colour was developed with diaminobenzidine (DAB) for 40 min at room temperature, counterstained with Mayer haematoxylin for 1 min. The expression of IL-6 and IL-6R was considered positive when >30% of cells showed cytoplasmic or membrane staining. The percentage of PCNA- stained nuclei was calculated by counting the number of stained nuclei out of 1000 cells per high-power field for 10 different tumor sections.

Statistical analysis

The data were expressed as mean ± SD. Groups were compared using the Mann-Whitney *U* test. χ^2 test was used for the frequency analyses. Simple linear regression test and Spearman's rank correlation test were used where appropriate. The cut-off values IL-6 and sIL-6R were calculated as the value of the maximized likelihood ratio (LR) obtained using the following formula: LR = probability of true positives + probability of true negatives/probability of false positives + probability of false negatives. $P < 0.05$ was considered statistically significant.

RESULTS

Table 1 shows the median, range and ratio of serum IL-6 and sIL-6R values in the 3 study groups, and also in the HCC group divided according to Okuda's classification. Analysis performed with the Mann-Withney *U* test showed that the HCC group had significantly higher IL-6 values than the LC ($z = 3.1$, $P < 0.002$) and control group ($z = 5.4$, $P < 0.0001$). However, the LC patients had significantly higher IL-6 serum levels than controls ($z = 2.3$, $P < 0.03$). A significant difference was found in sIL-6R serum levels between the HCC group and controls ($z = 2.1$, $P < 0.04$), but not between the HCC and LC groups.

Analysis of the values after division of the HCC group according to Okuda's classification showed that serum values of IL-6 were significantly higher in stage III patients than in stage II and stage I patients ($z = 2.7$, $P < 0.01$ and $z = 2.8$, $P < 0.005$, respectively). Moreover, IL-6 values in stage I HCC patients were also significantly higher than those in the LC patients ($z = 1.9$, $P < 0.05$). There was no significant difference in sIL-6R values between stage I and stage II patients and the LC patients. The only significant difference was found between the stage III HCC patients and the LC patients, stage I and stage II HCC patients ($P < 0.004$).

Spearman's rank correlation test showed a significant increase in IL-6 and sIL-6R levels from stage I to stage III ($r = 0.28$, $P < 0.02$; $r = 0.39$, $P < 0.0005$, respectively).

Table 2 shows the median and range of IL-6 and sIL-6R in the HCC and LC groups divided according to Child-Pugh classes. The median IL-6 value was higher in HCC group than in the LC group, but the difference was significant only in classes A and C ($P < 0.0005$ and < 0.04, respectively) when compared to the corresponding LC groups. The median sIL-6R value decreased from class A to C in the cirrhotic group, but increased in the HCC group. Consequently, in the LC group the median sIL-6R value was significantly higher in class A ($P < 0.04$), while in the HCC group it was higher in class C ($P < 0.03$).

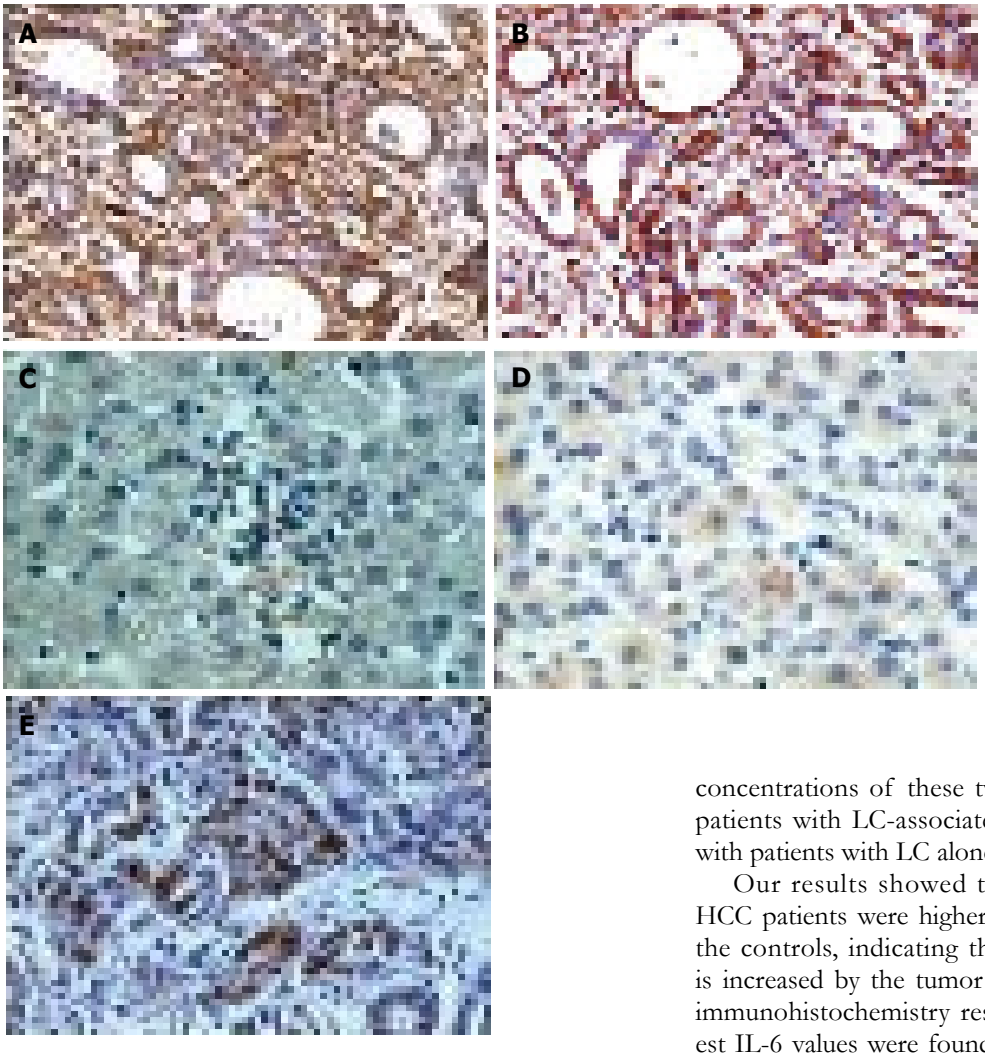

Figure 1 Results of immunohistochemical analysis of cytoplasmic and membrane reaction of IL-6 and IL-6R in HCC (**A, B**), normal liver (**C, D**), and cirrhotic (**E**) patients.

Figure 1 shows the results of the imunohistochemistry analysis. In the HCC group (ten cases), moderate to strong cytoplasmic and membrane reactivity for IL-6 and IL-6R respectively, was observed in at least 40% of cases (Figures 1A, 1B), whereas control cases of normal liver (two cases) were negative (Figure 1C) or showed a very mild and focal dot-like cytoplasmic reaction for IL-6R (Figure 1D). In liver cirrhosis group (ten cases) immunohistochemistry was negative on the whole or similar to control cases. PCNA immunoreactivity was observed in some nuclei of neoplastic cases with a mean value of 50% in the evaluated areas (Figure 1E).

DISCUSSION

Circulating IL-6 levels are elevated in patients with chronic viral[18-20] and alcoholic hepatitis[17]. Increased IL-6 is correlated with the stage of disease in liver cirrhosis [27]. Higher levels of IL-6 correlated with tumor size and cancer aggressiveness in patients with HCC[22].

At present, there are no data about the behaviour of circulating sIL-6R in patients with HCC and very few for other neoplasms. However, in multiple myeloma higher sIL-6R values correlate with a poor outcome of the disease[28]. Therefore, the aim of this study was to evaluate the expression and localization of IL-6 and sIL-6R in pathological liver tissue specimens as well as the serum concentrations of these two cytokines and their ratio in patients with LC-associated HCC and to compare them with patients with LC alone and healthy controls.

Our results showed that the median IL-6 levels in HCC patients were higher than those in LC patients and the controls, indicating that production of the cytokine is increased by the tumor cells. This is supported by the immunohistochemistry result and the fact that the highest IL-6 values were found in Okuda's stage III, in which the neoplastic mass is the most extensive. However, LC patients also had higher median IL-6 values than controls, but only for Child's classes B and C, while values in class A were close to control levels. In classes B and C, increased IL-6 serum levels compared to control values might reflect a response of the residual hepatic cells to cytolysis and to the attempt to recover the liver mass[25] associated with a clear impairment. In contrast, in HCC patients, whatever class of the disease, the median values were higher than those in LC patients, indicating that IL-6 serum levels increase when patients pass from cirrhosis to HCC. This was not true in the sIL-6R values, because the median value in HCC patients was significantly higher than that in controls ($P < 0.04$), and similar to that of LC patients. When the patients with HCC and LC were divided according to Child-Pugh's classification, the median serum values of sIL-6R tended to decrease from class A to class C in LC patients, while the opposite occurred in HCC patients, indicating that in cirrhotic patients sIL-6R production and release decrease as the disease progresses owing to the reduction of the liver parenchymal mass. On the other hand, in HCC patients, increased sIL-6R serum concentration might be due to the increasing tumor mass.

When the patients with HCC were divided according to Okuda's classification, IL-6 values significantly increased as the disease worsened. At all the stages the median IL-6 values were significantly elevated compared to the cirrhotic patients, indicating that neoplastic degeneration even in its

initial stages, causes variations in IL-6 levels, which could enable us to discriminate cirrhotic from HCC patients. Interestingly, sIL-6R levels were elevated only in patients with a more severe disease (stage III).

The high serum levels of IL-6 and sIL-6R were corroborated by the immunohistochemistry analyses, which showed the marked expression of both cytokines and their receptor in the tumor. This expression correlates to cell proliferation, as demonstrated by the concomitant presence of a high percentage of PCNA positive nuclei. The existence of such a relationship has also been reported for colorectal cancer[29]. On the whole, our results suggest that HCC cells, especially in advanced stages of the disease, may produce and secrete IL-6 and sIL-6R to stimulate their growth by an autocrine/paracrine mechanism as suggested by previous reports concerning cells derived from hepatomas or other types of cancer[30-34].

IL-6 production by tumor cells might also contribute to systemic complications such as induction of cachexia in the host[11] and local immunosuppression rather than immunopotentiation[35]. Thus, the present study might highlight the potential therapeutic benefits of approaches such as use of anti-sense nucleotides or IL-6R super antagonists, which can overcome the adverse effects of IL-6 in HCC, as in hepatoma cells, multiple myeloma, prostate cancer and other tumors[31, 34-36].

In conclusion, the IL-6 levels in patients with LC-associated HCC are higher than in patients with LC alone and controls, indicating that production of this cytokine is increased by tumor cells. This has been confirmed by the higher IL-6 values in stage III than in stages I and II of the disease. These results might be of help in differentiating cirrhosis from LC-associated HCC suggest that measures aimed at blocking adverse effects of IL-6 may be of potential clinical utility in HCC.

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RAPID COMMUNICATION

Rabeprazole test for the diagnosis of gastro-oesophageal reflux disease: Results of a study in a primary care setting

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Abstract

AIM: To determine the diagnostic value of the rabeprazole test in patients seen by general practitioners.

METHODS: Eighty-three patients with symptoms suggestive of GERD were enrolled by general practitioners in this multi-centre, randomized and double-blind study. All patients received either rabeprazole (20 mg bid) or a placebo for one week. The diagnosis of GERD was established on the presence of mucosal breaks at endoscopy and/or an abnormal esophageal 24-h pH test. The test was considered to be positive if patients reported at least a "clear improvement" of symptoms on a 7-point Likert scale.

RESULTS: The sensitivities of the test for rabeprazole and the placebo were 83% and 40%, respectively. The corresponding specificity, positive and negative predictive values were 45% and 67%, 71% and 71%, and 62% and 35%, respectively. A receiver operating characteristics (ROC) analysis confirmed that the best discriminatory cut-off corresponded to description of "clear improvement".

CONCLUSION: The poor specificity of the proton-pump inhibitor (PPI) test does not support such an approach to establish a diagnosis of GERD in a primary care setting.

Key words: Gastro-oesophageal reflux disease; Diagnostic tool; Rabeprazole; Proton pump inhibitors; Primary care

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INTRODUCTION

Gastro-esophageal reflux disease (GERD) is one of the most common disorders observed by primary care physicians. In this setting, an accurate, non-invasive and safe diagnostic test would be of great use. Proton-pump inhibitors (PPIs) are the most potent suppressors of gastric acid secretion and represent the mainstay of GERD treatment, with a therapeutic effect throughout the spectrum of the disorder. Therefore, in clinical practice, many physicians consider that rapid symptom relief after a short course of PPI therapy is a valuable marker for a diagnosis of GERD. This represents the basis for the development of so-called PPI tests, the value of which has previously been assessed by a number of different investigators, using various molecules which were tested in assorted referral populations, mainly in a secondary or tertiary care setting. The various PPIs and dosages, the duration of the PPI course and the way the results are interpreted are likely to be responsible for the conflicting results previously reported in the literature[1]. Rabeprazole is a more recently developed PPI with specific pharmacological properties such as a high pKa which may lead to both rapid accumulation in the acidic compartment of the parietal cell and more effective control of acidity during the first day of administration[2,3]. Similarly, when the target population for such PPI tests is considered, the lack of drug interference and the safety profile are both of most importance. In these respects, rabeprazole also displays some pharmacological advantages due to a partly non-hepatic metabolism and a linear response, which result in more predictable effects in terms of acid suppression[4,5].

We therefore aimed to determine the diagnostic value of the rabeprazole test in a population of patients followed up by general practitioners (GPs) for symptoms suspected to be reflux-related.

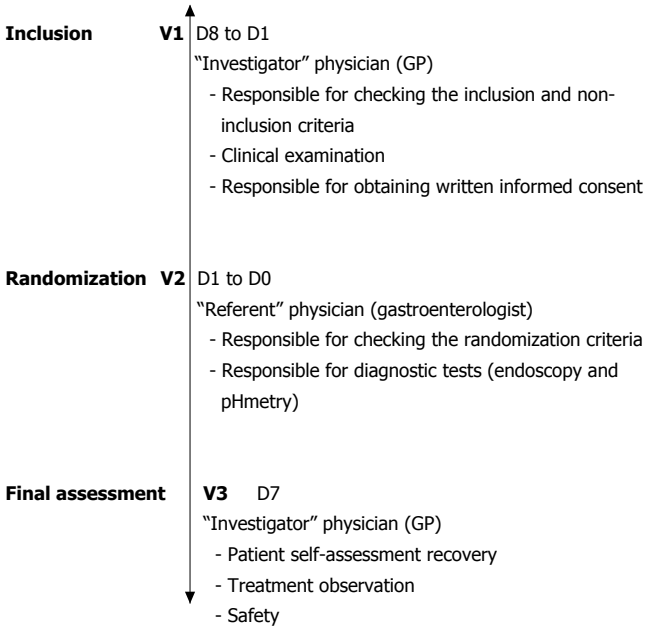

Figure 1 Diagrammatic illustration of the different phases of the study. Two visits were carried out by the investigator physician (V1: inclusion; and V3: final assessment). The second visit (V2) was carried out by the referent physician to check the inclusion criteria, to perform endoscopy (unless the patients had already undergone one endoscopy during the previous 6 months) and 24-h esophageal pH monitoring.

MATERIALS AND METHODS

Global design of the study

The study was conducted using a multi-centre, randomized and double-blind design. In order to maintain the double-blind nature of the study, each patient was examined by an "investigator" physician and a "referent" one. The investigator was a GP responsible for patient recruitment, inclusion and monitoring at the end of study assessment. The referent was a gastroenterologist with experience of endoscopy and esophageal pH monitoring, responsible for diagnostic testing, checking the randomization criteria, and prescribing treatment. The design of the study consisted three phases (Figure 1).

Phase 1 The investigator enrolled patients into the protocol according to the following inclusion criteria: (a) presence of at least 3 mo of typical (heartburn or regurgitation) or atypical (ascending burning epigastric pain, recurrent nausea, post-prandial digestive discomfort, dysphagia) gastrointestinal or extra-gastrointestinal symptoms suspected to be reflux-related; (b) occurrence of a particular symptom on at least 2 occasions during the 3 d prior to inclusion, the intensity of which being rated as "moderately uncomfortable or worse" using a 7-point Likert verbal analogue scale; (c) lack of previous investigations demonstrating esophagitis, such as esophageal pH monitoring and upper GI endoscopy; (d) absence of previous effective anti-reflux therapy, including PPIs, full-dose H_2-receptor antagonists or cisapride during the previous month. Conversely, the following patients were excluded from the trial: (a) women who were either pregnant, breast feeding or not using an effective method of contraception; (b) patients with a malignant condition or an uncompensated chronic disease, particularly uncompensated cardiac, liver or renal disease; (c) patients who had previously undergone a vagotomy or surgery that might alter gastric acid secretion; and (d) patients who were considered unable to comply with the conditions of the protocol, particularly with respect to follow-up and self-assessment questionnaires.

Written consent was obtained from all patients before inclusion. The study was approved by the Local Ethical Committee ('CCPPRB des Pays de la Loire n°2').

Patients were given a self-assessment form at the inclusion visit. On each day of the study, patients had to report their dominant symptom (i.e., the symptom which had led them to consult the investigator and that induced most discomfort for the patient). A consultation with the referral doctor was scheduled between 4 and 10 d after the inclusion visit. During this period patients were requested not to take any treatments that could be used to treat GERD, apart from the symptomatic treatment they had been given (Co-magaldrox, Maalox®).

Phase 2 The referent examined patients 4 to 10 d after their inclusion. The inclusion criteria were checked; notably the presence of at least two episodes of the dominant symptom in the 3 d prior to the consultation, rated as being at least "moderately uncomfortable" on the self-assessment form. Endoscopy was performed in all patients according to the usual practice of each centre. In patients with a normal endoscopy from the previous 3 mo, the results were considered not to contribute to a diagnosis of GERD and the investigation was not repeated. Twenty-four hour pH monitoring was performed immediately after the endoscopy. At the end of this second phase, patients who fulfilled the inclusion criteria and without any exclusion factors were randomly allocated to receive either a placebo or rabeprazole (20 mg bid) before breakfast and dinner for 1 wk. The investigators and patients were blinded to the administered treatments. Patients were not informed of the investigation results and the data was sent to a central database until the trial had been completed.

Phase 3 Patients were then received by the investigator at an end of study consultation after 7 ± 1 d of treatment. The investigator was unaware of the results of the endoscopy and pH monitoring. At this visit patients completed the response assessment form, rating any change in their symptoms. A rating according to 3 descriptions ("better", "roughly the same", or "worse") was firstly performed. Secondly, in cases where a symptom had improved, patients assessed the change using a Likert 7-point adjectival scale ("very slight improvement", "slight improvement", "clear improvement", "very great improvement", "near complete resolution", and "resolution"). The investigator recorded the number of tablets remaining, any adverse effects and any alteration in the patient's treatment over the period. According to the results of the rabeprazole test, the investigator classified the patient as a 'refluxer' or a 'non-refluxer'.

Esophageal pH monitoring

Esophageal pH monitoring was conducted using an ambulatory pH recording device (Synectics Mark II or III, Medtronics, Paris, France). The antimony electrode was positioned 5 cm above the cardia, located using the pH

step-up method[6] and an ambulatory recording was made. Patients were not given any particular lifestyle or dietary recommendations and were encouraged to behave as normally as possible. Patients were asked to record meals and sleeping periods, and the time of onset of symptoms. Patients returned to the referral centre 24 h later to stop the recording and remove the electrode. The data from the pH monitor were downloaded onto a computer, and the results were analyzed using a specific programme (EsopHogram Synectics software, Medtronics, Paris, France). For symptoms which had occurred during the recording period, analysis included symptom index determination (percentage of the total number of reported symptoms that were reflux-related) and probability of association as previously described[7,8]. Symptoms were considered to be reflux-related if they had occurred during the acid reflux event itself (pH < 4) or within 2 min after it had ended[9].

Evaluation and analysis of the results

Patients were classified as 'refluxers' or 'non-refluxers' according to the results of both upper GI endoscopy and pH monitoring. A diagnosis of reflux was established if one of the following criteria was present: (a) esophageal acid exposure (time below pH 4 during the 24-h period) greater than 4.2%; (b) statistically significant association between symptoms and reflux episodes ($P < 0.05$ or symptom index > 50%); and (c) presence of mucosal breaks at endoscopy.

The test (rabeprazole or a placebo) was considered to be positive or negative on the basis of the symptom response evaluated by the patient him/herself at the end of the one-week trial period. For this purpose, a 7-point adjectival Likert scale was used and the responses were dichotomised according to the cut-off descriptor of at least a "clear improvement". To further document the validity of that particular cut-off, a receiver operating characteristics (ROC) analysis of sensitivity was performed using different thresholds for the definition of a positive symptom response (CLINROC software - Metz Software, Chicago, IL, USA).

Sensitivity was defined as the proportion of 'refluxers' who had a positive test. Specificity was defined as the proportion of 'non-refluxers' for whom the test result was negative. The positive predictive value was defined as the proportion of 'refluxers' among patients with a positive test, whereas the negative predictive value was defined as the proportion of 'non-refluxers' among patients with a negative test.

Finally, all adverse events reported during the study period were also recorded for safety assessment.

Statistical analysis

As the study was mainly exploratory in nature, no prior forecast of subject number was made. The study period (January 2001 - May 2002) was also determined in order to reflect clinical practice in a primary care setting. We used mainly descriptive statistics and the results presented on the basis an intention-to-diagnose' (ITD) analysis. Student's *t*-test was used for quantitative variables and the Chi square test for qualitative variables. $P < 0.05$ was considered statistically significant.

Table 1 Characteristics of the intention-to-diagnose population in the placebo and rabeprazole groups (*n*, mean ± SD)

	Placebo (*n* = 39)	Rabeprazole (*n* = 33)	*P* value
Men/women (%)	46/54	33/67	0.269
Mean age (yr)(mean±SD)	47.1±11.8	49.1±11.9	0.4
BMI (kg/m^2) (mean±SD)	25.6±4.4	26.1±5.2	0.8
Smoker (%)	17.9	30.3	0.219
Previous endoscopy (%)	33.3	30.3	0.783
Time from first symptoms[1] (mo)	24.3±51.5	20.2±27.0	0.956
Post-prandial symptoms (%)	30.8	33.3	0.232
Nocturnal symptoms (%)	12.8	15.1	0.232
Hiatus hernia (%)	47.4	27.3	0.082
Esophagitis (%)	30.8	33.3	0.816
Barrett's oesophagus (%)	2.6	6.1	0.474
Diagnosis of GERD[2] (%)	67.6	62.1	0.642

[1]Suggesting the possibility of GERD; [2]Established by the presence of esophagitis and/or pathological exposure to acid during the 24-h period and/or significant association between symptoms and reflux.

RESULTS

Demographics and characteristics of population at inclusion

Ninety-one patients were selected. Of these patients, 83 were included, and 72 were randomized at the phase 2 visit. Among these 72 patients who completed the study and constituted the ITD population, 39 were in the placebo arm and 33 in the rabeprazole arm. Fourteen patients had at least one major deviation either at inclusion or during the course of the study (principally non-compliance with the intervals between visits and/or less than 6-d of treatment). The per-protocol population (PP), therefore, included 58 patients (33 in the placebo arm and 25 in the rabeprazole arm). In the two cohorts (ITD and PP), the most common predominant symptoms were epigastric pain, heartburn and regurgitation. At inclusion, the distribution of the predominant symptoms within the 2 groups was not significantly different. Approximately one third of patients in both groups had esophagitis, which was not of a severe grade in 90% of these subjects. None of the patients included had either stenosis or an ulcer at endoscopy. Compliance with treatment was good as 91% of tablets were taken; there was no significant difference between the two groups (Table 1). Since the results were very similar in the 2 cohorts, only the results from the ITD population are presented here. The characteristics of this population are shown in Table 1.

Six patients were unclassifiable due to pH monitoring technical failure. As a result, a definitive diagnosis of GERD or absence of GERD was made for 66 patients, 37 in the placebo group and 29 in the rabeprazole group. Forty-three were considered to be 'refluxers' (25 in the placebo arm and 18 in the rabeprazole arm) and 23 'non-refluxers' (12 in the placebo arm and 11 in the rabeprazole arm).

Diagnostic value of the rabeprazole test versus placebo test

In 'refluxers', the rabeprazole test was positive in 15 of 18 patients (sensitivity: 83.3%) whilst the placebo test was positive in 10 of 25 (sensitivity: 40.0%) ($P = 0.011$). In the 'non-refluxers', the rabeprazole test was negative in 5 of 11 patients (specificity: 45.5%) whilst placebo test was negative in 8 of 12 (specificity: 66.7%) ($P > 0.05$).

The positive and negative predictive values for the rabeprazole test were 71.4% and 62.5%, respectively. The corresponding predictive values for the placebo test were 71.4% and 34.8%, respectively ($P > 0.05$). The percentages of patients who were correctly classified by the rabeprazole test and the placebo test were 69.0% and 48.6%, respectively ($P > 0.05$).

The results of the ROC analysis are illustrated in Figure 2. The categories that were closest to the slope of the tangent to the 45° curve were those of "slight improvement" and "clear improvement". Nevertheless, the best compromise between sensitivity and specificity corresponded to the description "clear improvement" that was adopted for the former analysis.

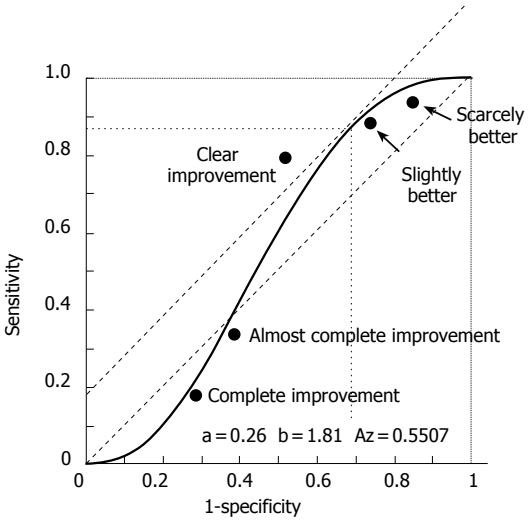

Figure 2 Receiver operating characteristics (ROC) curve. This was determined according to various satisfaction criteria (from "very slight improvement" to "resolution" of the symptoms) for patients with symptoms compatible with gastro-esophageal reflux. The symptoms were assessed after one week of treatment with a double dose of rabeprazole (20 mg bid) or a placebo.

Adverse events

Six patients (4 in the placebo arm and 2 in the rabeprazole arm) reported 7 adverse events (placebo: vomiting, diarrhoea, insomnia, dyslipidemia, urinary infection; rabeprazole: lymphadenitis, drug eruption) (non significant). No adverse event was considered to be serious.

DISCUSSION

The results of this study conducted in a primary care setting and with a placebo-controlled design showed that the rabeprazole test had high sensitivity (83.3%) but low specificity (45.5%). As compared with the placebo test, the rabeprazole test showed a superior sensitivity and negative predictive value, but non-significant differences with regard to specificity and positive predictive value.

This study is one of the first attempts to evaluate the diagnostic yield of a PPI test in primary care conditions with assessment of the results by GPs. The drug was administered for 7 d only for both practical and scientific reasons. In fact, a recent study using esomeprazole showed that although the sensitivity of the test increased each day following the start of the test, a plateau was reached after 5 d of administration, with no further improvement beyond that time point[10]. The reference tests (i.e. 24-h pH monitoring and endoscopy) were performed by independent physicians (referent) and the GP (investigator) remained blind to the results until response to PPI had been determined. In addition to acid exposure measurement, symptom analysis was also an important parameter of pH monitoring interpretation. This seems particularly relevant in a population of patients with normal endoscopy in approximately two thirds of cases. Indeed, nearly half of patients with endoscopy-negative GERD are known to have acid exposure within the normal range[11]. The use of symptom analysis permits an increase in the sensitivity of pH monitoring and therefore decreases the risk of missing genuine 'refluxers'. In addition, only patients with moderate to severe symptoms present on at least 2 occasions in the 3 d prior to inclusion were enrolled. The outcome of these rather rigorous inclusion criteria and design was a difficulty in fulfilling our initial goal of recruiting a large cohort of patients. Finally, from a number of candidate PPIs, we chose rabeprazole, since this drug has a number of potential advantages: (a) a rapid onset of action as shown by gastric pH monitoring studies[3,12,13]; (b) a good safety profile; (c) a lack of drug interactions due to its specificity in terms of hepatic and non-hepatic metabolic pathways[4,5]; and (d) its effectiveness in the treatment of some symptoms associated with GERD[14].

The results of our study are consistent with other studies of PPI tests which have without exception demonstrated the high sensitivity and poor specificity of this diagnostic approach[1]. This rather disappointing finding has recently been confirmed by the meta-analysis reported by Numans *et al*[15], which reached a similar conclusion concerning short-term trials of PPIs in GERD. The poor performance of PPI test is further reinforced by the comparison with the placebo-test which adequately 'classified' nearly half of the patients (random results). However, these results are not entirely surprising as a good placebo response has also been reported in short-term trials in GERD[10,16-18]. In addition, the above negative conclusions should be further balanced as far as negative predictive values are considered. Indeed, in the test conditions, a negative response to rabeprazole could exclude a diagnosis of GERD in 2 of 3 symptomatic patients (as compared to only one in three following the placebo). As the negative predictive value is influenced by the prevalence of the disease, our results may in fact be an underestimation and higher negative predictive values could be expected in a more representative sample of patients consulting for upper GI symptoms, supposing a 30% prevalence of GERD (i.e. approximately half of that observed in both the placebo and rabeprazole

arms of our study). Finally, our study design did not allow an appropriate evaluation of the cost-effectiveness of the PPI test as a whole and of the rabeprazole test in particular. The poor specificity of the test should, however, lead to some caution in terms of guidelines concerning long-term management strategy in GERD; there is a potential risk that 'non-refluxers' will continue to receive PPI treatment far beyond the initial test week if the symptomatic response is good. Whether such an empirical approach to acid-sensitive disorders is justified or potentially dangerous is presently unknown. Nevertheless, our data supports the conclusions of the French-Belgian consensus conference, which did not recommend the use of PPI tests for the diagnosis of GERD in clinical practice before the availability of further scientific information[19].

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RAPID COMMUNICATION

Progastrin-releasing peptide and gastrin-releasing peptide receptor mRNA expression in non-tumor tissues of the human gastrointestinal tract

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Abstract

AIM: To investigate the expression of gastrin-releasing peptide (GRP) and GRP-receptor mRNA in non-tumor tissues of the human esophagus, gastrointestinal tract, pancreas and gallbladder using molecular biology techniques.

METHODS: Poly A^+ mRNA was isolated from total RNA extracts using an automated nucleic acid extractor and, subsequently, converted into single-stranded cDNA (ss-cDNA). PCR amplifications were carried out using gene-specific GRP and GRP-receptor primers. The specificity of the PCR amplicons was further confirmed by Southern blot analyses using gene-specific GRP and GRP-receptor hybridization probes.

RESULTS: Expression of GRP and GRP-receptor mRNA was detected at various levels in nearly all segments of the non-tumor specimens analysed, except the gallbladder. In most of the biopsy specimens, co-expression of both GRP and GRP-receptor mRNA appeared to take place. However, expression of GRP mRNA was more prominent than was GRP-receptor mRNA.

CONCLUSION: GRP and GRP-receptor mRNAs are expressed throughout the gastrointestinal tract and provides information for the future mapping and determination of its physiological importance in normal and tumor cells.

Key words: Gastrin releasing peptide (GRP); Gastrin-releasing peptide receptor (GRPR); mRNA expression; Morphogenesis; Gastrointestinal tract

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INTRODUCTION

Gastrin-releasing peptide (GRP) is a member of the bombesin family of neuropeptides. Bombesin was originally isolated from the skin of the amphibian *Bombina bombina*, whereas GRP is the homologous peptide in mammals, including humans[1]. GRP and GRP-receptor are widely expressed in the central and enteric nervous systems (ENS). GRP is known to stimulate secretion of gastrin, gastric[2] and pancreatic juice[3] and hormones[4,5] to regulate the immune system[6], and to modulate smooth muscle contractility[7,8]. The direct expression of GRP and its receptor, and thus the exact mechanism behind its actions in gastrointestinal tissues, are only sparsely examined. Immunocyochemistry revealed the expression of GRP in submucosal cells of the ileum[9]. In colon, *in vitro* autoradiography showed the GRP-receptor expression in the myenteric, but not submucosal, plexus as well as on smooth muscle cells[10]. Examination of mucosal biopsies revealed GRP-receptor mRNA in cells lining the gastric antrum, but not in any other epithelial cells of the gastrointestinal tract[11].

GRP and GRP-receptor are frequently expressed in the gastrointestinal cancer cells, such as gastric adenocarcinoma[12,13], duodenal cancer[14], and colorectal cancer[15,16,17]. Cuttitta *et al*[18] have demonstrated that human cell lines derived from small-cell lung carcinomas of the lung (SCLC) proliferate in response to autocrine release of GRP. In gastrointestinal tumors, GRP-receptor activation only modestly increased tumor cell proliferation, but regulated tumor cell appearances or differentiation and, therefore, should be considered to act as a morphogen[1,16].

Based on these findings, it appears important to establish whether or not GRP and GRP-receptor mRNAs

Table 1 Biopsy specimens, sex, age, and β-actin, GRP and GRP-receptor PCR amplicons detected after exposure to X–ray films for one or five days

Experimental number	Tissue origin	Sex	Age	β-actin	GRP	GRPR	
				1 d	1 d	1 d	5 d
1	Esophagus	M	64	+	+	+	+
2	Ventricle	M	64	+	-	+	+
3	Ventricle	M	84	+	+	weak	weak
4	Duodenum	M	75	+	-	-	weak
5	Duodenum	M	63	+	+	-	weak
6	Ileum	F	78	+	+	+	+
7	Caecum	M	72	+	+	+	+
8	Colon ascendens	F	68	+	-	-	weak
9	Colon ascendens	M	83	+	-	-	weak
10	Colon ascendens	F	74	+	+	-	weak
11	Colon ascendens	M	72	+	+	-	weak
12	Colon transversum	M	79	+	+	+	+
13	Colon transversum	F	79	+	+	+	+
14	Colon transversum	F	79	+	+	+	+
15	Colon transversum	F	74	+	+	-	-
16	Colon descendent	M	79	+	+	+	+
17	Colon sigmoideum	M	69	+	+	-	-
18	Colon sigmoideum	M	86	+	+	-	weak
19	Colon sigmoideum	M	48	+	+	+	+
20	Colon sigmoideum	M	83	+	+	+	+
21	Rectum	M	81	+	+	-	weak
22	Rectum	F	54	+	-	-	-
23	Rectum	M	83	+	+	+	+
24	Gallbladder	M	58	+	-	-	-
25	Pancreas	Control		+	+	+	+
26	Stomach	Control		+	+	+	+

are expressed in non-tumor gastrointestinal tissues. In this study, we analyzed GRP and GRP-receptor mRNA expressions in the human esophagus, gastrointestinal tract, pancreas, and gallbladder by means of a reverse-transcription polymerase chain reaction (RT-PCR) technique and Southern blot analysis of the PCR amplicons.

MATERIALS AND METHODS

mRNA isolation and PCR amplification

The collection, origin and status of full thickness biopsies from the human gastrointestinal tract and surrounding tissues from surgically removed biopsies from patients undergoing surgery for gastric diseases has been described elsewhere in detail[19]. In all, 24 biopsy specimens and two control cDNAs (Table 1) were processed and analyzed for the expression of GRP and GRP-receptor mRNA. In subsequent PCR amplification experiments, mRNA and ss-cDNA preparations used were from a previous study and prepared as described recently[19].

β-actin amplification was performed in two rounds of PCR (30 and 25 cycles each time, respectively) with the same primers under cycle conditions as described above. Due to the positioning of the primers, cDNA and genomic DNA will yield β-actin fragments of different sizes (288 bp for cDNA and 400 bp for gDNA PCR amplicons, respectively). This allows monitoring for DNA contamination in ss-cDNA preparations and to assess for the integrity of the ss-cDNA used[19]. Control cDNA derived from pancreas and stomach mRNA was purchased from Clontech (BD-Biosciences, Clontech, Stockholm, Sweden).

PCR- amplification of GRP and GRP-receptor ss-cDNA

PCR was performed using a HotStarTaq Master mix kit (Qiagen, Hilden, Germany) in a final reaction volume of 25 μL. Each reaction contained 2 μL of the cDNA synthesis reaction as template. Quick-clone human pancreas and stomach cDNA (Clontech, BD Biosciences Stockholm, Sweden) were used as positive PCR amplification controls, whereas HotStar PCR amplification mix without ss-cDNA addition was used as a negative control. PCR amplification conditions, annealing temperature and primers used are shown in Table 2 and were taken from the study by Uchida *et al*[20]. For increased sensitivity, nested PCR amplifications were carried out for the detection of GRP and GRP-receptor cDNA. First round PCR amplicons were purified using a GFX PCR and Gel Band DNA Purification Kit (Amersham Biosciences, Uppsala, Sweden) and, subsequently, 1 μL was used in a nested PCR amplification. As positive PCR amplification controls, commercially available human stomach and pancreas cDNAs (Clontech, BD Biosciences Stockholm, Sweden) were included in the study. Negative PCR amplification controls (PCR mix without DNA template addition) were included to monitor possible contaminations.

Southern blot analysis of PCR amplicons

PCR amplicons were electrophoretically separated on a

Table 2 PCR-primers, expected fragment sizes and PCR amplification conditions

Primer	Sequence, 5′ to 3′ orientation	Size in bp	PCR conditions	
			[1]Cycles	Tannealing
hGRP-SE/1	AGTCTCTGCTCTTCCCAGCCTCT			
hGRP-AS/1	GCAGAACTCAGTCTCTTAGGGGT	558	30	55 ℃
hGRP-SE/2	CGTGCTGACCAAGATGTACC			
hGRP-AS/2	TCATTGCTGGTTCAGCTGGG	349	30	62 ℃
hGRPR-SE/1	AGCCCGGCATAGATCTTATCTTC			
hGRPR-AS/1	AGGGGGCAAAATCAAGGGTCAAT	1477	30	55 ℃
hGRPR-SE/2	CTCCCCGTGAACGATGACTGG			
hGRPR-AS/2	ATCTTCATCAGGGCATGGGAG	388	30	62 ℃
β-actin-SE	GCATGGAGTCCTGTCGCATCCACG			
β-actin-AS	CGTCATACTCCTGCTTGCTGATCCA	[2]288/400	30/25	55 ℃

[1]Numbers of cycles in first and second round PCR amplifications; [2]cDNA and gDNA PCR amplicon sizes, respectively.

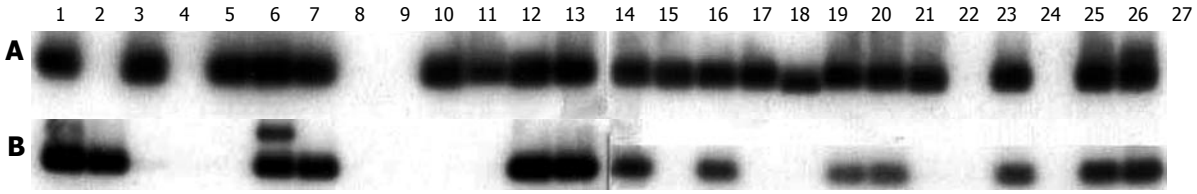

Figure 1 Southern blot hybridization analysis of nested PCR amplicons derived from ss-cDNA 1 to 26 as specified in Table 1. Lane 27 represents a negative PCR control (no ss-cDNA template added to the PCR master mix). Exposure to X-ray films was for 1 d using an intensifier screen at -70 ℃. **A:** hGRP-PCR amplicons; **B:** hGRP receptor PCR amplicons.

15 g/L agarose gel and Southern blot analysis was performed using 10 pmoles of [^{32}P]-5'-end-labelled nested primers hGRP-SE/2 and hGRP-AS/2 or hGRPR-1/SE and hGRPR-2/AS primers as hybridisation probes (Table 2) under the conditions described elsewhere[19].

RESULTS

Integrity of ss-cDNA

All analyzed ss-cDNA yielded fragments of the expected length (288 bp) after two rounds of β-actin PCR amplification and Southern blot analysis, indicating that the ss-cDNA used was essentially free of DNA contamination[19].

Differential tissue expression of GRP and GRP-receptor mRNA

Initially, first round and nested GRP and GRP–receptor PCR amplification conditions were optimized by means of annealing temperature and cycle conditions using two established, premade PCR amplification mixes (puReTaq Ready-To-Go PCR beads, Amersham Biosciences and HotStarTaq Master mix kit, Qiagen). Essentially, the HotStarTaq Master mix kit was used under the conditions described in Table 2.

Southern blot analysis (one-day exposure) of nested GRP-PCR amplicons derived from ss-cDNA revealed PCR bands of the expected size in 18 of 24 (75%) biopsy specimens and in the human stomach and pancreas control cDNA's. It appeared that GRP-PCR amplicons of two distinct sizes were present. These GRP-PCR amplicons were similar in sizes to earlier described GRP-PCR amplicons, derived from alternatively spliced GRP-mRNA[20]. However, no attempts were made to further investigate this point. Furthermore, 11 of 24 (46%) biopsy specimens and the human stomach and pancreas control cDNA yielded GRP-receptor PCR amplicons of the expected size (Figure 1, Table 1). After five-day exposure, an additional 9 of 24 (37%) biopsies yielded weak GRP-receptor PCR amplicons as judged by Southern blot analysis (Table 1), indicating a low level expression of GRP-receptor mRNA. Similarly, two tissues revealed the presence of an extra and larger GRP-receptor PCR amplicon. Its nature has not been further investigated.

No GRP and GRP-receptor PCR amplicons could be detected in the gallbladder tissue, indicating a lack of expression of these mRNAs. However, only one gallbladder biopsy was analyzed and, therefore, the result may not be conclusive since variable GRP and GRP-receptor mRNA expressions were observed in ventricle, duodenum, colon ascendens and rectum biopsies (Figure 1, Table 1). More specifically, GRP mRNA appeared to be expressed in 2 of 4 colon ascendens biopsies. In contrast, a weak band corresponding to GRP-receptor expression was detected in 4 of 4 colon ascendens biopsies after 5-d exposure, indicating a low level of GRP-receptor mRNA expression in these tissues (data not shown). Remarkably, PCR amplicons corresponding to GRP mRNA expression were detected in 4 of 4 transverse colon and 4 of 4 sigmoid colon biopsies (Figure 1). Similarly, GRP-receptor mRNA seemed to be co-expressed in 3 of 4

transverse colon and 3 of 4 sigmoid colon (Table 1).

DISCUSSION

Biopsy specimens were collected from various sites of the human gastrointestinal tract and surrounding tissues. Efforts were made to collect the biopsies from fresh, histologically normal tissues (Table 1). Our results showed that GRP and GRP-receptor mRNAs were widely expressed in the human gastrointestinal tract and surrounding tissues. It is tempting to speculate that the variation in GRP and GRP-receptor mRNA levels observed (Figure 1) may reflect a real-time mRNA expression situation. However, we can not exclude the possibility that artifacts based on sample selection (site of collecting and biopsy sizes) may contribute to the observed mRNA level variations.

Co-expression of GRP and GRP-receptor mRNA in the same tissue as observed could lend support to speculations about the existence of an autocrine and/or paracrine loop. For paracrine signaling, the communicating cells need to be in close proximity in order to establish such loops. Expressions of GRP and its receptor mRNA could be originating from different cell types in opposing parts of the tissue collected. To verify the existence of such an autocrine and/or paracrine loop, cellular co-expression of GRP and GRP-receptor mRNA and its subsequent translation into biologically active proteins must be confirmed. This could be achieved either by *in situ* hybridization or histochemistry techniques, using GRP and GRP-receptor specific hybridization probes or antibodies, respectively.

In lack of the results of such studies, the physiological importance of GRP and GRP-receptor mRNA expression in gastrointestinal tissues can only be speculated upon. However, based on earlier studies, it seems likely that GRP could act on the human colon via receptors on smooth muscle cells and gastric epithelial cells as well as on cells of the ENS[11,12]. GRP has been shown to be the primary transmitter of motor neurones to gastrin cells[2]. Pharmacological doses of GRP showed a concentration-dependent increase in the rhythmic activity of the ileocecum region[21] and evoked contractions of isolated muscle cells from jejunum[7]. Accordingly, inhibition of endogenous GRP delayed gastric emptying and gallbladder contraction[22]. In contrast, small bowel transit was prolonged by the same antagonist[22]. Thus, the effect on the small intestine may be important for mixing movements and not so much for the peristalsis. In our study, the presence of GRP and its receptor throughout the gastrointestinal tract, in addition to earlier studies that showed no expression of the peptides in the epithelial layer except gastric antrum[12] but on colonic smooth muscle cells and ENS[11], raises the hypothesis that the peptide may affect the motility along the entire GI tract. This is further underlined by the effects on smooth muscle cells[7,21]. Disturbed tissue levels of GRP have been described in patients with idiopathic intestinal pseudo-obstruction[23]. The physiology and pathophysiology behind intestinal motility and dysmotility are in many aspects unknown. It is difficult to study the physiological effect of one single peptide alone, as the ENS contains many different peptides with an important balance between them. However, GRP seems to be one of the interesting peptides in the regulation of gastrointestinal motility.

To best of our knowledge, this is the first study that describes the presence of GRP and its receptor in the human pancreas. It is in accordance with the observed effect of GRP on the secretion of pancreatic juice and pancreatic hormones[3-5]. Earlier animal studies have described that GRP is released from vagal, pancreatic nerves after stimulation[4,24]. GRP then acts by binding to a specific member of the 7 transmembrane spanning, G protein-coupled receptor superfamily where activation by GRP-receptors is coupled to phospholipase C and phospholipase D[24,25].

We were not able to detect GRP or GRP-receptor mRNA expression in the gallbladder. This may be explained by the fact that only one patient was examined. The earlier described effect of a GRP antagonist to inhibit gallbladder contraction suggests that GRP receptors are expressed in the gallbladder[22]. However, antagonists may antagonise more than one receptor, and the PCR technique in the present study was specifically examining the GRP-receptor, not similar receptors in the same family.

The effect of GRP in gastrointestinal carcinogenesis is unclear. Most of resected colon cancers aberrantly express GRP receptor mRNA[26], whereas immunohistochemically, less than three-quarters of human tumors express this protein[16]. Furthermore, not all of these receptors are functional when expressed, as only a minor amount of resected human colon cancers have been found to bind (^{125}I-Tyr^{4}) bombesin when studied pharmacologically[27]. The discrepancy between GRP-receptor mRNA and protein expression may be due to the frequency with which the coding sequence for this receptor is mutated[17]. Some authors suggest that GRP acts as a mitogen and increases tumor cell proliferation[18], while others have found that GRP/GRP-receptor co-expression in cancer promotes the development of a well-differentiated phenotype and is therefore more a morphogen than a mitogen[1]. Multiple studies suggest that the presence of these two peptides confers a survival advantage[14,16].

In conclusion, GRP and GRP-receptor mRNA appear to be expressed throughout the human gastrointestinal tract. This provides information for the future mapping of GRP and GRP-receptor expression at the cellular level, and thereby further determination of its physiological importance in normal and tumor cells.

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RAPID COMMUNICATION

Effects of ranitidine for exercise induced gastric mucosal changes and bleeding

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Abstract

AIM: To evaluate the effect of ranitidine on gastric mucosal changes and on GI bleeding in long distance runners.

METHODS: Twenty-four long distance runners (M: 16, F: 8, age: 18.2 ± 1.5 years) participated in this study. A symptom questionnaire, stool hemoccult test, and upper gastrointestinal (GI) endoscopy were performed on the subjects prior to the study. The subjects took oral ranitidine (150 mg, b.i.d.) for two weeks. The upper GI endoscopy and stool Hemoccult tests were repeated after the treatment.

RESULTS: Twenty-two of the 24 runners had at least one upper GI mucosal lesion before the medication. The Endoscopic improvements were seen in eleven of the 14 cases of erosive gastritis and four of the 5 cases of esophagitis. Six subjects were Heme occult positive prior to the study, but only one was positive after the medication.

CONCLUSION: Gastric mucosal lesions and GI bleeding in long distance runners seem to be associated to acid-related factors mediated by the high level of regular running. Ranitidine seems to be and effective prophylaxis to prevent gastric mucosal lesions and GI bleeding.

Key words: Endoscopy; Exercise; Gastrointestinal bleeding; Ranitidine

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INTRODUCTION

Long-distance running has become a popular exercise during the past decade, and the number of people participating in endurance racing has increased. It has been reported that troublesome gastrointestinal (GI) symptoms associated with endurance running events has been occurring more frequently[1,2]. Although the magnitudes of these symptoms are unknown, incidence rate varies from 10 to 81%, depending on factors such as the type, duration, and intensity of the exercise studied[3].

GI bleeding is not an uncommon finding after long distance races. Some of runners[3,4] who experienced macroscopic fecal blood and microscopic bleeding has been found in 8 to 87% of the runners[5,6]. Running-related GI mucosal changes such as gastric ulcer[7], hemorrhagic gastritis[8], erosive gastritis[9], ischemic colitis[10] were observed by endoscopy. These changes may contribute to runner's anemia and, if sustained, may possibly result in an iron deficiency that may potentially affect the recovery and performance of competitive runners. The mechanism by which hemorrhagic gastritis and ischemic colitis develop in runners is unknown, but it is usually attributed, in part, to ischemia. One report showed that running reduced visceral blood flow by up to 80% of pre-exercise level[11]. Another possible mechanism for the hemorrhagic gastritis is acid secretion[12]. Moderate-intensity running is not generally thought to alter gastric acidity[13] whereas heavier exertion paradoxically reduces acid secretion[14]. But some reports have suggested that acid reducing agents, i.e. cimetidine or ranitidine, might be useful in preventing running-associated

gastrointestinal bleeding and symptoms[6,15]. However, there has been only one case study regarding running related GI mucosal changes before and after treatment[15]. We, therefore, performed an observational trial to evaluate the effect of ranitidine on gastric mucosal changes and GI bleeding in long distance runners by endoscopy.

MATERIALS AND METHODS

Study subjects

Twenty-four professional long distance runners (16 male, 8 female, age range: 16-19 years) participated in this study. The Human Investigations Committee of Wonkwang University Hospital approved the protocol and each subject signed an informed consent prior to the study.

The subjects were well trained and had several years of experience in competitive running. They underwent a strict training program of 200 km of running per week and participated in three long-distance running competitions per year. All subjects were instructed to avoid nonsteroidal anti-inflammatory drugs, red meat, alcohol, iron and vitamin supplementation for at least 2 wk prior to the study

Symptom questionnaire

The questionnaire was designed with yes/no questions and a graded value scale (0: none = no symptom, 1: mild = intermittent symptoms but not interfere with the running, 2: moderate = want treatment for symptoms but rarely interfere with the running, 3: severe = want treatment for symptoms and interfere with the running) for the following symptoms: diarrhea, abdominal cramps, epigastric discomfort, epigastric pain, nausea, vomiting, and regurgitation related to running.

GI bleeding test

The presence of GI bleeding was tested before and after the study with hemeoccult test (LA Hemochaser, MIZUHO, Japan) by looking for the presence of agglutination after mixing the feces with a latex reagent within a 3 min.

Study protocols

Gastroscopy was performed on all subjects prior to the study. No pre-medication was used except for lidocaine (2 mL of viscous Xylocaine) as a local anesthetic. Two endoscopists performed the upper GI endoscopy with a flexible gastroscope (Pentax EG-2730, 9.0 mm diameter, Japan). Macroscopic observations of gastric mucosal damage were noted but not graded. *Helicobacter pylori* (*H pylori*) infection was evaluated by a rapid urease test of the one biopsy specimen obtained during endoscopy. The subjects with abnormal endoscopic findings took 150 mg of oral ranitidine (30 min postprandial, b.i.d.). The subjects commenced regular training for 2 wk while taking the medication. A second endoscopy was given to the subjects who had abnormal upper GI mucosal findings before the treatment.

Table 1 Gastrointestinal symptoms in runners (n = 24)

	None	Mild	Moderate	Severe	Percentage of symptomatic subjects (%)
Diarrhea	9	13	1	1	63
Abdominal pain	10	12	2		58
Epigstric discomfort	10	12	2		58
Epigastric pain	12	10	2		50
Nausea	14	8	2		42
Regurgitation	16	8			33
Vomiting	19	4	1		21

RESULTS

Symptom questionnaire

The results of the symptom questionnaire are summarized in Table 1. Only two subjects had no GI symptoms related to running. The most frequently experienced GI symptom was diarrhea (n = 15), followed by abdominal pain (n = 14), epigastric discomfort (n = 14), and epigastric pain (n = 12).

Gastroscopy findings

The results of the endoscopic examination before and after the treatment are listed in Table 2. Twenty-two of the 24 runners had at least one upper GI mucosal lesion, including erosive gastritis (n = 17), esophagitis (n = 8), alkaline reflux gastritis (n=8), and gastric ulcer (n = 1). Of the seventeen subjects with mucosal erosions, four subjects had stigmata of bleeding. Mucosal erosions were localized to the antrum (n = 11), gastric body (n = 8), fundus (n = 6), and gastric angle (n = 2). Sixteen out of 22 runners who had abnormal upper GI mucosal findings consented to have a second endoscopy after medication. Gastroscopic improvements were seen in eleven of the 14 cases of erosive gastritis, four of the 5 cases of esophagitis, but only one of the 5 cases of alkaline reflux gastritis.

GI bleeding

Six of the 24 participants were heme occult positive before the medication. Only one was positive after the medication (Table 2).

Helicobacter pylori status

Nine of the 24 participants were rapid urease test positive (Table 2). But there was no relation between *H pylori* status and endoscopic findings.

DISCUSSION

This study reported a high incidence of running related GI symptoms and the results confirmed the previous studies[2]. Moses reported that symptoms of the lower GI tract were more prevalent than those from the upper GI tract in most endurance events[16] and others found that frequency of GI symptoms were much higher during marathon running and/or triathlons than during other endurance sports,

Table 2 Endoscopic findings and hemoccult test before and after treatment with ranitidine

Subject/Sex	Before treatment (n = 24)			After treatment (n = 16)	
	Endoscopic findings	Hp status	OB	Endoscopic findings	OB
1/M	Alkaline reflux gastritis Erosive gastritis (upper body)	–	+	Procedure refused	–
2/M	Esophagitis, LA class A Alkaline reflux gastritis Erosive gastritis (fundus)	–	–	Erosive gastritis (antrum and body)	–
3/M	Esophagitis LA class A Erosion with blood clot (antrum)	–	+	Normal finding	–
4/M	Esophagitis, LA class A Alkaline reflux gastritis	+	+	Alkaline reflux gastritis	+
5/M	Erosive gastritis (fundus, body, antrum)	+	+	Normal finding	–
6/M	Esophagitis, LA class A Alkaline reflux gastritis	–	–	Procedure refused	
7/M	Erosive gastritis (fundus, body)	+	–	Procedure refused	
8/M	Gastric ulcer, Erosion with blood clot (midbody)	–	+	Normal finding	–
9/M	Erosive gastritis (fundus)	+	–	Normal finding	–
10/M	Erosive gastritis (fundus)	+	–	Erosive gastritis (body)	–
11/M	Esophagitis, LA class A Alkaline reflux gastritis	+	–	Procedure refused	
12/M	Erosive gastritis (body, antrum) Duodenitis	–	–	Procedure refused	
13/M	Normal finding	–	–	Procedure not performed	
14/M	Esophagitis, LA class A	+	–	Procedure refused	
15/M	Erosive gastritis (antrum)	–	–	Normal finding	–
16/M	Erosive gastritis (body, antrum)	–	–	Erosive gastritis (antrum)	–
17/M	Alkaline reflux gastritis, Erosive gastritis with blood clot (angle)	–	–	Alkaline reflux gastritis	–
18/F	Duodenitis	–	–	Normal finding	–
19/F	Esophagitis, LA class A Alkaline reflux gastritis Erosive gastritis (antrum)	–	–	Alkaline reflux gastritis	–
20/F	Erosion with blood clot (midbody)	–	+	Normal finding	–
21/F	Esophagitis, LA class A Erosive gastritis (fundus)	–	–	Esophagitis, LA class A	–
22/F	Alkaline reflux gastritis Erosive gastritis (antrum)	–	+	Alkaline reflux gastritis	–
23/F	Normal finding	+	–	Procedure not performed	
24/F	Erosive gastritis (antrum)	+	–	Normal finding	–

M, male; F, female; Hp, *Helicobacter pylori*; OB, occult blood; +, positive; -, negative; LA, Los Angeles.

i.e. cycling, rowing, and swimming[3,17].

The duration of the exercise can be an important factor. Peters *et al*[18] showed that during a protocol of alternately cycling and running, the number of subjects with GI symptoms increased exponentially with time. They also reported that running induced more GI symptoms when compared to cycling[18]. Another factor thought to influence the exercise induced GI symptoms is the intensity of the exercise. It has been reported that as intensity increases, gastric emptying is delayed[13,19] and splanchnic blood flow decreased[11]. Other factors such as mechanical trauma of the gut[20], intestinal permeability[21], and absorption[22] may also be affected by the intensity of the exercise.

The existence of gastrointestinal bleeding associated with endurance running has previously been demonstrated[2,23]. Sullivan[2] reported bloody stools after endurance events, and another study demonstrated the high frequency of occult GI bleeding, in which 23% of runners converted from blood test negative to blood test positive using the Hemeoccult card[23]. Stewart *et al*[24] reported that 83% of runners had positive stool occult blood after competitive running and the presence of iron-deficiency anemia possibly caused by multiple etiologies including GI bleeding.

While GI blood loss can be clearly attributed to running, its etiology or pathophysiology is not readily apparent. McMahon *et al*[25] suggested an ischemic etiology for the blood loss. They studied 32 runners after a marathon, and showed that six runners, who developed hemoccult-positive stools after a marathon race, were significantly younger and had faster race times when compared to those

with hemoccult-negative stool.

Another possible mechanism for the development of GI bleedings may be mucosal damages caused by gastric acid secretion[12]. Very little data exists regarding acid secretion during exercise and immediately following exercise due to the technical difficulties in such measurements during the exercise. Several reports have suggested that cimetidine or ranitidine might be useful in preventing running associated GI bleeding[6,8,15]. Our study found that six out of 24 participants had positive heme occult positive before medication and each of these 6 subjects had abnormal endoscopic findings before medication. After 2 wk of medication, five out of these 6 subjects became heme occult negative and had improved endoscopic findings (3 normal, 2 partial improvement, 1 dropout). These findings strongly suggest that GI bleeding induced by long distance running is closely related to gastric acid secretion, and reducing acid output with ranitidine seems to be an effective treatment.

With the wide spread popularity of this kind of sport, these complications might be more frequently observed than one would expect, and physicians should be aware of them when evaluating runners with occult GI bleeding with anemia.

The actual site of GI bleeding associated with running is uncertain, but some reports have identified bleeding lesions in the stomach and colon by endoscopy[7-10]. The most frequently reported endoscopic abnormalities in runners are erosive gastritis with bleeding stigmata in the stomach[8,9] and erosion with hemorrhage in the colon[10]. GI mucosal erosions have mainly been found in the corpus region of the stomach[9,15] where stress or shock associated gastritis is normally seen[26]. In contrast, one study has shown abnormal histological features in gastric antral mucosa with decreased mucosal secretion[27]. To our knowledge, only one case report using endoscopy has demonstrated that exercise induced hemorrhagic lesions were improved after taking a therapeutic course of cimetidine[15].

Our study shown that twenty-two of the 24 runners had at least one upper GI mucosal abnormality, of which seventeen subjects had mucosal erosions, and four had stigmata of bleeding. These mucosal erosions were found mainly in the antrum, but also in the gastric body, fundus, and angle. Sixteen of 24 runners had follow-up endoscopy after 2 wk medication of ranitidine. The endoscopic improvements were seen in eleven subjects with erosive gastritis.

Gastroesophageal reflux occurs more frequently with exercise than at rest[28]. Some GI symptoms occurring with exercise can be of esophageal origin, including chest pain and heartburn. These phenomena might be caused by reduced salivary secretion, which results in poor acid clearance[30]. In addition, esophageal ischemia, due to a decrease in visceral blood flow during exercise, may play an important role in development of esophagitis[11]. Esophageal acid exposure may be reduced by a histamine H2-receptor antagonist during running[29]. However, there are no reports describing exercise-induced esophagitis assessed by endoscopy. Long-term implications of gastro-esophageal reflux in the habitual runner or exerciser are also unknown. Our study showed that eight of the 24 runners had esophagitis before treatment and four out of 5 who received a second endoscopy had improvements after medication. This suggests that gastroesophageal reflux associated with running is also closely related to acid secretion.

In conclusion, this is the first study that was evaluated by endoscopy on running associated GI symptoms before and after acid reducing medication. Our results have shown that mucosal abnormalities, such as erosive gastritis and esophagitis in long distance runners are related to gastric acid. Therefore, acid suppressive agent is an effective treatment for running related GI symptoms.

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RAPID COMMUNICATION

Risk factor analysis for metaplastic gastritis in Koreans

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Abstract

AIM: To conduct a retrospective study to determine the risk factors for development of metaplastic gastritis in Korean population.

METHODS: The database of 113449 subjects who underwent a gastroscopy for the purpose of a regular check-up at center for health promotion, Samsung medical center during 5 years was collected and retrospectively analyzed. Among them, 5847 subjects who had endoscopically diagnosed as a metaplastic gastritis or 10076 normal as well as answered to questionnaire were included for present study. The subjects were divided into 2 groups; Group I, normal and Group II, metaplastic gastritis. Age, gender, *Helicobacter pylori* (*H pylori*) seropositivity, body mass index (BMI), family history of cancer, smoking, alcohol consumption, total daily calories, folate and salt intake and dietary habit (out-eating, overeating, irregular eating) were retrieved from questionnaire or electronic medical record and compared between group I and group II.

RESULTS: The prevalence of group II was 11% (13578/113449) increasing its prevalence with age ($P = 0.000$). But, there was no significant association between 2 groups in BMI, family history of cancer, alcohol consumption, total daily calories, folate and salt intake and dietary habit (out-eating, overeating, irregular eating). Old age ($P = 0.000$), male gender ($P = 0.000$), *H pylori* seropositivity ($P = 0.010$) and current smoker ($P = 0.000$) were significantly more common in group II at multiple logistic regression model.

CONCLUSION: Our data suggested that old age, male gender, *H pylori* seropositivity and smoking were risk factors for metaplastic gastritis, precancerous lesion of gastric cancer.

Key words: Intestinal metaplasia; Risk factors

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INTRODUCTION

Gastric cancer was recognized as the first leading cause of cancer death in Korea[1] and attention turned to epidemiologic associations between risk factors and gastric cancer. In the early 1970s, Correa formulated a multi-step model of gastric cancer, which postulated a temporal sequence of pathologic changes that led from chronic gastritis to atrophic gastritis, intestinal metaplasia and dysplasia and the eventual development of gastric cancer[2]. Chronic gastric inflammation seems to be the critical common cause of gastric cancer[3].

The purpose of this paper was to determine the risk factors for development of metaplastic gastritis, precursor of gastric cancer in Korean population

MATERIALS AND METHODS

Data collection

The database of 113449 subjects who underwent a gastroscopy for the purpose of a regular check-up at center for health promotion, Samsung medical center from January 2001 through June 2004 was collected and retrospectively analyzed. Among them, 13578 subjects were endoscopically diagnosed as a metaplastic gastritis and 10521 subjects was endoscopically diagnosed as a normal. But, only 5847 subjects who had endoscopically diagnosed as a metaplastic gastritis were answered to questionnaire whereas 10076 subjects who had endoscopically diagnosed as a normal were answered to questionnaire. Both 5847 metaplastic gastritis and 10076 normal were included for present study. Subjects with peptic ulcer or erosion of stomach were excluded in this study population. The subjects were divided into 2 groups; Group I, normal and Group II, metaplastic gastritis. Demographic data [age, gender, body mass index (BMI), family history of cancer] and life style data [smoking, alcohol consumption, total daily calorie intake, folate intake, salt intake, dietary habit (out-eating, overeating, irregular eating)] were retrieved from question-

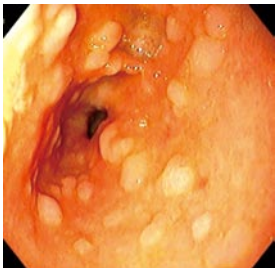

Figure 1 Endoscopic finding showed that atrophic mucosa with surface nodularity was generally diagnosed as a metaplastic gastritis.

naire or electronic medical records and compared between group I and group II. Those who have family history of any cancer were those who had a reply to yes in the question ("Do you have a blood relation who has a experience of diagnosis as cancer by a doctor?"). Data including smoking, alcohol consumption (frequency and amount of alcohol), dietary habit (out-eating, overeating, irregular eating) were collected from self administered questionnaire. Patients were asked whether they were active, past or never smoker. The subjects were divided into two groups (current smoking group and non-smoking group) by current smoking status. Nonsmoking group is composed of past or non-smoking. The subjects were divided into two groups by frequency and amounts of liquors (namely, *so-ju)* consumption in questionnaire. Over or equal 3-4 frequencies a week and $\geqslant$ 80 g once alcohol consumption is defined as the group of heavy alcohol consumption. Below 3-4 frequencies a week or $<$ 80 g once alcohol consumption is classified as the other group. Total amount of calorie, folate intake and fat intake were obtained in diet surveys. *Helicobacter pylori (H pylori)* seropositivity was retrieved from electronic medical record. *H pylori* infection was determined by measuring serum *H pylori* IgG antibodies. Specific anti- *H pylori* antibodies were measured with an enzyme-linked immunosorbent assay (ELISA) kit using an antigen (RADIM SpA, Pomezia, Italy). The sensitivity and specificity of this assay was reported to be 79% and 83%, respectively[4].

Criteria for metaplastic gastritis

Atrophic mucosa with surface nodularity on the endoscopic finding was diagnosed as metaplastic gastritis (Figure 1). 3354 individuals who showed endoscopically metaplastic gastritis had also a presence of metaplasia histologically on the updated Sydney system.

Statistical analysis

Logistic regression analysis was conducted by fixing the each group as a dependent variable and risk factors as independent variables. Continuous variables such as age, BMI, total daily calorie intake, folate intake and salt intake were analyzed by *t*-test. Gender, smoking, alcohol consumption, family history of cancer, dietary habit (out-eating, overeating, irregular eating) and *H pylori* seropositivity were analyzed by χ^2 test. The relative risk to develop metapalstic gastritis was calculated with odds ratio with 95% confidence interval. Risk factors were examined by multiple logistic regression analysis. Statistical significance was assumed at $P<0.05$. Statistical analyses were performed with SAS version 8.1 (SAS Institute Inc, Cary, NC, USA).

Table 1 Comparison of characteristics of normal and metaplastic gastritis (simple logistic regression model)

	Normal (%)	Metaplastic gastritis (%)	*P* value
Age	42.46±10.15	53.20±9.00	0.000
Sex			0.000
Men	3804 (36)	3983 (68)	
Women	6272 (64)	1864 (32)	
BMI[1]	24.52±2.36	23.97±2.86	0.208
Family history of cancer	160 (1)	125 (2)	0.065
Smoking			0.000
None or past	2933(84)	3308 (74)	
Current	550 (16)	1130 (26)	
Alcohol			0.059
Heavy alcoholics[2]	553 (15)	747 (16)	
Out-eating	2477 (23)	1535 (26)	0.110
Over eating	1743 (16)	912 (15)	0.598
Irregular eating	1824 (17)	1152 (19)	0.228
Total calories	2102.94±470.17	2226.46±486.22	0.515
Folate	246.34±91.24	289.51±108.55	0.211
Salts	22.48±7.27	23.02±4.97	0.319
H pylori seropositivity[3]	4165 (39)	3566 (60)	0.007

[1]BMI, body mass index.
[2]Over or equal 3-4 frequencies a week and $\geqslant$ 80 g once alcohol consumption is defined as the group of heavy alcoholics.
[3]*Helicobacter pylori* is abbreviated as *H pylori*.

RESULTS

Characteristics of study subjects (Table 1)

The prevalence of group II was 11% (13 578/113 449) increasing its prevalence with age ($P=0.000$). But, there was no significant association between 2 groups in BMI, alcohol consumption and family history of cancer. Male gender was a risk factor for metaplastic gastritis and current smokers were more likely to have metaplastic gastritis than none or past smokers. Neither dietary composition (folate, salts, calories) nor dietary habits (out-eating, overeating, irregular eating) was associated with metaplastic gastritis. *H pylori* seropositivity was more common in the group II.

Multiple logistic regression analysis (Table 2)

Finally, we conducted stepwise multiple logistic regression analysis in which above significant variables were used as independent variables. Number of subjects entered into the stepwise multiple logistic regression model were 4438 in group II and 3483 in group I. Entered variables were age, gender, BMI, family history of cancer, smoking, alcohol consumption, total daily calorie intake, folate intake, salt intake, dietary habit (out-eating, overeating, irregular eating), and *H pylori* seropositivity.

Old age ($P=0.000$), male gender ($P=0.000$), *H pylori* seropositivity ($P=0.010$) and current smoker ($P=0.000$) were significantly more common in the group II at multiple logistic regression model.

DISCUSSION

Gastric cancer was recognized as the first leading cause of

cancer death in Korea[1] and many epidemiologic studies about risk factors for gastric cancer were reported[5,6]. So far, we had no a large-scale epidemiologic studies about metaplastic gastritis, precursors of gastric cancer in the Korean population.

In the early 1970s, Correa formulated a multi-step model of gastric cancer, which postulated a sequence from chronic atrophic gastritis, intestinal metaplasia, dysplasia and gastric cancer[2]. Chronic gastric inflammation leads to repetitive injury and repair resulting in hyperplasia[3]. Whereas acute injury and inflammation associated with healing are usually self-limited, chronic injury or inflammation leads to a sustained expansion of tissue proliferation[7-10]. Sustained tissue proliferation is generally accepted as a risk factor for cancer[3]. As is well known, metaplastic gastritis is precursors of gastric cancer.

Our understanding of gastritis and cancer underwent a marked shift with rediscovery of *H pylori*[7-13]. *H pylori* is now thought to account for most of gastritis[13] whereas *H pylori* infection is not an only important factor for development for gastric cancer[5]. But, it is not clear whether *H pylori* infection is also important for development for metaplastic gastritis. Our study demonstrated that *H pylori* seropositivity is an independent risk factor for the metaplastic gastritis.

In the intestinal type of gastric cancer, environmental factors other than *H pylori* infection seem to play a part in the carcinogenesis[12]. Environmental factors may facilitate the development of atrophic gastritis and intestinal metaplasia[12,14]. Based on epidemiologic studies of dietary histories, the first step in the Correa pathway was believed to be initiated by a diet rich in salt and nitrates/nitrites as well as deficiencies in fresh fruits and vegetables[5]. Dietary factors and continued effects of chronic inflammation were felt to be responsible for the progression from gastritis to atrophy, metaplasia, dysplasia and carcinoma[12]. Ingestion of sodium chloride is thought to promote gastric carcinogenesis[14]. Exposure to N-nitroso compounds probably facilitates advancement of chronic atrophic gastritis and intestinal metaplasia in adulthood[5]. Our study showed that neither dietary habits, salts nor folate intakes is a risk factor for the metaplastic gastritis. The major limitation of our study is that diet survey used in our study is carried out by not-validated questionnaire.

Both superficial and chronic atrophic gastritis are common in alcoholics[6]. Alcohol consumption can also cause acute gastritis[15]. Our study demonstrated that alcohol is not a risk factor for the metaplastic gastritis. Male dominance of the metaplastic gastritis can be explained, in which male gender tends to have more dangerous environmental factors such as smoking. But, our study demonstrated that male gender and smoking are independent risk factors for the metaplastic gastritis, respectively.

First, the limitation of present study is selection-bias. The substantial numbers of subjects did not have information about all items of the questionnaire. Numbers of subjects entered into the multiple logistic regression model were 4438 (4438/13578, 32%) in group II and 3483 (3483/10521, 33%) in group I. The other limitation of our study is inter-examiner or intra-examiner bias of endoscopic diagnosis about metaplastic gastritis and normal. To ascertain the precision in endoscopic diagnosis of gastritis, we undertook a pilot study on 10 individuals. Endoscopic finding was obtained 2 times on 10 individuals by 2 examiners. We examined the inter-examiner bias between 2 examiners and intra-examiner bias between 2 examinations by same examiner. Kappa value of intra-examiner was 1.0 ($P=0.002$), 0.737 ($P=0.016$), respectively. Kappa value of inter-examiner was 0.875 ($P<0.001$).

Our data suggested that old age, male gender, *H pylori* seropositivity and smoking were risk factors for metaplastic gastritis, precancerous lesion of gastric cancer.

Table 2 Multiple logistic regression analysis about risk factors for development of metaplastic gastritis

Variable[1]	Odds ratio (95% CI[2])	*P* value
Age	8.945 (7.204-11.105)	0.000
Male gender	1.144 (1.133-1.155)	0.000
Current smoking	2.137 (1.693-2.697)	0.000
H pylori[3] seropositivity	1.223 (1.049-1.426)	0.010

[1]All variables in the model are as follows: age, sex, body mass index, family history of cancer, smoking, alcohol consumption, total daily calorie intake, folate intake, salt intake, dietary habit (out-eating, overeating, irregular eating), and *H pylori* seropositivity.
[2]CI, confidence interval.
[3]*Helicobacter pylori* is abbreviated as *H pylori*.

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RAPID COMMUNICATION

Gastric cancer patients at high-risk of having synchronous cancer

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Abstract

AIM: To identify patients with a high-risk of having a synchronous cancer among gastric cancer patients.

METHODS: We retrospectively analyzed the prospective gastric cancer database at the National Cancer Center, Korea from December 2000 to December 2004. The clinicopathological characteristics of patients with synchronous cancers and those of patients without synchronous cancers were compared. Multivariate analysis was performed to identify the risk factors for the presence of a synchronous cancer in gastric cancer patients.

RESULTS: 111 of 3291 gastric cancer patients (3.4%) registered in the database had a synchronous cancer. Among these 111 patients, 109 had a single synchronous cancer and 2 patients had two synchronous cancers. The most common form of synchronous cancer was colorectal cancer (42 patients, 37.2%) followed by lung cancer (21 patients, 18.6%). Multivariate analyses revealed that elderly patients with differentiated early gastric cancer have a higher probability of a synchronous cancer.

CONCLUSION: Synchronous cancers in gastric cancer patients are not infrequent. The physicians should try to find synchronous cancers in gastric cancer patients, especially in the elderly with a differentiated early gastric cancer.

Key words: Synchronous cancer; Gastric cancer; Colorectal cancer

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INTRODUCTION

Gastric cancer is the most common form of cancer in Korea[1]. The overall age-standardized incidence rates of gastric cancer in 2002 were 69.6 per 100 000 among males and 26.8 per 100 000 among females. Despite the improved prognosis of gastric cancer resulting from early diagnosis, radical operations, and the development of adjuvant therapy, gastric cancer remains the second most common cause of cancer death worldwide[2,3]. In 1995, early gastric cancer (EGC) accounted over 30% of patients who underwent gastric cancer surgery in Korea and this percentage continues to increase[4]. As the prognosis of patients with EGC is excellent and age at diagnosis for gastric cancer is increasing, there is a greater risk that patients will also have a second primary cancer[5,6].

Second primary cancer influences the prognosis of gastric cancer patients, and because primary or secondary prevention is the best way to cure cancer, some investigators have focused on the characteristics of second primary cancers in gastric cancer patients[7-10]. However, few studies have been performed in this regard, and most of these studies are limited to metachronous cancers or the treatment-related second primary malignancies of gastric cancer patients[11,12]. The detection of synchronous cancers gives us the opportunity to treat both cancers simultaneously using less invasive techniques and thus to beneficially influence the prognosis and quality of life of these patients.

The aim of this study was to find a means of identifying gastric cancer patients at risk of having a synchronous cancer.

MATERIALS AND METHODS

Subjects

We retrospectively analyzed the prospective gastric cancer database at the National Cancer Center (NCC), Korea from December 2000 to December 2004. A total of 3291 gastric

Table 1 Clinicopathological features of patients with or without synchronous cancer

Characteristics	Synchronous cancer With *n* (%)	Synchronous cancer Without *n* (%)	*P* value
Age (yr, mean±SD)	64.6±9.5	59.4±12.5	<0.001
Sex			0.002
Male	90 (81.1)	2144 (67.4)	
Female	21 (18.9)	1036 (32.6)	
Multiplicity			0.009
Single	106 (95.5)	3146 (98.9)	
Multiple	5 (4.5)	34 (1.1)	
Preoperative Stage			<0.001
Early gastric cancer	62 (55.4)	1135 (35.7)	
Advanced gastric cancer	50 (44.6)	2045 (64.3)	
Differentiation			<0.001
Differentiated	94 (84.7)	1383 (43.4)	
Undifferentiated	17 (15.3)	1797 (56.6)	

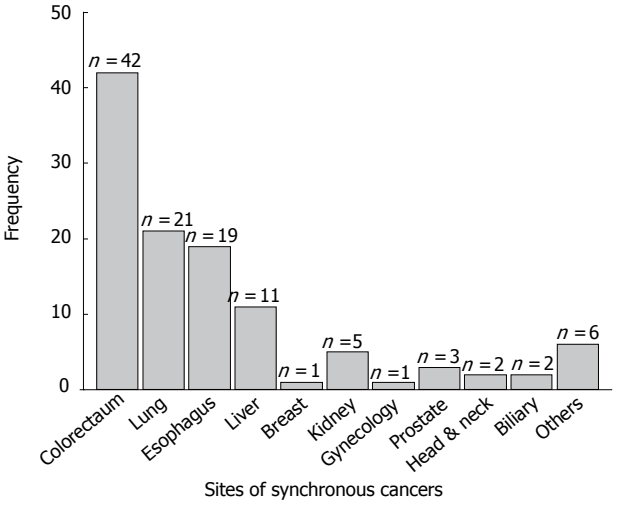

Figure 1 Site distribution of synchronous cancers.

cancer patients were registered (the registered patients were consecutive patients who had ever visited out patient clinic in the Center for Gastric Cancer with a diagnosis of gastric cancer or who were diagnosed as having gastric cancer at our center) during the study period. Gastric and synchronous cancers were all pathologically confirmed.

Methods

Synchronous cancers were defined as cancers detected in organs other than the stomach, which were diagnosed at the time of or within 6 mo of the first cancer diagnosis[13]. For each synchronous cancer we ruled out of the possibility that synchronous cancer represented metastasis of gastric cancer by histologic examination. Clinicopathological characteristics including age, sex, histological classification, a preoperative diagnosis of early or advanced gastric cancer, and multiplicity of gastric cancer were compared for patients with and without synchronous cancers. As for histological classifications, tubular carcinoma and papillary adenocarcinoma were classified as differentiated types, whereas poorly differentiated adenocarcinoma, mucinous adenocarcinoma, and signet ring cell carcinoma were classified as undifferentiated types[14]. The characteristics of synchronous cancers including operability and when they were diagnosed were separately analyzed.

Statistical analysis

All statistical analyses were performed using the statistical software 'Statistical Package for Social Sciences' (SPSS) version 10.0 for Windows (SPSS, Inc, Chicago, IL). Inter-group (for patients with and without synchronous cancer) comparisons of clinicopathological variables were made using the Student t-test for continuous variables and using the two-tailed Chi-square test for discrete variables.

Risk factors influencing synchronous cancers were determined by logistic regression analysis. Hazard ratio as determined by multivariate analysis, was defined as the ratio of the probability that an event (with synchronous cancer or not) would occur to the probability that it would not occur. The predicting powers of covariates were expressed by calculating hazard ratios with a 95% confidence interval. The accepted level of significance was $P < 0.05$.

RESULTS

Clinicopathological characteristics of the patients

Among the 3291 gastric cancer patients, 111 patients (3.4 %) had a synchronous cancer other than in the stomach; 2 patients (2/111 patients, 1.7%) had three cancers and the others had a single synchronous cancer. The mean age of patients with a synchronous cancer was higher than that of patients without (64.6 ± 9.5 *vs* 59.4 ± 12.5, t = -0.453, $P < 0.001$) and male patients were more common among those with synchronous cancers (χ^2=9.81, P=0.002). Differentiated type and early gastric cancer were more common among patients with synchronous cancers (χ^2=73.97, $P < 0.001$, χ^2=19.65, $P < 0.001$ respectively, Table 1).

Characteristics of synchronous cancers

The most common synchronous cancer was colorectal cancer (42 cases, 37.2%), followed by the lung (21 cases, 18.6%), esophagus (19 cases, 16.8%), and liver cancer (11cases, 9.7%) (Figure 1, Table 2). Gastric cancer was the most common form of cancer among colorectal cancer patients with a synchronous cancer. Of the 2 triple cancer patients, one patient had esophageal cancer and prostate cancer and the other patient had lung cancer and colorectal cancer. Forty-three patients (38.1%) were diagnosed with gastric cancer and synchronous cancer simultaneously and 45 patients (39.8%) were diagnosed with synchronous cancers before gastric cancer, and 25 cases (21.1%) were diagnosed after receiving a diagnosis of gastric cancer.

Operation of gastric cancer

Of the 111 patients with synchronous gastric cancers, 73 (64.6%) underwent gastric cancer surgery. Despite having a potentially resectable gastric cancer, 38 patients (34.2%) were treated non-surgically because of advanced

Table 2 Distribution of synchronous cancer according to gastric cancer location and diagnosis

Location	Time interval between the diagnoses of synchronous and gastric cancers			
	6 mo ~ (n=45)	Simultaneous (n=43)	~ 6 mo (n=25)	Total (n=113[1], %)
Colorectal	28	6	8	42 (37.2)
Lung	3	11	7	21 (18.6)
Esophagus	8	11	0	19 (16.8)
Liver	2	8	1	11 (9.7)
Breast	0	0	1	1 (0.9)
Kidney	0	2	3	5 (4.4)
Gynecologic	0	1	0	1 (0.9)
Prostate	1	0	2	3 (2.7)
Head and neck	1	1	0	2 (1.8)
GB, bile duct	0	2	0	2 (1.8)
Others	2	1	3	6 (5.3)

[1]Two patients have double synchronous cancers.

synchronous cancer (19 patients, 50.0%), an advanced gastric cancer (6 patients, 15.8%), or patient refusal (11 patients, 28.9%), and other causes (2 patients).

Logistic regression analysis of risk factors

Logistic regression analysis identified age at gastric cancer diagnosis, differentiation, and early or advanced gastric cancer as independent risk factors of synchronous gastric cancer. The incidence of synchronous cancer in elderly patients (≥60 years) with differentiated early gastric cancer was 9.3% (43/418 patients), while that in other patients was 2.4% (68/2 830 patients). Moreover, the incidence of colon cancer in elderly patients (≥60 years) with differentiated early gastric cancer was 3.5% (15/418 patients, Table 3).

Table 3 Logistic regression analyses of risk factors

Covariate	β	SE	RR (95% CI)	*P* value
Age, yr (< 62 *vs* ≥ 62)	0.471	0.223	1.601 (1.035-2.477)	0.035
Sex (Male *vs* Female)	-0.430	0.250	0.650 (0.398-1.061)	0.085
Histology (Diff. *vs* Undiff.)	- 1.691	0.276	0.184 (0.107-0.316)	< 0.001
Multiplicity (Single *vs* Multiple)	0.288	0.412	1.334 (0.595-2.994)	0.484
Stage (EGC *vs* AGC)	-0.431	0.202	0.650 (0.437-0.966)	0.033

SE: Standard error; RR: Relative risk; CI: Confidence interval.

DISCUSSION

The main findings of this study are; 1) That synchronous cancer has an incidence of 3.4% (111/3 291 patients) in gastric cancer patients; 2) That the most common type of synchronous cancer in gastric cancer patients is colorectal cancer (42 patients, 37.2%), followed by lung cancer (21 patients, 18.6%); and 3) That age at diagnosis, a differentiated gastric cancer, and early gastric cancer are risk factors of synchronous cancer in gastric cancer patients. The incidence of synchronous cancer has been reported to vary from 0.7% to 3.5%[7-12]. Reasons for these wide ranges of incidence are attributed to different study populations and methods. The lowest incidence of synchronous cancers in gastric cancer patients reported was 0.7%, and this study was a population-based study, whereas, the other studies were institution-based, like the present study. The relatively high incidence of synchronous cancer in our study (3.4%), despite the inclusion of patients with EGC and AGC, may be due to time. Most studies were performed before 2000, whereas the patients included in this study were diagnosed as having gastric and synchronous cancer from 2001. Thus, radiologic diagnostic tools, such as, computed tomography (CT) and positron emission tomography (PET) were considerably developed and diagnostic accuracy improved over the intervening period[15,16]. Another possible explanation might be that this study was conducted as a retrospective analysis of a prospective database, and that our institution is a cancer center and as such diagnostic efforts are more focused on the detection of cancers.

In the present study, colorectal cancer was the most common cancer among gastric cancer patients with a synchronous cancer. On the contrary, gastric cancer was the most common form of cancer among colorectal cancer patients with a synchronous cancer. This association between colorectal cancer and gastric cancer may be incidental; however, there is some basis to support the existence of such a relation. It is well known that gastric cancer is the second most common extra-colonic malignancy associated with hereditary non-polyposis colorectal cancer (HNPCC) syndrome[17]. Moreover, a defect in the mismatch repair system has been suggested to play a role in the development of multiple cancers, but mechanistic basis for the development of synchronous cancers is unclear[18]. Preoperative endoscopy is a routine procedure in the Center for Colorectal Cancer in NCC for the patients with a colorectal cancer.

Most patients with synchronous colorectal cancers in this study were diagnosed within one month prior to receiving a diagnosis of gastric cancer, or were diagnosed while gastric cancer diagnosis. Patients with colorectal cancer frequently have symptoms of obstruction or bleeding, whereas the symptoms of gastric cancer patients are rare and vague[19,20]. In addition, esophagogastrodu odenoscopy (EGD) in colorectal cancer patients is one of our institutional policies. Thus, it is frequently the cases that the stages of gastric cancer and associated colorectal cancer are early and advanced, respectively. The incidences of most cancers are higher in men than in women and tend to increase with age, and patients with synchronous cancer in this study were more commonly elderly males. These findings are consistent with those of previous studies and might be associated with recent trends in gastric cancer epidemiology in Korea and characteristics of gastric cancer[1,2,4]. The high incidence of synchronous cancers found among early gastric cancer patients in the present study might be associated with the increasing incidence of early gastric cancer in Korea, and the fact that patient diagnosed as having colorectal cancer undergo routine gastroscopy. However, the reason for the higher incidence of differentiated

type in gastric cancer patients with synchronous cancer is unclear, and requires further investigation.

The incidence of synchronous cancer in those with multiple gastric cancers tended to be higher than for those with a single gastric cancer. Thus, genetic instability such as microsatellite instability might be involved in the development of synchronous cancer and multiple gastric cancers. However, the multiplicity of gastric cancer proved not to be a risk factor of synchronous cancer by multivariate analysis.

Regardless of the fact that they had potentially curable gastric cancer, 38 (35.4%) patients did not undergo an operation, and the presence of an advanced synchronous cancer was a principal reason. The major factor influencing treatment plans was cancer stage. Therefore, urgent efforts should be made to identify those at high-risk of having a synchronous cancer.

Multivariate analysis showed that age at gastric cancer diagnosis of, tumor differentiation, and preoperative stage are risk factors for the presence of synchronous cancer in gastric cancer patients. Elderly patients ($\geqslant$ 60 years) with differentiated type of early gastric cancer had a synchronous cancer incidence of 9.3% and a synchronous colon cancer incidence of 3.5%. This result suggests that elderly patients with differentiated type of early gastric cancer should be assessed for the presence of a synchronous cancer. Most synchronous cancers, such as, colorectal, lung, esophageal, and liver cancers can be discovered during routine preoperative staging work up using EGD and radiologic examinations, such as, chest X-ray or abdomino-pelvic CT. However, the early detection of colorectal cancer, which proved to be the most common synchronous cancer type, requires colonoscopy.

Despite the fact that this study was conducted based on an analysis of a prospective database, not all patient information was recorded, because almost one third of the patients were not treated at our institution. Therefore, the preoperative stage was not always the same as the pathological stage. However, considering that the accuracy of preoperative staging for EGC or AGC is $>80\%$, we believe that our results would have been comparatively unchanged had we adopted pathological stage[21].

Colonoscopy is not routinely performed at our center in gastric cancer patients scheduled to undergo an operation or endoscopic mucosal resection. Because the indications for endoscopic mucosal resection are a differentiated cancer of <2cm in diameter, those patients that undergo EMR present a group at high-risk of having a synchronous cancer[22]. Moreover, it is likely that the incidence of synchronous colon cancer may have been higher if we had performed routine colonoscopy in these patients. We now plan to perform a routine colonoscopic examination on those determined by this study to be at high-risk.

In conclusion, synchronous cancers in gastric cancer patients are not infrequent. Considering the trend that the peak age of gastric cancer patients and the incidence of early gastric cancer are increasing, the present study cautions that physicians should try to find synchronous cancers in gastric cancer patients at risk, such as, in elderly patients with differentiated early gastric cancer.

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RAPID COMMUNICATION

An inherent acceleratory effect of insulin on small intestinal transit and its pharmacological characterization in normal mice

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Abstract

AIM: To study an inherent effect of insulin on small intestinal transit and to explore involvement of various systems/mechanisms in normal mice.

METHODS: Insulin at the doses of 2 μU/kg, 2 mU/kg, 2 U/kg or vehicle was subcutaneously administered to four groups of overnight fasted normal male mice. Blood glucose (BG) levels were measured 2 min before insulin administration and 2 min before sacrificing the animals for the measurement of small intestinal transit (SIT). Charcoal meal was administered (0.3 mL) intragastrically 20 min after insulin administration and animals were sacrificed after 20 min and SIT was determined. For exploration of the various mechanisms involved in insulin-induced effect on SIT, the dose of insulin which can produce a significant acceleration of SIT without altering BG levels was determined. The following drugs, atropine (1 mg/kg), clonidine (0.1 mg/kg), ondansetron (1 mg/kg), naloxone (5 mg/kg), verapamil (8 mg/kg) and glibenclamide (10 mg/kg), were administered intravenously 10 min prior to the administration of insulin (2 μU/kg).

RESULTS: The lower doses of insulin (2 μU/kg and 2 mU/kg) produced a significant acceleration of SIT from 52.0% to 70.7% and 73.5% without lowering blood glucose levels ($P < 0.01$), while the highest dose of insulin (2 U/kg) produced a fall in blood glucose levels which was also associated with significant acceleration of SIT ($P < 0.01$). After pretreatment of insulin (2 μU/kg) group with atropine, insulin could reverse 50% of the inhibition produced by atropine. In clonidine-pretreated group, insulin administration could reverse only 37% of the inhibition produced by clonidine and inhibition of SIT was significant compared with vehicle + insulin-treated group, i.e. from 74.7% to 27.7% ($P < 0.01$). In ondansetron-pretreated group, insulin administration could produce only mild acceleration of SIT (23.5%). In naloxone-pretreated group, insulin administration could significantly reverse the inhibition of SIT produced by naloxone when compared with naloxone *per se* group, i.e. from 32.3% to 53.9% ($P < 0.01$). In verapamil-pretreated group, insulin administration could only partially reverse the inhibition (65%). In glibenclamide-pretreated group, insulin administration produced further acceleration of SIT (12.2%).

CONCLUSION: Insulin inherently possesses an acceleratory effect on SIT in normal mice. Adrenergic and cholinergic systems can play a significant role. Calcium channels and opioidergic system can play a supportive role; in addition, enhancement of endogenous insulin release can augment the effect of exogenously administered insulin on SIT.

Key words: Adrenergic system; Blood glucose levels; Ca^{2+} channels; Cholinergic system; Insulin; Intestinal transit; Opioid system; Serotonergic system

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INTRODUCTION

Insulin is the drug of choice in the management of elevated blood glucose level in type 1 and sometimes in type 2 diabetes mellitus[1]. In addition to its effect on glucose metabolism, insulin is reported to act as a neuromodulator in the central nervous system[2] and as a

mild analgesic[3]. Takeshita and Yamaguchi[4] characterized an inherent antinociceptive response for insulin when administered subcutaneously in mice. Their study has also shown that the antinociceptive response is independent of hypoglycemic action of insulin.

Gastrointestinal (GI) disorders are common in diabetic patients[5]. About 75% of these patients suffer from GI disorders due to GI neuropathy leading to considerable morbidity[6,7]. These GI disorders include nausea, gastric stasis, constipation, fecal incontinence and diarrhea[8,9]. Clinical studies are available that report insulin therapy increases the gastric emptying through hypoglycemic effect[10,11], but no detailed report is available in normal experimental animals on its effect on small intestinal transit and mechanism involved. In this study, we, therefore, investigated an inherent effect of insulin on small intestinal transit in normal mice and explored the involvement of possible mechanisms. The outcome of this experiment may provide valuable insights into the effect of insulin on normal intestinal transit.

MATERIALS AND METHODS

Animals

Adequate number of randomly bred normal/healthy adult Swiss albino male mice, weighing between 20-25 g, were obtained from Central Animal House, JIPMER, Pondicherry. One week before the study, the animals were housed at Departmental Animal House in polypropylene cages under standard laboratory conditions. Animals were fasted overnight prior to the experiment in cages with mesh bottom and had free access to water. Experiments were performed during the day time (09:00 to 18:00). The experimental protocol was approved by JIPMER Institutional Animal Ethics committee.

Drugs and chemicals

Atropine injection IP (S K Parenterals Pvt. Ltd., Tetali), clonidine HCl (C.H. Boehringer Sohn Ingelhein, Germany), glibenclamide (Hoechst India Ltd., Bombay), gum acacia IP (Hikasu Chemicals, Mumbai), insulin injection IP (purified bovine insulin, 40 U per milliliter; Knoll Pharmaceuticals Ltd., Aslai, India), naloxone HCl (Endo Labs, USA), ondansetron (Cipla Ltd., Mumbai), verapamil (Torrent Laboratories Pvt. Ltd., Ahmedabad), wood charcoal (SD Fine Chemicals, Boisar) were used in this study. All the drugs were dissolved in sodium chloride injection IP except glibenclamide which was dispersed in 50 g/L Tween 80 in water for injection before administration.

Administration of insulin

Mice were randomly divided into four groups, each group consisted of 6 mice. Each group was subcutaneously (sc) administered insulin 2 μU/kg, 2 mU/kg, 2 U/kg or vehicle. Blood glucose level was recorded before insulin administration. Charcoal meal was administered 20 min after insulin administration and SIT was determined after 40 min.

Measurement of small intestinal transit

The small intestinal transit (SIT) was determined by identifying leading front of intragastrically (ig) administered marker in small intestine of an animal[12,13]. Charcoal meal marker was freshly prepared by dispersing 10 g of wood charcoal in 50 g/L gum Acacia mucilage. After 20 min of insulin administration, each mouse received 0.3 mL of this suspension intragastrically using metallic oral cannula. After 20 min, animals were sacrificed by intravenous administration of sodium pentobarbital (100 mg/kg), abdomen opened, the leading front of marker was identified in the small intestine and tied immediately to avoid movement of marker. The entire length of small intestine was isolated by cutting at pyloric and ileocaecal ends. The distance travelled by the charcoal meal and the total length of the intestine were measured in cm. The SIT was expressed as percentage (%) of the distance travelled by the charcoal meal to length of the intestine. This was carried out in the animals 40 min after insulin administration.

Measurement of blood glucose

Blood glucose (BG)[14] was measured by placing a drop of blood obtained by tail venipuncture, over an appropriate glucostix, read by Advantage Glucometer (Boerhinger Mannheim Corporation, Indianapolis, USA) and expressed as %, change in the glucose level considering the initial value of that animal as 100. This estimation was done 2 min before insulin/vehicle administration and 2 min before sacrificing the animal for measuring small intestinal transit.

Mechanisms of insulin-induced acceleration of small intestinal transit

Insulin at 2 μU/kg dose significantly accelerated (35.7%) small intestinal transit without affecting the blood glucose level; hence this dose was selected to evaluate the mechanism of insulin-induced intestinal hypermotility (Table 1). For exploring the various systems/mechanisms involved in insulin-induced effect on SIT, the antagonists or agonists (agents) of the following systems were attempted (Figure 1).

Cholinergic system

Involvement of cholinergic system was evaluated by using a well studied non-specific cholinergic antagonist atropine. The dose selection was based on a study reported by Chaudhuri *et al*[15]. Atropine (1 mg/kg) was injected intravenously (iv) 10 min before insulin administration (2 μU/kg sc) to one group of animals. After 40 min of insulin administration, the animals were sacrificed to measure SIT. Another group was treated similarly but with vehicle + insulin (Figure 1).

Adrenergic system

Involvement of adrenergic system was evaluated by using clonidine (0.1 mg/kg iv). The dose selection was based on studies reported by Donoso *et al*[16] and DiTullio

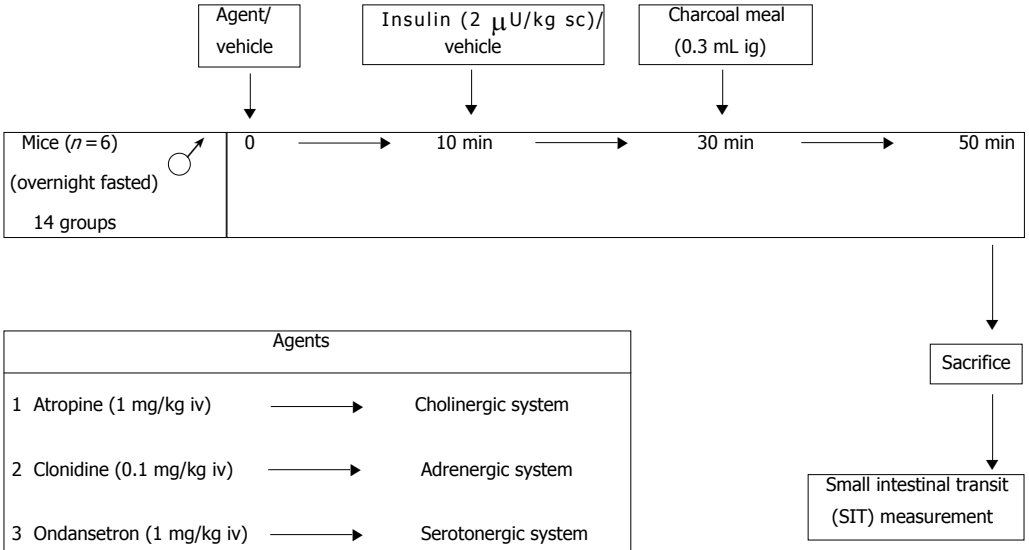

Figure 1 Experimental design carried out to explore various systems/mechanisms involved in insulin-induced acceleration of small intestinal transit in normal mice.

Table 1 Effect of insulin administration on small intestinal transit and blood glucose levels in normal mice

Treatment Insulin (U/kg sc)	% SIT[1]	Blood glucose (%)[2]
Vehicle	52.06 ± 1.48	103.73 ± 4.19
2 μ	70.77 ± 0.62[a]	99.47 ± 1.59
2 m	73.53 ± 0.63[a]	94.54 ± 4.62
2	80.10 ± 1.93[a]	42.09 ± 1.78[a]

Each value represents the mean ± SE (n = 6) sc: subcutaneous; SIT: small intestinal transit; [a]P < 0.01 *vs* vehicle treated group; [1]Values are % SIT considering the total length of the intestine as 100, SIT was determined 40 min after insulin administration; [2]Blood glucose was estimated 2 min before insulin/vehicle administration and 2 min before sacrificing the animals for the measurement of small intestinal transit (SIT), final blood glucose expressed as % considering the initial blood glucose of each animal as 100.

et al[17]. Clonidine (0.1 mg/kg iv) was injected 10 min before insulin administration (2 μU/kg sc) to one group of animals. After 40 min of insulin administration, the animals were sacrificed to measure SIT (Figure 1).

Serotonergic system

Involvement of serotonergic system was evaluated by using ondansetron (1 mg/kg iv). The dose selection was based on a study reported by Nagakura *et al*[18]. Ondansetron (1 mg/kg iv) was injected 10 min before insulin administration (2 μU/kg sc) to one group of animals. After 40 min of insulin administration, the animals were sacrificed to measure SIT (Figure 1).

Opioidergic system

Involvement of opioidergic system was evaluated by using naloxone (5 mg/kg iv). The dose selection was based on studies reported by Stein *et al*[19] and Peana *et al*[20]. Naloxone (5 mg/kg iv) was injected 10 min before insulin administration (2 μU/kg sc). After 40 min of insulin injection, the animals were sacrificed to measure SIT (Figure 1).

Calcium channels

Involvement of calcium channels was evaluated by using verapamil (8 mg/kg iv). The dose selection was based on studies reported by Ramaswamy *et al*[21] and Amos *et al*[22]. Verapamil (8 mg/kg iv) was injected 10 min before insulin administration (2 μU/kg sc). After 40 min of insulin administration, the animals were sacrificed to measure SIT (Figure 1).

Insulin secretogogue

Influence of elevated endogenous insulin levels on SIT was evaluated by using glibenclamide (10 mg/kg iv). The dose selection was based on studies reported by Ramaswamy *et al*[23] and Ojewole *et al*[24]. Glibenclamide (10 mg/kg iv) was injected 10 min before insulin administration (2 μU/kg sc) to one group of animals. After 40 min of insulin administration, the animals were sacrificed to measure SIT (Figure 1).

Statistical analysis

Results are expressed as mean ± SE and analyzed statistically using ANOVA, followed by Dunnett's multiple comparisons test. A P < 0.05 was considered statistically significant.

RESULTS

Effect of insulin on small intestinal transit

Insulin administration at lower doses (2 μU/kg and 2 mU/kg) produced a significant acceleration of SIT without altering the blood glucose levels (P < 0.01 Table 1), while the higher dose of insulin (2 U/kg) produced a profound fall in blood glucose levels (P < 0.01) which was associated with an acceleration of SIT.

Cholinergic system

Atropine (1 mg/kg) *per se* produced an attenuation of SIT by 47.0% when compared with vehicle treated group. In

atropine-pretreated group, insulin administration (2 μU/kg) could not completely reverse the inhibition produced by atropine. Insulin could overcome only 50% of the inhibition produced by atropine (Table 2 and Figure 2).

Adrenergic system

Clonidine (0.1 mg/kg) *per se* produced an inhibition of SIT by 72.0%. Conversely, in clonidine-pretreated group, insulin administration (2 μU /kg) could partially reverse (37%) the inhibition produced by clonidine but the inhibition of SIT was still significant (P <0.01) when compared with vehicle + insulin-pretreated group (Table 2 and Figure 2).

Serotonergic system

Ondansetron (1 mg/kg) *per se* could not alter the SIT when compared with vehicle-treated group. Conversely, in ondansetron-pretreated group, insulin administration (2 μU/kg) could produce only mild acceleration of SIT (23.5%) (Table 2 and Figure 2).

Opioidergic system

Naloxone (5 mg/kg) *per se* produced a significant inhibition of SIT by 41.2% when compared with vehicle-treated group. In naloxone-pretreated group, insulin administration (2 μU/kg) could significantly reverse the inhibition produced by naloxone (P < 0.01) when compared with naloxone *per se* group (66.4%). However, the reversal of inhibition was still significantly lower than insulin *per se* group (P <0.01) (Table 2 and Figure 2).

Calcium channels

Verapamil (8 mg/kg) *per se* produced a significant deceleration of SIT by 26.0% when compared with vehicle-treated group. In verapamil-pretreated group, insulin administration (2 μU/kg) could only partially reverse the deceleration produced by verapamil (65%) (Table 2 and Figure 2).

Insulin secretogogue

Glibenclamide (10 mg/kg) *per se* produced a significant acceleration of SIT by 43.8% when compared with vehicle-treated group. In glibenclamide-pretreated group, insulin administration (2 μU/kg) produced a further acceleration of SIT by 12.2% (Table 2 and Figure 2).

DISCUSSION

Recent experiments in streptozotocin (STZ)-induced diabetes in rats have demonstrated deleterious effects on the neuromuscular junction as well as on muscle itself. Actions at both sites may contribute to neuropathy and functional alterations in muscle contractile properties[25,26]. Abnormalities in gastric emptying and small intestinal motor function were also reported in STZ-treated rats[27,28]. This may be ascribed to the ability of STZ, which not only destroys β-cells of pancreas leading to diabetic state, but also affect the nervous system function which maintains the tone and motility of GI smooth muscles. Hence, STZ-induced experimental diabetic model became untenable to explore the effect of any agent on SIT, instead normal or healthy animals are appropriate for exploring inherent effect of any substance on SIT. Therefore, the data obtained can reflect the true changes and are devoid of the influence of degenerative changes induced by STZ in laboratory animals.

Our study with insulin administration demonstrated an interesting finding that after 40 min of its administration in normal mice, lower doses of insulin (2 μU/kg and 2 mU/kg) significantly accelerated the small intestinal transit (P< 0.01) without lowering blood glucose levels. On the other hand, the highest dose of insulin (2 U/kg) produced a significant fall in blood glucose levels which was also associated with an acceleration of SIT (P<0.01). The available literature indicated that insulin-induced hypoglycemia accelerates gastric emptying of solids and liquids in long-standing type 1 diabetes[29] or stimulates gastric vagal activity causing an increase in gastric emptying in healthy volunteers[30]. These effects on gastric emptying were dependent on blood glucose levels[29,30]. Moreover, these findings were observed with normal or higher doses of insulin. In our experiment, the blood glucose levels were not affected by the lower doses of insulin (2 μU/kg or 2 mU/kg) but produced a significant acceleration of SIT. The sub-hypoglycemic doses of insulin were used to avoid the hypoglycemic effect on intestinal motility. We suggest that the blood glucose levels may not play a significant role in accelerating SIT at least in the lower doses of insulin, indicating an inherent acceleratory effect of insulin on SIT. Our findings are partly in agreement with Takeshita and Yamaguchi[4] who reported antinociceptive effect of insulin was independent of blood glucose level in normal mice. It seems that insulin therapy may bring about an additional benefit in diabetic patients by normalizing the derangement of the gastrointestinal motility.

The gastrointestinal tract is in a continuous state of contraction, relaxation and secretion. These functions are controlled by neurohumoral systems, which in turn are regulated by various receptor systems, such as cholinergic, adrenergic, serotonergic, opioidergic and cell surface channels[31]. Many drugs affect GI transit by acting as agonists or antagonists at specific cellular receptors[32,33]. Acceleration of GI motility can be achieved by direct stimulation of gastrointestinal muscle, by activation of excitatory neural pathways or by inhibition of inhibitory pathways. Deceleration can be produced by direct relaxant effect on smooth muscle, by inhibiting the excitatory neural pathways or by activation of inhibitory pathways. Insulin's inherent acceleration of SIT can be evaluated by exploring the following systems.

Cholinergic system

Atropine is frequently used as a tool for identifying mechanisms involving cholinergic pathways[34]. It is a non-specific competitive antagonist of acetylcholine for muscarinic receptors and abolishes the effects of acetylcholine completely on the GI tract. Both in normal subjects and in patients with GI diseases, full therapeutic doses of atropine (0.5-1 mg) produce definite and prolonged inhibitory effect on the motor activity of the

Table 2 Influence of various systems / mechanisms on insulin-induced acceleration of small intestinal transit in normal mice

Pretreatment Agent/vehicle (mg/kg iv)[1]	Treatment Insulin (2 μU/kg sc)[2]	% SIT mean ± SE	% Acceleration	% Inhibition
Vehicle	Vehicle	55.10 ± 1.46	--	--
Vehicle	Insulin	74.76 ± 1.40[a]	35.68[3]	--
Atropine (1)	Vehicle	29.19 ± 3.28[a]	--	47.02[3]
Atropine (1)	Insulin	37.32 ± 3.58[b]	--	50.08[4]
Clonidine (0.1)	Vehicle	15.50 ± 1.46[a]	--	71.86[3]
Clonidine (0.1)	Insulin	27.71 ± 4.78[b]	--	62.93[4]
Ondansetron (1)	Vehicle	51.69 ± 1.13	--	6.18[3]
Ondansetron (1)	Insulin	63.84 ± 5.88	--	14.60[4]
Naloxone (5)	Vehicle	32.39 ± 2.56[a]	--	41.21[3]
Naloxone (5)	Insulin	53.90 ± 2.95[b]	--	27.90[4]
Verapamil (8)	Vehicle	40.72 ± 1.72[a]	--	26.09[3]
Verapamil (8)	Insulin	48.61 ± 3.27[b]	--	34.97[4]
Glibenclamide(10)	Vehicle	79.23 ± 1.52[a]	43.79[3]	--
Glibenclamide(10)	Insulin	88.88 ± 3.52	18.88[4]	--
Glibenclamide(10)	Insulin	88.88 ± 3.52	61.30[3]	--

Each value represents the mean ± SE (n = 6) or %.
[a]P < 0.01 *vs* vehicle + vehicle group.
[b]P < 0.01 *vs* vehicle + insulin group.
[1]Agent/vehicle administered 50 min before SIT measurement.
[2]Insulin/vehicle administered 40 min before SIT measurement.
[3]compared with vehicle + vehicle group.
[4]compared with vehicle + insulin group.

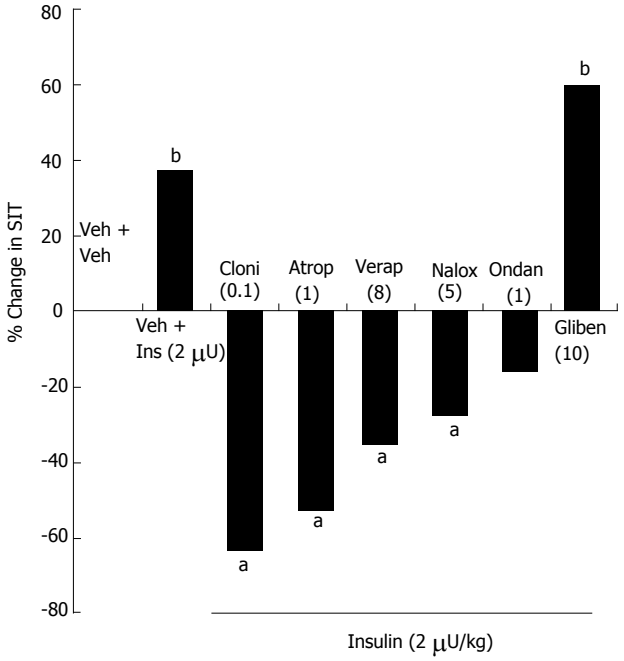

Figure 2 Involvement of various mechanisms in insulin-induced acceleration of SIT in normal mice. Each value represents % acceleration of SIT. [b]P < 0.01 *vs* vehicle + vehicle-treated group [insulin (2 μU/kg); glibenclamide (10 mg/kg)] or % inhibition of SIT. [b]P < 0.01 *vs* vehicle + insulin-treated group (2 μU/kg) (data was derived from Table 2). Cloni (0.1): clonidine (0.1 mg/kg); Atrop (1): atropine (1 mg/kg); Verap (8): verapamil (8 mg/kg); Nalox (5): naloxone (5 mg/kg); Ondan (1): ondansetron (1 mg/kg).

stomach, duodenum, jejunum and ileum[35]. Our study also confirmed inhibitory effect of atropine on SIT in normal mice. When atropine-injected group (1 mg/kg) was treated with insulin (2 μU/kg), insulin failed significantly to reverse the inhibition of SIT induced by atropine when compared with insulin *per se* treated group (Table 2). This finding indicates that insulin acts directly through muscarinic receptors to accelerate the SIT. Since atropine at the given dose could produce 50% inhibition of the insulin effect on SIT, this indicates a possibility that insulin could partly produce acceleratory effect by some other pathways in addition to cholinergic pathways as atropine could not completely prevent the acceleratory effect of insulin (Figure 2).

Adrenergic system

Clonidine has presynaptic α_2 receptor agonistic activity. Stimulation of α_2 receptors which are present on excitatory cholinergic intramural neurons in the intestine[36,37] attenuates the release of acetylcholine presynaptically, thereby producing depression of intestinal motility. In our study, we observed a significant attenuation of SIT in clonidine (0.1 mg/kg) *per se* treated group when compared with vehicle-treated group (P < 0.01), thus confirming the inhibitory effect of clonidine on SIT in mice.

When clonidine-injected group (0.1 mg/kg) was treated with insulin (2 μU/kg), the SIT was inhibited by 63% as compared with vehicle + insulin-treated group (Figure 2), thereby indicating that even though insulin could partially reverse the SIT (37%), the inhibitory effect of clonidine was still significant when compared with insulin *per se* treated group (P < 0.01) (Table 2). This finding indicates α-adrenergic pathways may be dominant in producing acceleratory effect of insulin on SIT. Insulin might have interfered with presynaptic α_2 receptors and facilitated the release of acetylcholine from excitatory neurons in the intestine. Since clonidine could not completely inhibit the SIT, we suggest that in addition to α-adrenergic pathways, insulin action may be associated with any other pathways but playing a minor role.

Serotonergic system

5-Hydroxy tryptamine (5-HT) receptors are broadly classified into five subtypes: 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4 and 5-HT_7[38]. Of these principal receptors, 5-HT_3 receptor has been suggested to play a role in the regulation of gastrointestinal motor function in many animal species and humans[39]. In the mouse ileum, 5-HT_3 receptors modulate neuronal activity within the enteric nerves leading to the contraction of the smooth muscle. The selective antagonists of 5-HT_3 receptors block the depolarizing actions of 5-HT on vagal afferents in gastrointestinal tract and have been proved to be a major breakthrough in the control of chemotherapy and radiotherapy-induced emesis[40,41]. In our study, a selective 5-HT_3 receptor antagonist, ondansetron (1 mg/kg), *per se* could not alter the normal SIT. Indeed, we selected the dose of ondansetron (1 mg/kg) which had significantly increased whole gut transit time in mice[18]. On the other hand, in ondansetron-pretreated group, insulin administration (2 μU/kg) slightly accelerated SIT (23.5%) (Table 2). This finding indicates that at this dose, ondansetron might rather partially inhibit the insulin-induced acceleration on SIT (14.6%) (Figure 2). Ondansetron (1 mg/kg) might have a prominent effect on the large intestine of mice. Hence, we propose that dose-

dependent studies are required to show the involvement of serotonergic systems with ondansetron in small intestinal motility and to evaluate insulin's acceleratory effect on SIT in this animal model.

Opioidergic system

Opioid neurons constitute the largest population of peptide-containing neurons in the myenteric plexus of the gut[42]. Opioid receptors are present on enteric nerves, epithelial cells and muscle cells[43,44]. It is known that opioid agonists slow intestinal transit particularly at the proximal portion and also reduce luminal secretions[45]. The administration of naloxone will antagonize or reverse the actions of opioids and of similar agents[44]. Therefore, the use of naloxone is expected to produce normal SIT, followed by administration of any opioids. In contrast, we observed that naloxone (5 mg/kg) *per se* produced significant attenuation of SIT ($P < 0.01$) in this animal species. This finding indicates that naloxone acts similar to opioids (Table 2).

In support of this finding, we came across similar contrasting reports but on gastric emptying. Champion *et al*[46] reported that naloxone (2 mg) delayed gastric emptying of radio-opaque material in healthy volunteers. They suggested that naloxone inhibited endogenous opiate system which normally stimulates gastric emptying and they had used the dose of naloxone two to three times greater than those usually given to reverse narcotic-induced respiratory depression and in large doses, naloxone itself may inhibit gastric emptying. Similarly, Asai and Power[47] also reported naloxone (0.01-10 mg/kg) *per se* significantly inhibited gastric emptying in rats. These studies revealed effect of naloxone on gastric emptying, but our study reveals a similar finding in the small intestine. Our study with naloxone *per se* may also suggest that naloxone may inhibit a subset of endogenous opiate system which normally stimulates movement of the contents of the intestine in this animal species. We used higher dose of naloxone to block all the receptors, thereby masking the effects of endogenous opioids, and to explore whether any subset of opioid receptors were involved in insulin-induced acceleration of SIT.

When naloxone-injected group (5 mg/kg) was treated with insulin, the SIT was significantly reversed as compared with naloxone *per se* treated group ($P < 0.01$) (Table 2). However, the naloxone-induced inhibition of SIT (28%) in insulin-treated group was still significant when compared with insulin *per se* treated group (Figure 2). This finding suggests that insulin may act through a subset of opioid receptors in the intestine to accelerate the SIT and that might be inhibited by naloxone treatment.

Calcium channels

Calcium is involved in the initiation of contraction of smooth muscle[48]. The visceral smooth muscle has a poorly developed sarcoplasmic reticulum and the increase in intracellular calcium concentration is primarily due to Ca^{2+} influx from the extracellular fluid via voltage-gated Ca^{2+} channels[49]. The L-type calcium channel is present in many cells and it is the main source of Ca^{2+} for contraction of smooth muscle[50]. This channel is blocked by dihydropyridines such as nifedipine, and other drugs such as verapamil and diltiazem[50]. Verapamil, a phenylalkylamine derivative, blocks the calcium channels on the surface of smooth muscle cells and relaxes the smooth muscle, thereby attenuating the intestinal motility[45,51]. The objective of this experiment was to evaluate the involvement of Ca^{2+} in insulin-induced acceleration of SIT. Verapamil at the given dose (8 mg/kg) *per se* significantly inhibited SIT, indicating the involvement of Ca^{2+} channels in normal physiology of small intestinal motility (Table 2). In verapamil-pretreated group, insulin administration could reverse the inhibition produced by verapamil by 65%. This finding may indicate that as insulin could not completely reverse inhibition of calcium channels, some other systems are also involved in acceleratory effect of insulin action on SIT.

Insulin secretogogue

Glibenclamide, an oral hypoglycemic drug, acts by stimulating insulin release from β-cells of pancreas[1]. The objective of this experiment was to find whether endogenously released insulin can potentiate the action of exogenously administered insulin effect on SIT. Glibenclamide (10 mg/kg) *per se* produced a significant acceleration of SIT by 43.8% (Table 2). This finding may indicate that endogenous release of insulin by glibenclamide increases the SIT.

When insulin-injected group was pretreated with glibenclamide, the SIT was further accelerated by 12% (Figure 2). This observation indicated that insulin from endogenous sources might have contributed to the additional acceleration of SIT by exogenously administered insulin.

Figure 2 indicates the significant involvement of following systems in decreasing order of acceleratory effect of insulin on SIT: adrenergic system > cholinergic system > calcium channels > opioidergic system ($P < 0.01$). In addition, release of endogenous insulin augments the effect of exogenously administered insulin on SIT.

In conclusion, the sub-hypoglycemic doses (2 μU/kg or 2 mU/kg) of insulin accelerate the small intestinal transit in normal mice without markedly changing blood glucose levels. It can be assumed that therapy of type 1 diabetes with insulin can simultaneously relieve at least one of the diabetic GI complications, such as constipation or sometimes may aggravate the diabetic neuropathy-associated diarrhoea. Furthermore, we explored influence of various systems, channels and endogenous insulin in acceleratory effect of exogenously administered insulin. Based on these observations, we report that adrenergic and cholinergic pathways play a significant role in hypermotility of small intestine induced by insulin administration in mice. Calcium channels and opioidergic pathways play supportive role; in addition, enhancement of endogenous insulin release can augment the effect of exogenously administered insulin on SIT.

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RAPID COMMUNICATION

Comparison of clonogenic assay with premature chromosome condensation assay in prediction of human cell radiosensitivity

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Abstract

AIM: To determine whether the number of non-rejoining G2-chromatid breaks can predict the radiosensitivity of human cell lines.

METHODS: Cell lines of human ovary carcinoma cells (HO8910), human hepatoma cells (HepG2) and liver cells (L02) were irradiated with a range of doses and assessed both of cell survival and non-rejoining G2-chromatid breaks at 24 h after irradiation. Cell survival was documented by a colony assay. Non-rejoining G2-chromatid breaks were measured by counting the number of non-rejoining G2 chromatid breaks at 24 h after irradiation, detected by the prematurely chromosome condensed (PCC) technique.

RESULTS: A linear-quadratic survival curve was observed in three cell lines, and HepG2 was the most sensitive to γ-radiation. A dose-dependent linear increase was observed in radiation-induced non-rejoining G2-PCC breaks measured at 24 h after irradiation in all cell lines, and HepG2 was the most susceptible to induction of non-rejoining G2-PCC breaks. A close correlation was found between the clonogenic radiosensitivity and the radiation-induced non-rejoining G2-PCC breaks ($r = 0.923$). Furthermore, survival-aberration correlations for two or more than two doses lever were also significant.

CONCLUSION: The number of non-rejoining G2 PCC breaks holds considerable promise for predicting the radiosensitivity of normal and tumor cells when two or more than two doses lever is tested.

INTRODUCTION

An important goal of current research in radiation oncology is the development of an assay or a combination of assays to predict the radiation response of individual human tumors and normal tissues. Accurate prediction of response to conventional therapy could lead to treatment modification when necessary. The important radiobiological factor that has a significant effect on a tumor's response to radiotherapy is intrinsic radiosensitivity, which, determined with a colony assay, is significantly associated with treatment outcome after radiotherapy[1,2]. However, it is unlikely to use colony formation as a routine predictive assay, as it takes several weeks to complete. This is usually too long for patients to wait for appropriate treatment. Other assays should therefore be developed which can be used routinely in a clinical setting. Such assays should give a direct or indirect measure of cell killing, and chromosomal radiosensitivity is one of the potential endpoints for assessing cellular intrinsic radiosensitivity.

Researchers have studied the metaphase chromosome aberrations using conventional techniques, and found a close relationship between radiation-induced chromosome aberrations and cell kill[3,4]. However, it is difficult to obtain enough metaphase for cytogenetic analysis because of the cell cycle delay or interphase cell death after irradiation. These problems might be solved by studying the interphase cell aberration directly. Thus, PCC technique using phosphatase inhibitors is optimal. Because the method involves non complex procedures and generates results in the non-cycling state, it is an advantage for measuring chromatin damage by qualitatively different types of radiation because one can observe chromatin

damage in the situation in which the effects on an LET-dependent cell-cycle delay and interphase cell death are removed, and PCC technique is very useful for measuring the radiation-induced chromatid breaks in all of the cell cycles, especially in G2 phase[5-8].

It has been shown a linear dose response in the same dose range used for cell-survival experiments in different *in vitro* cell systems using PCC assay to measure chromosomal damage[9-12]; and the repair kinetics of radiation-induced chromatid breaks has been studied[9,13,14]. Furthermore it was demonstrated that there is a good correlation between the induction of non-rejoining PCC breaks in interphase cells and cellular radiosensitivity[15-17]. However, it is well established that the radio-sensitivity is dependent of cell cycle progression, and among each stage of interphase, G2 phase is the most radiosensitive, G1 phase the second radiosensitive, and S phase the least radiosensitive; while G0 phase is radio-resistant for lack of oxygen and enough repairing time[18-20]. Previous studies led to the conclusion that G2 phase is a very important stage. However, there were only a few reports on the chromosome aberrations in G2 phase and little is known of the relationship between G2 chromosomal aberration and cellular clonogenic radiosensitivity.

The purpose of this study was to explore the chromosome aberrations in G2 phase. The non-rejoining G2-PCC breaks after 24 h of post-irradiation incubation by γ-rays of two human tumor cell lines were measured using PCC technique. In order to evaluate the potential of the PCC assay as a predictor of cellular radiosensitivity, the correlation between radiation-induced non-rejoining G2-PCC breaks and the cellular clonogenic radiosensitivity was studied. Furthermore, the results were analyzed together with the author's previous results of human normal liver cells L02[21].

MATERIALS AND METHODS

Cell culture and irradiation

Human ovary carcinoma cells (HO8910) and human hepatoma cells (HepG2) were grown in RPMI-1640 medium supplemented with 100 mL/L foetal calf serum, 100 kU/L penicillin and 100 g/L streptomycin at 37 ℃ in a 50 mL/L CO_2 atmosphere with 950 mL/L humidification.

Irradiation was generated from ^{60}Co source (Radiology Department, Affiliated No.1 Hospital, Lanzhou University, Lanzhou). HO8910 cells and HepG2 cells were irradiated at doses of 0, 0.5, 1.0, 2.0, 4.0, 6.0 or 8 Gy, with a dose rate of 0.2 Gy/min.

Survival assay and PCC induction

After irradiation, cells were plated at a density of about 100 surviving cells per 6 cm culture dish and incubated for about 14 d, and then fixed and stained with a solution of Giemsa.

Calyculin A, a specific inhibitor of protein phosphatase types 1 and 2A, which can induce PCC in various types of cells with high efficiency[5,22], was purchased from Wako Chemicals (Osaka, Japan), dissolved in 100% ethanol as a 1 mmol/L stock solution and stored at -20 ℃. After irradiation, cells were incubated at 37 ℃ in a 50 mL/L CO_2 atmosphere. Twenty-four hours later calyculin A was added (to give a final concentration of 50 nmol/L) to the cell cultures and cells were further incubated for 30 min. Chromosome spreads were then harvested by cell swelling in 75 mmol/L KCl for 20 min at 37 ℃ and fixed with Carnoy's fixation. A final wash and fixation in the same fixative was completed before dropping cells onto a glass slide and hot humidity drying.

Table 1 Parameters of cell survival and non-rejoining G2-chromatid breaks of the three cell lines after exposure to γ-rays irradiation

Cell line	$\alpha(Gy^{-1})^1$	$\beta(Gy^{-2})^1$	$SF2^2$ (mean ± SE)	$Slope^3$ (mean ± SE)
HepG2	0.2	0.075	0.455±0.06	2.17±0.225 (r=0.974)
HO8910	0.08	0.065	0.652±0.07	1.83±0.013 (r=0.987)
L02	0.04	0.05	0.863±0.09	1.134±0.06 (r=0.992)

[1]α and β values were determined from the linear quadratic equation.
[2]Survival fraction at 2 Gy was measured from the raw data.
[3]Slope from the fitted linear regression represents non-rejoining G2-chromatid breaks per cell per unit dose.

Observation and data evaluation

Colonies of more than 50 cells were counted as survivors. The number of colonies per dish was counted and the surviving fractions were calculated as the ratio of plating efficiencies for irradiated and non-irradiated cells. Plating efficiency was defined as the colony number divided by the number of cells plated. The survival data were fitted to the linear-quadratic model: $LnS = -\alpha D - \beta D^2$, where S is the survival fraction and D is the radiation dose.

Chromosome was stained with 50 g/L Giemsa for 20 min. Fifty well-spread G2 phase cells were scored under oil immersion with a light microscope for each dose point according to the standard criteria[23]. Briefly, chromatid discontinuing, misalignment of the distal to the lesion, or a non-stained region longer than the chromatid width were classified as a break. Iso-chromatid breaks were scored two breaks. The total chromatid breaks were calculated by summing the production of chromatid-type breaks and iso-chromatid breaks. The mean aberration values for the three separate experiments were obtained. These mean values and the standard error of the means were plotted and fitted by linear regression, and the slopes of the dose-response curves were calculated.

RESULTS

Cell survival

Cell survival parameter from the linear quadratic model fitted to the survival data is shown in Table 1. SF2 was measured from the raw data also shown in Table 1. For radiosensitivity, three cell lines displayed the different potency, in the order of, HepG2 > HO8910 > L02.

Non-rejoining G2-chromatid breaks

All cell lines showed a dose-related increase in the induction of non-rejoining G2-chromatid breaks (Figure 1). The dose-response curves were fitted by linear regression and the slopes of the dose-response curves were evaluated.

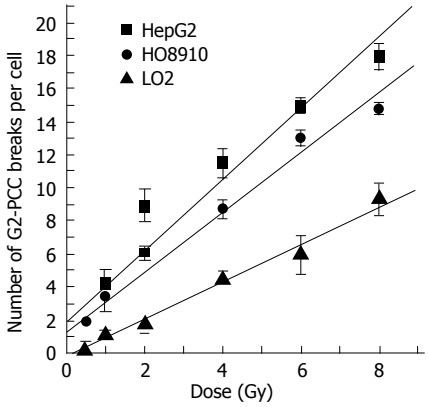

Figure 1 Dose-response curves for the induction of non-rejoining G2-PCC breaks 24 h after irradiation.

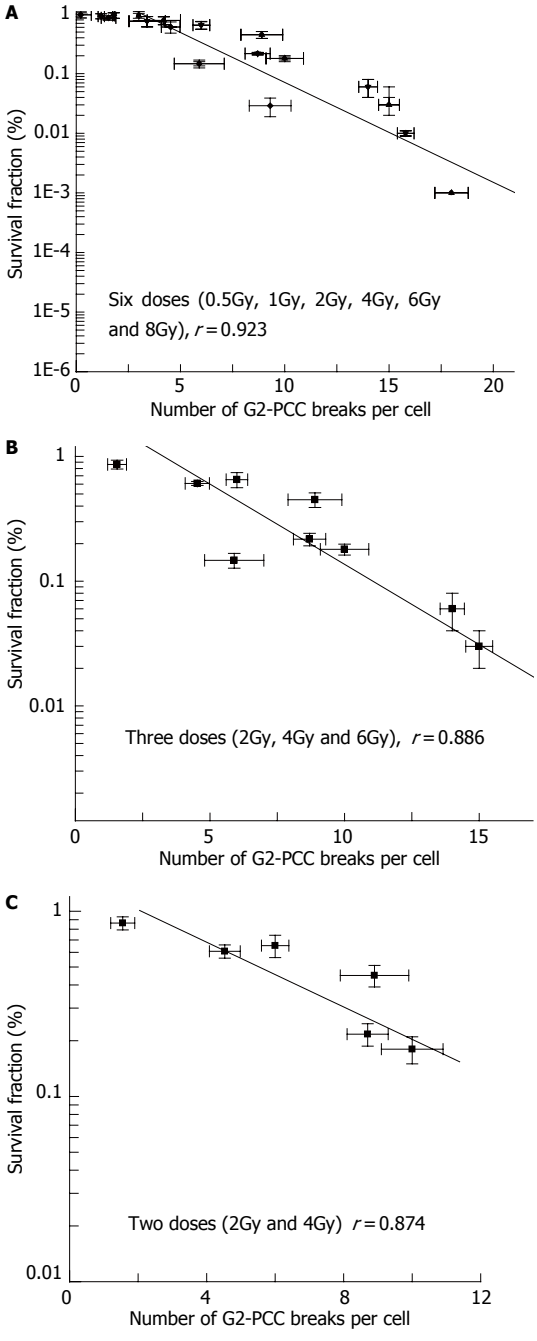

Figure 2 The relationship between survival fraction and non-rejoining G2-PCC breaks 24 h after irradiation.

Table 2 Correlation between non-rejoining G2 chromatid breaks and cell survival

	Dose (Gy)	*r*	*P*
Multiple	2+4	0.874	0.023
	2+4+6	0.886	<0.01
	0.5+1+2+4+6+8	0.923	<0.001

The slope of HepG2 was the steepest, and that of HO8910 the second and L02 the least. Namely, the order of the slopes was HepG2 > HO8910 > L02. The values for the slopes of the curves are given in Table 1.

Correlation between cell survival fraction and non-rejoining G2 chromatid breaks

To examine the correlation between clonogenic radiosensitivity and the non-rejoining G2-chromatid breaks, the non-rejoining G2 chromatid breaks were plotted against the surviving fractions (Figure 2A). Correlation coefficient was 0.923 for three cell lines. The relationships for non-rejoining G2 chromatid breaks at different multiple doses (Figure 2) were investigated. Correlation coefficients are shown in Table 2. It is clear that increasing combining doses improves the correlation coefficients. This is true for any combination of two doses or more than two doses (data not shown).

DISCUSSION

Consistent with previous studies[21], fitted cell survival curve of three cell lines were linear quadratic in the present study. Among the three cell lines, survival fraction of L02 cells was the highest after absorbing same dose of radiation. These suggested that human normal liver cells were of much lower sensitivity to γ-rays than both human ovary carcinoma cells and human hepatoma cells. These may be because reductive glycogens synthesized in normal liver cells which interact with free radicals reduce the radiation injury[21]. In addition, the theoretic basis of radiotherapy is that the normal cells are of low radiosensitivity, and our results further confirmed this.

It has been shown that the rejoining of radiation-induced DNA double strand breaks (dsbs) was saturated within 2-4 h in mammalian cell lines[24,25] and that the number of residual dsbs did not change between 4 and 20 h using the neutral filter elution[26]. So it is considered that the most important parameter related to clonogenic radiosensitivity is the residual dsbs for 4 h or more after irradiation[27]. The PCC technique measures the chromatid breaks caused by the dsbs[28,29], and the processes of chromosomal breaks rejoining contain a fast component and a slow component[5]. The G2-PCC breaks were rejoined with a half-time of 5 min for the fast component and about 3 h for the slow component after irradiation[5], implying that the rejoining of chromosome breaks occurred mainly during the early hours after irradiation. Furthermore, Borgmann[29] reported that the number of PCC fragments induced by radiation plateaued at 4-6 h after irradiation, and did not change for about 10 h. In

addition, Suzuki[13] reported that the repairing process of radiation-induced chromosome aberration occurred during the early 10 h after irradiation. Based on previous studies, most kinds of cells end their spontaneous repair process within 24 h if they are injured, and after 24 h, injured and normal cells without any artificial factors will be stable and continue synthesizing, mitosis processes. Therefore, it is reasonable that the non-rejoined excess fragments examined after incubation for 24 h after irradiation may be the most important parameter related to clonogenic radiosensitivity. Based on these, the non-rejoining G2-PCC breaks were examined after incubation for 24 h post-irradiation in this study, and a good linear relationship was found between dose and the non-rejoining G2 chromatid breaks after the cells were exposed to ^{60}Co γ-rays (Figure 1). In addition, we found that cells which were more sensitive to the cell killing were similarly more susceptible to induction of non-rejoining G2 chromatin breaks (Table 1 and Figure 1). Suzuki[15-17] and Borgmann[29] found similar results. Additionally, a good linear relationship was also found between dose and the initial chromatid breaks[7,21,30]. It is easy to study the radiation-induced chromatid damage with the PCC technique[11,31,32], and the data obtained thereby may accurately reflect the radiation damage for the chromatin level without the competition as a result of cell cycle and/or interphase cell death. Therefore the PCC technique is very useful for detecting chromatin damages by qualitatively different types of radiation. Furthermore, it is faster in obtaining the result by the PCC technique than by most of other techniques.

In the present study we have attempted to validate the use of radiation-induced non-rejoining G2 chromatid breaks as a measure of cellular radiosensitivity. One of the prerequisites for the potential use of such a rapid assay would be that the relationship with clonogenic survival, the Gold Standard and most relevant assay for tumor control, should be high. In this research, a good correlation (r=0.923) was found between cell survival and the number of non-rejoining G2-chromatid breaks in the three cell lines (Figure 2A). It implies that radiosensitivity can be determined by the induction of non-rejoining G2-PCC breaks.

To our knowledge, few researches have looked at the relationship between non-rejoining G2 chromatid breaks and cell killing, and only a few studies have investigated the relationship between the induction of non-rejoining chromatid breaks in interphase detected by PCC technique and cell killing. Ofuchi[17] showed a strong correlation between non-rejoining chromatid breaks and cell survival in human hepatoma cells. Suzuki[11] showed a good correlation between induction of non-rejoining PCC breaks and cell death in normal human cells, in human tumor cell lines[12], and in 6 primary cultured cells[15]. All these suggest one possibility that radiosensitivity can be determined by the induction of non-rejoining PCC breaks.

Dependent on the amount of primary tumor material (biopsy), it might not be feasible to obtain a complete dose-response curve consisting, for example, of four or more different doses. It is therefore more likely that only one or two doses could be studied with any statistical significance. Because of the limited number of cell lines in this study, it was not reliable to analyze the correlation between cell survival and the induction of non-rejoining G2-chromatid breaks for individual doses. For multiple doses, highly significant and good correlation were observed if two or more doses were used (Figure 2 and Table 2). Coco Martin[3] found similar correlation coefficients as reported here for 13 human tumor cell lines at two or more radiation doses using metaphase cytogenetic techniques. It indicates that two doses can be applied on fresh tumor tissue to obtain a relative high correlation. As for the individual doses, it is necessary to expand the number of cell lines to confirm the correlation between cell survival and the induction of non-rejoining G2-chromatid breaks.

In conclusion, it is suggested that the PCC technique is useful for determining chromosome aberration in G2 cells, and the amount of non-rejoining G2-PCC breaks induced by radiation can evaluate the cellular clonogenic radiosensitivity. To assess the general applicability of this approach, further studies are needed to expand the number of human cell lines.

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RAPID COMMUNICATION

Clinical characteristics of remote Zeus robot-assisted laparoscopic cholecystectomy: A report of 40 cases

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Abstract

AIM: To summarize the performing essentials and analyze the characteristics of remote Zeus robot-assisted laparoscopic cholecystectomy.

METHODS: Robot-assisted laparoscopic cholecystectomy was performed in 40 patients between May 2004 and July 2005. The operating procedures and a variety of clinical parameters were recorded and analyzed.

RESULTS: Forty laparoscopic cholecystectomy procedures were successfully completed with Zeus robotic system. And there were no post-operative complications. Total operating time, system setup time and performing time were 100.3 ± 18.5 min, 27.7 ± 8.8 min and 65.6 ± 18.3 min, respectively. The blood loss and post-operative hospital stay were 30.6 ± 10.2 mL and 2.8 ± 0.8 d, respectively. Camera clearing times and time used for operative field adjustment were 1.1 ± 1.0 min and 2.0 ± 0.8 min, respectively. The operative error was 7.5%.

CONCLUSION: Robot-assisted laparoscopic cholecystectomy following the principles of laparoscopic operation has specific performing essentials. It preserves the benefits of minimally invasive surgery and offers enhanced ability of controlling operation field, precise and stable operative manipulations.

Key words: Zeus; Robotic surgical system; Laparoscopic cholecystectomy

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INTRODUCTION

During the past two decades, the benefits of laparoscopic surgery for the patients are less trauma and pain, a shorter post-operative hospitalization, a better cosmetic result and a faster return to normal activity[1]. However, although there are clear benefits to the patients, surgeons face distinct disadvantages. Working through fixed entry points limits maneuverability of the instruments inside the body cavity to five degrees of freedom (DOF). Looking at a two-dimensional screen, surgeons are handicapped by the loss of the visual perception of depth, and the need for a human assistant to hold and move the camera makes surgeons loss the independent ability of controlling operation field [2]. Reduced dexterity and impaired visual control are considered the major burdens of laparoscopic surgery and the attempts in developing robotic telemanipulation systems aim at overcoming these problems. Nowadays both Zeus and da Vinci robotic telemanipulation systems are available to patients. After the success of the first laparoscopic cholecystectomy with Zeus robotic surgical system of our hospital on 26 April 2004, we performed the same operation in the other 40 patients between May 2004 and July 2005, and acquired some experience.

MATERIALS AND METHODS

Patients

Between May 2004 and July 2005, forty patients (24 females and 16 males), with average age 36 years (range 14-50 years) underwent the robot-assisted laparoscopic cholecystectomy in our hospital. Six cases were cholecystopolyp and the others were cholecystolithiasis combined with chronic cholecystitis. Informed consents were obtained from all patients before operation.

Operation

Zeus robotic surgical system (Computer Motion Com-

pany, IEC 60601, Class III, Type CF) consists of Aesop (automatic endoscopic system optimal position), Hermes (acoustic center), Zeus surgical system (three separate robotic arms, operator console, optic console), and Socrates (remote co-operating system).

All the patients in supine position were under the total intravenous combined inhalation anesthesia. The left and right robotic arms were placed at each side of the patient's head. Aesop arm was placed beside the left hip. All robotic arms were attached to the sidebars of the operating table. The movable lower limits of the three arms were set at an optimal distance that a palm was able to insert between the robotic arm and the patient's abdominal wall, and then the instrument adapter was installed onto its robotic arms. Pneumoperitoneum was established after the puncturation with Verres needle at the umbilicus and the cavity pressure was maintained at 12 mmHg. The first 10-mm trocar was introduced at the umbilicus and the 10-mm 30º laparoscope was inserted to explore the abdominal cavity. Under laparoscopic vision, another 10-mm trocar and a 5-mm trocar were respectively introduced at 5-cm below the xiphoid process and 5-cm below the right costal margin in anterior axillary line. A dissection clamp and a grasper were inserted through the trocars. The laparoscope, a grasper and a dissection clamp were engaged with the instrument adapters of Aesop arm, left robotic arm and right robotic arm. Then the three robotic arms were adjusted in a double 90º position, i.e., the angle between the forearm and the upper arm of a robotic arm was 90º, and the angle between the laparoscopic instruments in the adapters and the forearm was also 90º. The condition of double 90º offered the largest range of motion. The surgeon wearing a microphone sat before the operator consol which was placed about 5 m far from the operating table, and steered two egg-shaped manipulators to control the left or right robotic arm. In the mean time, Aesop was acoustically controlled to move up and down, left and right, forward and backward, so as to offer the optimal operative field. An assistant standing by the operating table prepared to adjust the tiny regulator of Aesop or another two robotic arms. An instrument nurse also stood by the table to replace the instruments at any moment. The surgeon steered the grasper of the left robotic arm to retract the neck of gallbladder to expose the Calot's triangle and manipulated the dissection clamp of the right robotic arm to dissect the cystic duct and cystic artery. The cystic artery and duct were sheared after double ligation with clips separately, and then gallbladder was cut down from the liver with the diathermy hook. The gallbladder was pulled out by the assistant from the port below xiphoid process[3].

All the operation procedures were recorded on videotape for later analysis. Time parameters included total operative time, system setup time and performing time. The total operative time was defined as the time from disinfection of the operative field to skin closure. The system setup time was defined as the time from disinfection to the start of grasping the neck of gallbladder. The performing time was defined as the time from the start of grasping the neck of gallbladder to the moment the gallbladder was completely freed from the liver. Minimally invasive parameters included the blood loss, post-operative complications and post-operative hospital stay. Parameters of operative field included camera clearing time and time used for operative field adjustment. Camera clearing time was defined as the time that the laparoscope was taken out to clear the contaminant on the camera during operation. Time used for operative field adjustment mean the time which was taken for the surgeon to stop perform to adjust the operative field or clear the camera. The rate of operative error mean the percent that the cases in which operative errors, such as hepatic laceration or gallbladder perforation happened, occupied among the 40 cases. Conversion cases mean the number of procedures converted to other procedures, such as open procedure or conventional laparoscopic procedure.

RESULTS

Forty laparoscopic cholecystectomy procedures were successfully completed with Zeus robotic system and there were no conversion cases. Total operating time, system setup time and performing time were 100.3 ± 18.5 min, 27.7 ± 8.8 min and 65.6 ± 18.3 min, respectively. The blood loss and postoperative hospital stay were 30.6 ± 10.2 mL and 2.8 ± 0.8 d, respectively. Camera clearing time and time used for operative field adjustment were 1.1 ± 1.0 and 2.0 ± 0.8 min, respectively. The operative error was 7.5%. All the patients began to intake diet at 6-9 h after operation. Their wound healed well without oozing blood and infection, and there were no post-operative complications in any of the patients.

DISCUSSION

At the beginning of 1999, two US companies, Computer Motion and Intuitive Surgical, received European CE accreditation for the clinical application of Zeus and da Vinci robotic surgical systems that were independently invented. In 2000, the two companies received FDA accreditation too, which indicated that robotic surgical systems began to be formally available to patients in the world[4]. In 2001 September 7, a medical team led by French doctor Marescaux accomplished the famous Zeus robot-assisted cholecystectomy, cross over Atlantic Ocean, named as "Lindbergh operation", which created a precedent of remote operation[5]. Up to now, the safety and feasibility of robotic surgical systems applied in general surgery, thoracic/vascular surgery and gynaecology/urology have been demonstrated. Until 2000, more than 6000 robot-assisted procedures were performed, thirty-seven percent among them were in general surgery, mainly cholecystectomy[6]. In the late two years, the technique of robot-assisted operation became more and more mature and wide, and the number of procedures increased quickly. After the success in the first Zeus robot-assisted laparoscopic cholecystectomy in Chinese mainland by our hospital, the other 40 cases were accomplished between May 2004 and July 2005. Based on these, the robot-assisted operative characteristics and performing essentials were analyzed.

Robotic surgical system is a new achievement which is resulted from the medical application of highly developed automatic technique, computer image technique and con-

trol technique. The advanced system possesses the enormous superiority over conventional laparoscopic surgery and forms its characteristics. During Zeus robot-assisted procedures, there was no need for a human assistant to hold the camera, who often provides error and unstable operative field or contaminates camera by touching tissue. The surgeon directly control the camera engaged with Aesop arm by voice, which restores his capability to master operative field as open operation. Moreover, the arm cannot shake and has the ability to memory the previous position. Therefore, the adjustment of operative field is quick and convenient and the operative field is direct-viewing and stable[7]. In our study, camera clearing time and time used for operative field adjustment were merely 1.1 ± 1.0 and 2.0 ± 0.8 min, respectively. There was no vibration of laparoscopic instruments in all the 40 procedures, which benefits from the mechanism and working principle of Zeus robot. First, with the surgeon sitting at a remote and ergonomically designed workstation, Zeus robotic system eliminates the need to twist and turn in awkward positions, which is in favour of the long and precise operation[8]. Second, it is the 12- to 15-fold magnification of image by the camera of Zeus system that conduces to accurate operation[9]. Third, it provides adjustable motion scaling and tremor reduction. Motion scaling reduces the surgeon' s motion at the console to finer movements within the patient. When the system is set to 5:1, a 5-cm sweep by surgeon's hand is a 1-cm sweep within the abdomen. This promotes the accuracy of operation. Tremor reduction can completely eliminate any tremor from the surgeon's hand, which increases the stability of operation, thereby decreasing operative errors[10]. The operative error was lower than that of early conventional laparoscopic surgery. The endowrist at the tip of instrument provides the surgeon with six degrees of freedom inside the patient's body. The additional degrees of freedom increase the dexterity and create the sense of actually having the surgeon's hand within the abdominal cavity during laparoscopic surgery. This vastly simplifies tasks such as suturing, tying and complex dissection, all of which are extremely challenging for most surgeons with standard laparoscopic equipment[11]. Nio *et al*.[12] selected 20 medical students without any surgical experience to perform at random a set of laparoscopic tasks either manually or robot-assisted (Zeus). This task consisted of dropping beads into receptacles, running a 25-cm rope, capping a hypodermic needle, suturing, and performing a laparoscopic cholecystectomy on a cadaver liver of a pig. The dropping beads exercise and the laparoscopic cholecystectomy required more time when performed with robotic assistance, as compared with manual performance. Grasping the beads, the rope, and either the needle or the cap were tasks that required fewer actions to complete when performed with robotic assistance. As compared with the robot-assisted rope-passing exercise, more failures were made in the manually performed procedure, mainly caused by unintentional dropping of the rope. Therefore, robot-assisted laparoscopic surgery by participants without any surgical experience might require more time, but actions can be performed more precisely as compared with manual laparoscopic surgery. Zeus robot-assisted laparoscopic cholecystectomy preserves the benefits of minimally invasive surgery. Robotic surgical system overcomes the technical limit of conventional laparoscopic surgery and expends the field of minimally invasive surgery into cardiac surgery. Kappert *et al*.[13] performed 29 off-pump totally endoscopic coronary bypass (TECAB) on a beating heart with the Da Vinci system and an endoscopic stabilizer (Intuitive Surgical Company). Patients were operated upon via four 1-cm chest incisions using the da Vinci robot for the internal thoracic artery (ITA) harvesting and for performance of anastomoses on the beating heart. In this series, they had a 100% survival rate. Conversion rate to a median sternotomy was 3.4%; time of harvesting was 26 min; time of anastomosis was 29 min; and operating time was 130 min. Post-operative time in ICU was 17 h; and post-operative hospital stay was 7 d. In robotic surgery, extra time is needed to setup and position the robotic arms and instruments before starting of the actual dissection[14]. However, the setup time decreased from 40-50 min to 20-30 min with increasing experience. If the robotic systems are placed at independent operating room and arms are fixed on the operating table the total operative time will reduce.

Zeus robot-assisted cholecystectomy is performed by the surgeon who controls the robotic arms via manipulators to accomplish the operation. It is not a job fulfilled 'automatically' by the robot according to a certain input program. The surgeon should follow the operative principle of conventional laparoscopic cholecystectomy, be familiar with the working rational of robotic systems, and continue to summarize the performing essentials as the accumulation of experience: (1) Installation of the robotic arms. Installation should depend on the body height and body type of the individual patient. Generally, the left and right robotic arms were placed at each side of the patient's head. Aesop arm was placed beside the left hip. The movable lower limits of the three arms were set to insert a palm between the robotic arm and the patient' s abdominal wall. The three robotic arms are adjusted in a double 90º position; (2) Place of trocar ports. The left trocar port should be placed in anterior axillary line 5 cm below the right costal margin, lower than that of conventional laparoscopic cholecystectomy, so as to favor the left robotic arm to retract gallbladder from various angles; (3) Tactile feedback is compensated by visual feedback. During robot-assisted procedures the surgeon cannot touch both the tissue and the instruments and lose the tactile feedback, which makes performing and judging more difficult. But, high-resolution and vivid three-dimensional operative field provided by robotic surgical system make it possible for the surgeon to observe the tiny morphological change of tissue. Thus the surgeon should utilize the high-quality visual feedback to compensate the lost tactile feedback, such as observing the deformation of tissue under pressure to judge strength[15]; (4) Conversion of the performing habits. During robot-assisted procedures, basic surgical manipulations, such as incision, separation, hemostasis, suture and so on, are converted to squeeze, relax, or rotate the egg-shaped manipulators. Due to these entirely changes of operative maneuvers, the surgeon is forced to form new habits through special training and practice.

Though the experience through the 40 Zeus robot-as-

sisted laparoscopic cholecystectomies was preliminary, we understood the advanced technical advantages of robotic surgical system, such as independent fine visualization, restored dexterity, *etc*. With the experience accumulated and performing craft enhanced, these advantages will convert to superior therapeutic efficacy, and the system will become a new therapeutic technical platform for the surgeons.

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RAPID COMMUNICATION

Three-dimensional conformal radiotherapy combined with FOLFOX4 chemotherapy for unresectable recurrent rectal cancer

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Abstract

AIM: To investigate the effect of three-dimensional conformal radiotherapy (3-DCRT) in combination with FOLFOX4 chemotherapy for unresectable recurrent rectal cancer.

METHODS: Forty-eight patients with unresectable recurrent rectal cancer were randomized and treated by 3-DCRT or 3-DCRT combined with FOLFOX4 chemotherapy between September 2001 and October 2003. For the patients without prior radiation history, the initial radiation was given to the whole pelvis by traditional methods with tumor dose of 40 Gy, followed by 3-DCRT for the recurrent lesions to the median total cumulative tumor dose of 60 Gy (range 56-66 Gy); for the post-radiation recurrent patients, 3-DCRT was directly given for the recurrent lesions to the median tumor dose of 40 Gy (36-46 Gy). For patients in the study group, two cycles chemotherapy with FOLFOX4 regimen were given concurrently with radiotherapy, with the first cycle given simultaneously with the initiation of radiation and the second cycle given in the fifth week for patients receiving conventional pelvis radiation or given in the last week of 3-DCRT for patients receiving 3-DCRT directly. Another 2-4 cycles (average 3.6 cycles) sequential FOLFOX4 regimen chemotherapy were given to the patients in the study group, beginning at 2-3 wk after chemoradiation. The outcomes of symptoms relieve, tumor response, survival and toxicity were recorded and compared between the study group and the control group.

RESULTS: For the study group and the control group, the pain-alleviation rates were 95.2% and 91.3% ($P > 0.05$); the overall response rates were 56.5% and 40.0% ($P > 0.05$); the 1-year and 2-year survival rates were 86.9%, 50.2% and 80.0%, 23.9%, with median survival time of 25 mo and 16 mo ($P < 0.05$); the 2-year distant metastasis rates were 39.1% and 56.0% ($P = 0.054$), respectively. The side effects, except peripheral neuropathy which was relatively severer in the study group, were similar in the the two groups and well tolerated.

CONCLUSION: Three-dimensional conformal radiotherapy combined with FOLFOX4 chemotherapy for unresectable recurrent rectal cancer is a feasible and effective therapeutic approach, and can reduce distant metastasis rate and improve the survival rate.

Key words: Rectal neoplasms; Radiotherapy; Chemotherapy

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INTRODUCTION

Despite all previous efforts at radical curative resection and multidisciplinary treatment, locally recurrent rectal cancer (LRRC) occurs in up to one third of patients[1-3]. A curative treatment is possible only when local recurrences represent limited disease that may be amenable to surgical re-excision[4,5]. Unfortunately, most patients with LRRC will be excluded from curative surgery on the basis of medical fitness, the presence of distant metastasis, locally unresectable disease or an unwilling to accept the considerable associated morbidity and mortality. In these patients, palliative intervention still may be required[6].

Patients with unresectable LRRC are often treated with nonsurgical palliation, including radiation therapy, chemotherapy and chemoradiation. Radiation has been confirmed as an effective method to palliate the symptoms and dose-response relationship between radiation doses and subjective response of LRRC has been revealed by some studies[7-10]. The strategy to elevate the radiation dose by adopting

new techniques, such as three-dimensional conformal radiotherapy (3-DCRT) or intensity-modified radiotherapy (IMRT), is needed to improve the local control of LRRC. It is also reasonable to combine chemotherapy into the multi-modality treatment for LRRC patients because systemic metastasis is a common problem. We designed herein a randomized, controlled study to compare the efficacy and the toxicities of exclusive 3-DCRT and 3-DCRT combined with FOLFOX4 chemotherapy for patients with unresectable LRRC.

MATERIALS AND METHODS

Patients and characteristics

Between September 2001 and October 2003, 48 patients with unresectable LRRC were randomized and treated by 3-DCRT at the Radiation Oncology Department, Sir Run Run Shaw Hospital. Twenty-five cases among them were treated by exclusive 3-DCRT and defined as the control group, while the other 23 cases received 3-DCRT combined with FOLFOX4 regimen and were defined as the study group. Table 1 shows the clinical data and pathologic characteristics with the initial operation. The diagnosis of local recurrence of rectal cancer mainly depended on imaging exam. Of 44 patients, presacral masses were detected by pelvis B-ultrasound, computerized tomography (CT) or magnetic resonance imaging, 10 cases were confirmed by biopsy pathological result. Thirty-five cases among them had solitary presacral mass, 9 cases (5 cases in the study group and 4 cases in the control group) had multiple masses or accompanied with adjacent lymph nodes metastases, but all the lesions can be dealt as a whole target. Of the other 4 cases, solitary masses in the pelvic sidewall were found by imaging exam. All the cases were consulted by radiologists and surgeons and evaluated as unresectable. Systemic examination was carried out to exclude distant metastasis. The median interval between local recurrence and initial operation was 15 (range 7-42) mo. Eighteen cases among the study group and 20 cases among the control group had received peri-operative radiotherapy with dose of 40-50 Gy. Twenty cases among the both groups had received 5-flurouracil (5-Fu)-based peri-operative chemotherapy.

Table 1 Clinical and pathologic characteristics of the patients

Variables	Control group (n = 25)	Study group (n = 23)
Age (yr): Median (range)	62 (36-70)	62 (40-72)
Sex		
Male	17	14
Female	8	9
Dukes' stage of initial lesion		
A	1	1
B	7	5
C	17	17
Tumor pathologic type		
Well and moderately differentiated adenocarcinoma	19	17
Mucinous adenocarcinoma	4	3
Signet ring cell carcinoma	2	3
Recurrent sites		
Presacral	23	21
Pelvic sidewall	2	2

No significant differences in clinical or pathologic variables between the two groups were observed.

Treatment

For the patients without prior radiation history, the initial radiation was given to the whole pelvis by traditional methods with tumor dose of 40 Gy, followed by 3-DCRT for the recurrent lesions to the median total cumulative tumor dose of 60 Gy (range 56-66 Gy); for the post-radiation recurrent patients, 3-DCRT was directly given for the recurrent lesions to the median tumor dose of 40 Gy (36-46 Gy). The entire pelvis was irradiated with 10 MV photons using AP/PA portals or PA portal and two lateral wedged portals. The schedule was once daily, 5 times a week, using 200 cGy fractions, to a final dose of 40 Gy. Belly board was used to reduce the volume of small bowel irradiated. For 3-DCRT, all patients had a CT scan in the treatment position immobilized by thermoplastic molds for treatment planning purposes. Using the CT data set, the clinical target volume defined as the gross tumor volume with 5-mm margin was delineated and confirmed by radiologists, radiation oncologists and radiotherapy physicians. An additional 5-10 mm was added for planning target volume. Radiotherapy treatment planning was performed using Pinnacle3 3-D conformal radiation treatment planning system. Three to seven fields with individualized blocks derived from beam's-eye-view were used to implement the 3-DCRT. PTV was surrounded by 90% isodose curvature. Three-dimensional CRT was delivered with 10 MV photon and conventional fractionization: once daily, 5 times a week, 200 cGy per fraction. For patients in the study group, two cycles chemotherapy with FOLFOX4 regimen were given concurrently with radiotherapy, with the first cycle given simultaneously with the initiation of radiation and the second cycle given in the fifth week for patients receiving conventional pelvis radiation or given in the last week of 3-DCRT for patients receiving 3-DCRT directly. Another 2-4 (average 3.6) cycles sequential FOLFOX4 regimen chemotherapy were given to the patients in the study group beginning at 2-3 wk after chemoradiation. FOLFOX4 regimen comprised of intravenous injection of oxaliplatin at a dose of 85 mg/m^2 on d 1, intravenous injection of leucovorin at a dose of 300 mg/m^2 and intravenous injection of 5-Fu at a dose of 400 mg/m^2 and continuous intravenous injection of 5-Fu at a dose of 600 mg/m^2 on d 1 and d 2.

Evaluation of patients

Patients were observed at 3-mo intervals for 18 mo after the completion of therapy and every 6 mo for 3 years. All patients were followed up till December 2004, with follow-up duration of 6-39 (median 23) mo, except two who were lost to follow-up and presumed dead. Assessment of pain was scored from 0 (no pain) to 10 (as bad as you can imagine) by numeric rating scale. If the pain score decreased more than a half, good pain palliation was considered. Assessments of tumor dimensions by CT scan were performed before the start of treatment and repeated

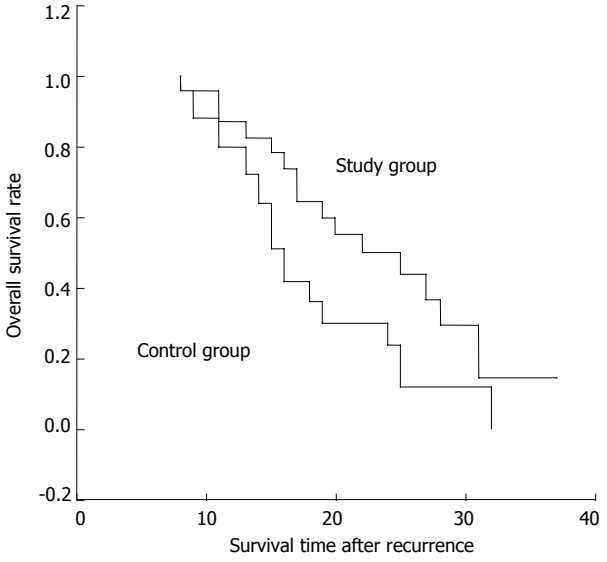

Figure 1 Overall survival curves of patients in the two groups. Five patients survived in the control group, and 8 patients survived in the study group.

1 mo after the end of 3-DCRT. Tumor objective responses were classified as complete response, partial response, stable disease and progression disease based on standard World Health Organization (WHO) criteria. The data of toxicity were ranked according to the WHO evaluation.

Statistical analysis

Survival estimates were calculated using Kaplan-Meier curves and a two-sided log-rank test was used to compare survival curves. χ^2 test was used to determine the difference of pain palliation rates, objective response rates and distant metastasis rate. P value < 0.05 was considered statistically significant.

RESULTS

Good pain palliation rate was 95.2% (20/21) in the study group and 91.3% (21/23) in the control group, with median palliation time of 13 d (range 6-58 d) and 15 d (range 8-65 d), respectively ($\chi^2=0.261$, $P=0.609$).

The overall objective response rate in the study group was 56.5% (1 complete response, 12 partial responses), while that in the control group was 40.0% (0 complete response, 10 partial responses) ($\chi^2=1.283$, $P=0.257$).

Figure 1 shows overall survival of the two groups. In the study group, 1-year and 2-year overall survival rates were 86.9% and 50.2%, while those of the control group were 80.0% and 23.9% with median survival time of 25 mo and 16 mo, respectively (Log-rank = 4.01, $P = 0.045$).

Interestingly, 2-year distant metastasis rates of the two groups were 39.1% and 56.0% with median distant metastatic time of 16 (range 7-26) mo and 10 (range 3-23) mo, respectively ($\chi^2=3.715$, $P=0.054$).

Toxicities of patients were scored according to WHO scale. A detailed description of acute toxicities was given in Table 2. The main toxic reactions of the control group patients included diarrhea, rectal tenesmus, perianal area skin reaction and bone marrow suppression. The main toxicities of the study group patients were similar but relatively severer peripheral neuropathy. No toxicity-related death was observed in both the groups. In the study group, radiotherapy of 3 patients was interrupted and delayed for 2-4 d due to severe diarrhea and rectal tenesmus, and the 2nd cycle chemotherapy of 1 patient was canceled due to severe bone marrow suppression. In the control group, radiotherapy of 2 patients was interrupted and delayed for 2 and 3 d, respectively, due to severe diarrhea and rectal tenesmus. No severe late toxicity was observed in both groups in the follow-up duration.

Table 2 WHO scale for acute toxicities

Toxicity	Grade	Control group	Study group	χ^2 value
Leukopenia	I	6	10	
	II	2	8	3.41
	III	0	3	
	IV	0	0	
GI tract	I	8	3	
	II	5	10	
	III	5	6	0.66
	IV	1	2	
Proctitis	I	10	9	
	II	6	8	
	III	3	3	0.01
	IV	0	0	
Skin	I	5	3	
	II	8	9	
	III	12	11	0.00
	IV	0	0	
Peripheral neuropathy	I	0	10	
	II	0	4	1.09
	III	0	1	
Bladder	I	12	11	
	II	6	8	
	III	2	1	0.27
	IV	0	0	

$P>0.05$. P value was calculated by subgroups for toxicity grade $\geqslant 3$.

DISCUSSION

The optimal treatment for locoregionally recurrent rectal cancer after curative surgery has not yet been defined[5,11,12]. Most of the recurrent rectal cancers are not resectable and require nonsurgical approaches. Multimodality treatment would probably offer the best result[13]. In the past, patients with locally unresectable recurrent rectal cancer were assumed incurable and received mostly palliative therapy. Traditional radiotherapy and chemotherapy given with palliative goal have been confirmed good palliation result of symptoms, such as local pain and bleeding with unknown effect on survival. Noticeably, several series have found a dose-response relationship of radiotherapy for symptoms control[9,14,15]. Some of them revealed the relationship also for local control and survival rate[9]. Sanfilippo *et al*[16] reported that despite aggressive multimodality therapy, a high rate of pelvic recurrence occurred in patients with clinically staged T_4 disease, and regional disease recurred almost exclusively in the radiation field. Under such circumstances, there is a significant need to adopt new techniques, such as intraoperative radiation therapy, brachytherapy and 3-DCRT, to safely deliver tumoricidal dose of radiation in an attempt to improve the local control[17-20].

Moreover, 3-DCRT allows more accurate definition of target volume and anatomy of critical normal structures. This technique focuses radiation to specific sites of disease, thereby minimizing injury to normal tissues. Higher doses of irradiation can be delivered by this technique to produce better tumor control without increasing the probabilities of particular sequela. It is controversial that whether previously irradiated LRRC patients could receive reirradiation and whether the reirradiation is of any value. Several investigators reported that high doses of reirradiation could be delivered with acceptable risks without prohibitive long-term side effects in patients with LRRC and could result in surgical salvage and long-term survival in selected patients[10,21,22]. This may be related to the location of local recurrence. A multicenter analysis of 123 patients with recurrent rectal cancer within the pelvis revealed that recurrent tumors were mainly situated in the posterior part of the bony pelvis and patients received abdominoperineal resection had a significantly more extension of recurrent tumors in the inferior parts of the pelvis comparing to those patients received low anterior resection[23]. There are fewer organs at risk in the lower and posterior pelvis. The usual local recurrent location of rectal cancer and 3-DCRT technique make it feasible to deliver higher radiation dose comparing with conventional radiation or to reirradiate with high dose for patients suffered from LRRC. In this study, we treated all patients with 3-DCRT or 3-DCRT boost to relatively higher dose, resulting in good palliation of pain in 93.2% (41/44) patients, the objective response rate in 47.9% (23/48) patients, and well tolerated toxicities. Thus we can roughly draw a conclusion that 3-DCRT for LRRC patients is feasible and effective.

It is now generally accepted that exclusive radiotherapy plays a minor role in improvement in survival unlike its major role in palliation of symptoms and improvement of local control for rectal cancer. Combined modality treatment is the recommended standard adjuvant therapy for patients with locally advanced rectal cancer. Currently, most adjuvant therapy includes chemotherapy[24]. Traditional chemotherapy or chemoradiation focuses on 5-Fu-based regimens, which have been confirmed to be effective. In our previous study, we have reported that preoperative radiotherapy combined with full course chemotherapy (LV + 5-Fu + 5'DfuR) is effective and safe[25]. Because of the clinical appliances of oxaliplatin during the recent years, substantial progress has been made in chemotherapy of rectal cancer. Chemotherapy with oxaliplatin combined with 5-Fu, such as FOLFOX4 regimen, is more effective and has become the standard treatment for advanced stage colorectal cancer[26]. In the United States, using similar chemotherapy regimens as adjuvant therapy has been approved. The advantages of oxaliplatin, such as its mild toxicities in gastrointestinal tract and bone marrow suppression, make it feasible to combine it with radiotherapy, especially when new radiation techniques, such as 3-DCRT, are applied. Local recurrence of rectal cancer is more common in the locally advanced patients, who have received 5-Fu-based chemotherapy in primary treatment, as exhibited in this study. For these patients, chemotherapy using more effective new drugs without cross-resistance is mandatory. In this study, we attempted to adopt 3-DCRT combined with FOLFOX4 chemotherapy for unresectable LRRC. The tumor response rates were similar in the both groups, but the 1- and 2-year overall survival rates and median survival time of the study group were better than those of the control group. Further analysis of the data revealed that the distant metastatic rate and median distant metastatic time of the study group marginally surpassed those of the control group ($P = 0.054$). We postulate that for LRRC patients receiving radiation and the combination of chemotherapy with FOLFOX4 regimen can reduce distant metastatic rate, delay the occurrence of distant metastasis and then influence the overall survival rate, even majority of the patients have received full course 5-Fu-based chemotherapy. In summary, 3-DCRT combined with FOLFOX4 chemotherapy appears to be a feasible and effective treatment for unresectable LRRC. Larger-scale studies are needed to evaluate the potency of this kind of therapeutic strategy.

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RAPID COMMUNICATION

Wnt/β-catenin signaling pathway is active in pancreatic development of rat embryo

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Abstract

AIM: To elucidate the role of Wnt/β-catenin signaling pathway in pancreatic development of rat embryo.

METHODS: The mRNAs of β-catenin, APC, cyclin D1 genes were amplified by means of semiquantitative reverse transcription polymerase chain reaction (RT-PCR) from embryonic pancreas in different periods and normal pancreas of rat, respectively. Protein expression of these genes in embryonic pancreas of E14.5-E18.5 was examined by immunohistochemical method.

RESULTS: In embryonic pancreas of E14.5, the transcript amplification of β-catenin and cyclinD1 genes was detected. In embryonic pancreas of E18.5, the transcription levels of β-catenin and cyclinD1 genes became much higher than in other periods. But in adult rat pancreas the transcription of cyclinD1 gene could not be observed. Only until E18.5, the transcript amplification of mRNA of APC gene could be detected. Surprisingly, the transcription level of APC gene became much higher in adult rat pancreas than in embryonic pancreas. By means of immunohistochemical staining, identical results were obtained to the above by RP-PCR, except for β-catenin protein in adult rat pancreas.

CONCLUSION: Active Wnt/β-catenin signaling occurs in rat embryonic pancreas and is probably important for pancreatic development and organ formation.

Key words: Wnt/β-catenin signaling; Pancreas; Development; Embryo

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INTRODUCTION

The fetal pancreatic development begins at the end of fourth week of gestational period in human being. The pancreatic bud comes from entodermal epithelium in the foregut. In rat embryo the pancreatic bud occurs at 9.5 d after mating (E9.5)[1]. With pancreatic islet arising, the rat embryonic pancreas began to become a special organ including external secretory portion and internal secretory portion. E14.5 - E18.5 is a crucial period for rat pancreatic cell proliferation, differentiation and structure formation[2-3]. Wnt/β-catenin signaling is involved in many developmental processes such as proliferation, differentiation, cell fate decisions, and morphogenesis[4]. However, little is known about Wnt/β-catenin signaling during pancreas development. β-catenin serves not only as a structural component of the E-cadherin-mediated cell-cell adhesion system, but also a signaling molecule of the Wnt/β-catenin pathway. In present study, we tried to investigate the role of Wnt/β-catenin signaling pathway in rat pancreatic development by means of RT-PCR and immunohistochemical method for collecting more data on diagnosis and therapy of pancreatic diseases.

MATERIALS AND METHODS

Experimental animals and specimen preparation

Healthy SD rats (male 10 and female 20), weighing 200-250 g, were purchased from the experimental animal center in Huaxi University of Medical Sciences, Chendu, China. Every two female rats and one male rat was put into one cage after these animals were fed for a week. Assessment of the embryonic age of the fetuses was based on the plug date, defined as embryonic d 0 (E0). The embryos were harvested from female rats with E14.5, E15.5, E16.5, E17.5 and E18.5, respectively. A part of pancreases was rapidly frozen in liquid nitrogen as soon as they were dissected from embryos under microscope.

Reagents and antibodies

Kits used in this study were as follows: Total RNA extraction kit (W6701, Watson, Shanghai), reverse transcription kit (RevertAid HMinus First Strand cDNA Synthesis Kit, Fermentas), and PCR reaction kit (DRR01AM, TaKaRa, Japan).

Table 1 Primers for RT-PCR

Genes	Sequence of primer (5′→3′)	Length of production (bp)	Annealing temperature (Tm)
	F: ACAGCACCTTCAGCACTCT		
β-catenin	R: AAGTTCTTGGCTATTACGACA	168	58.2
	F: CGGAACATGCATGACTGAGAC		
APC	R: GTCACGAGGTACGACCTCAGAT	310	60
	F: CAGAAGTGCGAAGCTTAGGTCT		
cyclin D1	R: GTAGCAGGAGAAGTTGTTGG	470	58
	F: CATGTGCAAGGCCGGCTTCG		
β-actin	R: GTAGCAGGAGAAGTTGTTGG	665	60

The following antibodies were used in this study: Rabbit polyclonal antibody against β-catenin, mouse monoclonal antibody against cyclin D1, rabbit polyclonal antibody against APC (Santa Cruz, USA).

Semiquantitative RT-PCR

Semiquantitative PCR was performed to determine the levels of the mRNA transcripts encoding β-catenin, APC and cyclinD1 genes in embryonic pancreas on E14.5-E18.5. The published sequences of the primers for amplification of β-catenin, APC, cyclinD1 and the housekeeping gene β-actin are showed in Table 1. To determine the optimum number of cycles required for the amplification of these genes or β-actin, an aliquot of first strand cDNA generated from normal rat pancreas was amplified with the respective primers using an increasing number of PCR cycles (20-36). To avoid primer-dependent artifacts, the reaction mixtures were denatured at 95 ℃ for 5 min prior to the addition of the Taq polymerase. The subsequent cycling programs consisted of denaturation at 95 ℃ for 30s, annealing at 60 ℃ (Table 1) (β-actin for 30 seconds) and extension at 72 ℃ for 1 min, followed by a final extension at 72 ℃ for 8 min. A linear relationship between the band intensity of the PCR products and the number of amplification cycles performed was observed. Based upon these observations, the optimum numbers of cycles for the amplification of these genes were 28-30 cycles and of β-actin was 28 cycles. PCR reactions in which the first strand cDNA were omitted served as negative controls and cDNA generated from endometrial samples were used as positive controls for these studies. To avoid technical error, each PCR experiment has been repeated twice. The PCR products were separated on 1.5% agarose gels, stained with ethidium bromide, and photographed using Gel Imaging System (BioRad, USA). The intensity of the bands specific for each target gene or β-actin was quantified using the Phoretix Gel Analysis Software Version 3.01 (NonLinear Dynamics, UK). The relative mRNA levels of these genes for each sample obtained from embryonic pancreas of E14.5-E18.5 were normalized to the corresponding β-actin levels.

Immunohistochemical detection of β-catenin, APC and cyclin D1 proteins

The avidin-biotin peroxidase complex (ABC) technique was used for immunohistochemical staining. Sections were cut at 5μm thickness, deparaffinized in xylene, rehydrated and washed with water. They were treated with 3% hydrogen peroxidase for 20 min to quench endogenous peroxidase and heated in a citrate buffer solution (0.1 mol/L sodium citrate, pH 6.0) at 95℃ for 10 min. After pre-incubation with 10% normal goat serum to block non-specific binding, sections were incubated with the primary antibodies against β-catenin, APC and cyclin D1 at 4℃ overnight. Alternatively, the sections were incubated with biotinylated anti-rabbit or mouse IgG (dilution of 1 : 200) for 40 min at room temperature and with ABC (dilution of 1 : 200) for 30 min at room temperature. Between incubations, sections were washed with 0.1 mol/L PBS (pH7.4). Color was developed with diaminobenzidine tetrahydrochloride supplemented with 0.04% hydrogen peroxidase and counterstained with Mayer's hematoxylin.

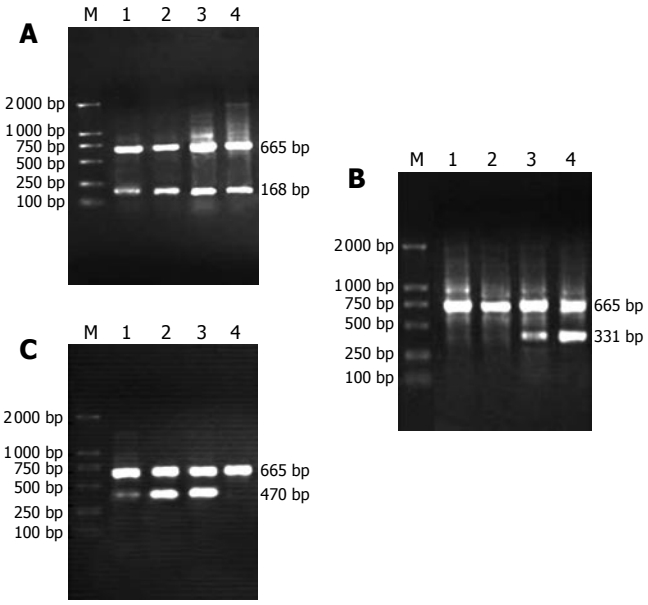

Figure 1 (**A**-**C**): Representative RT-PCR results of β-catenin, APC and cyclin D1 genes in embryonic pancreas of E14.5-E18.5 and adult rat. **A**: RT-PCR results of β-catenin gene (low) and β-actin gene (top, same in other lanes); **B**: RT-PCR results of APC gene (low); **C**: RT-PCR results of cyclinD1 gene (low). M: Molecular -weight marker, lane 1-4: amplification result of RT -PCR for β-catenin, APC and cyclin D1 genes from embryonic pancreas of E14.5, 16.5, 18.5 d and adult rat, respectively.

Statistical analysis

The results of semiquantitative RT-PCR are presented as the ratio of the mean relative absorbance of β-catenin, APC and cyclin D1 to corresponding β-actin respectively for at least three independent experiments of each sample. Statistical differences among different periods of embryo were assessed by *t* test. A $P < 0.05$ was considered significant.

RESULTS

Expression of β-catenin, APC and cyclinD1 mRNA in different pancreas

As shown in Figure 1(A-C), the amplification bands of mRNA of β-catenin and cyclinD1 genes could still be detected in rat embryonic pancreas from E14.5-E18.5. In

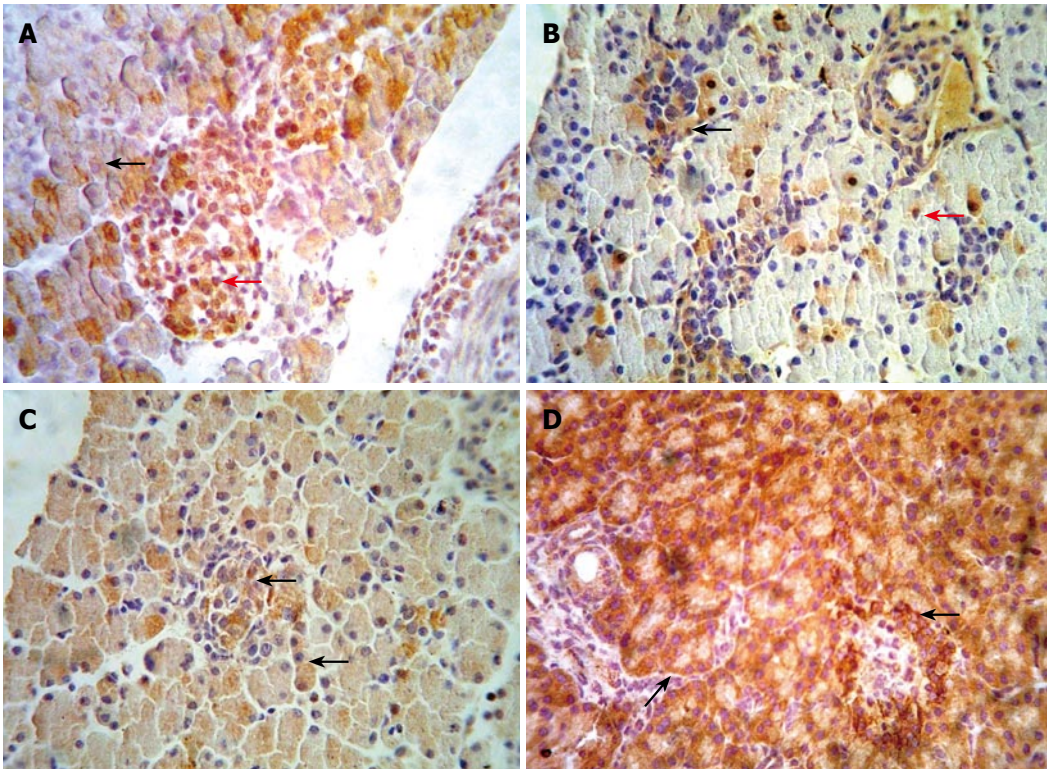

Figure 2 (**A-D**) Representative immunohistochemical results of β-catenin, APC and cyclin D1 proteins in embryonic pancreas of E14.5-E18.5 and adult rat. Immunohistochemical staining was performed using an anti-β-catenin APC and cyclin D1antibodies respectively. Slides were detected at ×400 magnification under a light microscope. **A:** showed staining of β-catenin at cytoplasm (black arrow) and nucleus (red row) in embryonic pancreas of E18.5. **B:** showed staining of cyclin D1 within cytoplasm (black arrow) and/or nucleus (red row) in embryonic pancreas of E16.5. **C:** showed staining of APC within cytoplasm (black arrow) in embryonic pancreas of E18.5. **D:** showed staining of APC within cytoplasm (black arrow) in adult rat pancreas.

adult rat pancreas the transcription of cyclinD1 gene could not be observed while the transcription of β-catenin gene could be found. Only in pancreas of E18.5, the transcript amplification of mRNA of APC gene could be detected. Surprisingly, the transcription level of APC gene was higher in adult rat pancreas than in embryonic pancreas.

Immunohistochemical staining of β-catenin, cyclinD1 and APC

We examined the β-catenin, cyclinD1 and APC protein in embryo from E14.5-E18.5 by immunohistochemical staining. Representative results are shown in Figure 2 (A-D). In the embryo of E14.5, β-catenin staining appeared at the cytoplasm of pancreatic cells. The cells with positive staining showed a diffuse distribution in embryonic pancreas. In the embryo of E18.5, we could observe full pancreas including external and internal secretion portions in morphology. At the same time, our results showed that β-catenin could be detected frequently both in external and internal secretion portions. The positive cells of cyclinD1 with diffuse distribution were observed in the pancreas of E14.5. The positive staining located in the cytoplasm and/or nuclei of pancreatic cells. In pancreas of E18.5, the expression of cyclinD1 protein was significantly higher than that in other period. However, none of positive cells expressing cyclinD1 protein was observed in adult pancreas. Expression of APC has not been observed in the early embryonic pancreas until E18.5. In contrast, the positive cells of APC staining scattered throughout the pancreas in adult rat.

DISCUSSION

At present, morbidity rate of diabetes has an ascending tendency in the world while high morbidity and mortality of pancreatic carcinoma is becoming one formidable topic for surgeon[5]. In order to treat these diseases effectively, fully understanding of the development and differentiation of embryonic pancreas, as well as the underlying molecular mechanism of pancreatic diseases is needed.

Wnt/β-catenin signaling pathway is highly conservative during embryonic development and tumorigenesis of human being and animal[6]. Recent studies have suggested that activation of Wnt/β-catenin signaling pathway may play an important role in hematonosis[7], gastroenteric tumor such as colon cancer[8], and development of embryo[9]. In this study, we have investigated the transcription of gene and expression of protein of β-catenin, APC and cyclin D1 which are three important components in Wnt/β-catenin signaling pathway.

β-catenin serves not only as a structural component of the E-cadherin-mediated cell-cell adhesion system, but also as a signaling molecule of the Wnt/β-catenin signaling pathway. The β-catenin protein is at the core of the canonical Wnt/β-catenin signaling pathway. Wnt stimulation leads to β-catenin accumulation, nuclear translocation and interaction with T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors to regulate genes important for embryonic development and proliferation. In a word, accumulation of ββ-catenin protein in the cytoplasm and/or nuclei of cell are popularly considered a hallmark of activation of the canonical Wnt/β-catenin signaling pathway[7,10]. At this study, we have observed the transcription of β-catenin gene by the RT-PCR and positive stain of β-catenin protein with immunohistochemical techniques in pancreas of E14.5-E18.5. Moreover the positive cells of β-catenin existed in not only the external secretion portion but also internal secretion portion. Therefore we presumed that Wnt/β-catenin signaling pathway may be very important for morphogenesis of pancreas. In pancre-

as of E18.5, the positive stain of β-catenin protein could be found in nucleus especially in pancreatic islet. We have known that pancreatic islet began to mature during late period of embryo[11]. So β-catenin may be essential for proliferation and differentiation of pancreas islet in embryo. Dessimoz *et al*[12] examined the role of β-catenin gene in development of pancreas using an animal model with the conditional gene knockout.They found a reduction in endocrine islet numbers after deleting the β-catenin gene in the epithelium of the pancreas. This result in some degree coincided with our conclusion. On contrast, Murtaugh *et al*[13] confirmed β-catenin is essential only for pancreatic acinar not for islet development. Obviously the development process of pancreas is too complicated to be understood only by Wnt/β-catenin signaling.

In the study, we investigated simultaneously cyclin D1 gene which is one of target genes of Wnt/catenin signaling pathway[14]. The changed tendency of cyclin D1 on the transcription level of mRNA and protein expression is almost in step with β-catenin in pancreas of E14.5-E18.5. It is known that cyclin D1 is a key regulator of the G1 phase of cell cycle[15]. Expressing of cyclin D1 protein in the cytoplasm and/or nuclei indicated that pancreatic cells should be in the condition of proliferation and differentiation. We therefore had come to another conclusion that cyclin D1 exactly is one of activated target genes of Wnt signaling pathway in development of embryonic pancreas.

It has been reported that APC is a negative regulatory factor as well as Axin in Wnt signaling pathway[16]. Experiments in Drosophila ultimately revealed that genetic ablation of APC indeed resulted in upregulation of β-catenin signaling[17]. In pancreas of E14.5-17.5, we could not observe the amplification band of mRNA and protein expressing of APC gene. Only in pancreas of E18.5, could we first find positive stain of APC protein as well as transcription of mRNA of APC gene. In adult rat the positive cells of APC protein spread throughout external secretion portion and internal secretion portion of pancreas. We have known that the proliferation level of pancreas is very low in adult rat particularly in diabetes[18]. This is coincident with the negative role of APC in Wnt/β-catenin signaling pathway.

In this study the cell with positive stain of β-catenin couldn't be found in adult rat pancreas although the transcription of β-catenin gene could be detected by RT-PCR. The reasons are discussed below. First the destruction complex including GSK3、Axin and APC can degradate the free β-catenin in the cytoplasm in time in adult rat pancreas[19]. Secondly the quantity of β-catenin protein which are attached to membrane with E-cadherin may be too little to be detected by immunohistochemical techniques used in present experiments[20].

Although this signaling pathway was active, we had known little about the role of Wnt /β-catenin signaling pathway in development of embryonic pancreas indeed. Which are the upperstream signals[21-22]? Which target genes beside of cyclin D1 are activated? How to crosstalk with other signaling pathway[23]? All these topics need us to investigate further.

Meanwhile, some workers have investigated the Wnt/β-catenin signaling in tumor of pancreas. They suggested the notion that Wnt/β-catenin signaling pathway had been activated in adenocarcinoma, cystocarcinoma and solid tumor of pancreas[24-26]. We will believe that the more we know the Wnt/β-catenin signaling in embryonic pancreas and tumor of pancreas, the better the therapeutic measures for patient with diabetes and tumor of pancreas clinically.

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CASE REPORT

A rare complication of a common disease: Bouveret syndrome, a case report

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Abstract

This is a case report of an 85-year old patient who presented with abdominal pain, nausea and vomiting associated with altered liver function test. The plain X-rays and CT scan showed pneumobilia with an ectopic gallstone. The patient was diagnosed with Bouveret syndrome and managed surgically. The report is followed by a discussion about Bouveret syndrome.

Key words: Bouveret syndrome; Gall bladder; Stone; Fistula; Gastric outlet obstruction

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INTRODUCTION

Gallstone ileus is a rare complication of gallstones but it accounts for almost 25% of non strangulated intestinal obstruction in patients above 65 years of age. Mortality has improved but remains at 15%-18% due to the age and co-morbidity of patients[1]. Bouveret syndrome, first described in 1896, is a subgroup of gallstone ileus in which a cholecystoduodenal fistula allows the passage of a stone which impacts in the duodenum and causes gastric outlet obstruction. It is rare with 175 cases reported in the literature up to the year 2000[2]. We present a case of Bouveret syndrome, with typical radiological findings, that was successfully managed surgically.

CASE REPORT

An 85-year old lady presented with a five-day history of right upper quadrant (RUQ) abdominal pain radiating to her back, associated with nausea and vomiting. On examination she had tenderness, guarding and rebound in the RUQ and epigastrium. The leukocyte count was 20.6 (predominantly neutrophils), the amylase 1039 and urea and creatinine were elevated. Plain abdominal X-ray showed a radio-opaque shadow in the right side of the abdomen, air in the gall bladder and biliary tree but no evidence of small bowel obstruction (Figure 1). She was assumed to have pancreatitis and a suspected cholecystoenteric fistula.

CT scan with oral contrast revealed a large amount of air in the biliary tree, a fistula between the second part of duodenum and the gall bladder and a large stone in the third part of duodenum. A small amount of contrast outlined the stone and reached the small bowel (Figure 2). This impacted stone (7.5 cm × 4 cm × 4 cm) was removed surgically from the junction of the second and third part of duodenum through a small jejunostomy after mobilization of the duodenum and the DJ flexure. Her post operative recovery was uneventful.

DISCUSSION

Cholecystoenteric fistulae occur in less than 1% of patients with gallstones. Most (60%) are cholecystoduodenal fistulae, but cholecystocolic, cholecystogastric and choledochoduodenal fistulae have been described. Large stones passing through the fistula may cause intestinal obstruction, especially in the terminal ileum[1,3]. A plain abdominal X-ray is diagnostic in about 50% of cases[3] and may demonstrate intestinal obstruction, pneumobilia, an ectopic gallstone, alteration in the position of the previously observed stone or two air fluid levels in the right upper quadrant secondary to air in the gall bladder.

Patients with Bouveret syndrome usually present with symptoms of gastric outlet obstruction, though presenting with other complications of gall stone disease or upper gastrointestinal bleeding has been reported[4]. A plain abdominal X-ray may show a dilated stomach, and CT may demonstrate pneumobilia, intestinal obstruction and an ectopic gallstone[5]. Open surgery, endoscopic removal[6] and laparoscopic or laparoscopic assisted enterolithotomy[7] have all been used successfully for stone removal. A stone in the duodenum can be difficult to access laparoscopically but may be reached endoscopically[6].

Whether the gallbladder should be disturbed is controversial but the recurrence of gallstone ileus following enterolithotomy is rare, and complications related to the persistence of a cholecystoenteric fistula are unusual[3,8].

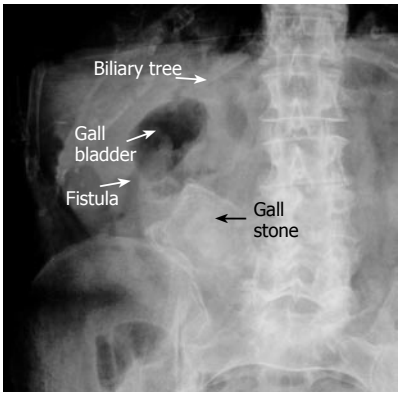

Figure 1 Plain X-ray showing air in the biliary tree and an ectopic bizarre-shaped gall stone suggesting the diagnosis of cholecystoenteric fistula.

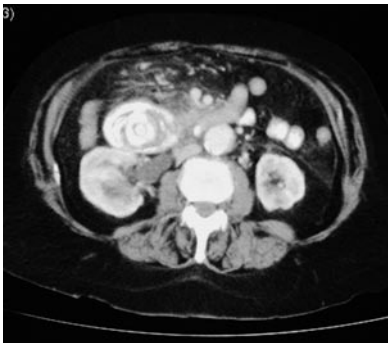

Figure 2 CT abdomen with contrast showing the gall stone in the duodenum with some contrast passing distal to the stone.

Bouveret syndrome is a rare complication of cholelithiasis with a relatively high mortality. In the patient has a pre-operative diagnosis of Bouveret syndrome, endoscopic disimpaction should be attempted. Failing this laparotomy is likely to be required but laparoscopy may allow identification of the stone's position minimizing subsequent skin incision.

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CASE REPORT

Intestinal Behcet's disease with esophageal ulcers and colonic longitudinal ulcers

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Abstract

Intestinal Behcet's disease in a 38-year-old woman was diagnosed because of the history of recurrent oral aphthous ulcers, erythema nodosum-like eruptions, genital ulcer, and endoscopic findings of esophageal and ileocolonic punched-out ulcers with colonic longitudinal ulcers. Esophageal lesions and colonic longitudinal ulcers are rarely seen in intestinal Behcet's disease. The ulcers of esophagus and ileocolon healed with 3 wk of treatment with prednisolone and mesalazine without any adverse effect. Mesalazine may decrease the total dose of prednisolone required to treat the disease.

Key words: Colonic longitudinal ulcer; Esophageal ulcer; Intestinal Behcet's disease; Mmesalazine

Fujiwara S, Shimizu I, Ishikawa M, Uehara K, Yamamoto H, Okazaki M, Horie T, Iuchi A, Ito S. Intestinal Behcet's disease with esophageal ulcers and colonic longitudinal ulcers. *World J Gastroenterol* 2006; 12(16): 2622-2624

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INTRODUCTION

In Japan, Behcet's disease, when accompanied by intestinal involvement, is called intestinal Behcet's disease, which primarily affects the terminal ileum, caecum, or ascending colon[1]. However, esophageal aphthous ulcerations and colonic longitudinal ulcers are rare in intestinal Behcet's disease. Intestinal lesions in Crohn's disease tend to be longitudinal ulcers with a cobblestone appearance, while those in Behcet's disease are round and oval "punched-out" ulcers. Moreover, epithelioid granuloma is one of the pathological characteristics of Crohn's disease, whereas it is uncommon in intestinal Behcet's disease. Another feature of Behcet's colitis is lymphocyte venulitis, which is a disorder of vasculitis. Despite these differences, it can be difficult on occasions to make a differential diagnosis between these two diseases. We present a case of intestinal Behcet's disease with esophageal and colonic longitudinal ulcers.

CASE REPORT

The patient was a 38-year-old woman who was admitted to our department in December 2003 for epigastralgia and melena that occurred after transient fever. For the previous two days, she was in good health, except for numerous recurrences of aphthous stomatitis. Physical examinations on admission revealed the presence of multiple oral aphthous ulcers, in addition to slight tenderness in the epigastrium. Ophthalmologic and neurologic examinations showed no remarkable findings despite the presence of erythema nodosum-like eruptions in the bilateral inferior limbs and genital ulcers. Negative results were obtained by a prick test of the skin. Clinical laboratory tests showed the following: white blood cells, 7 830/μL; red blood cells, 360×10^4/μL; hemoglobin, 9.6 g/dL; hematocrit, 29.7%; platelets, 61×10^4/μL; C-reactive protein, 10.9 mg/dL; total protein, 8.6 g/dL; AST, 36 IU/L; ALT, 64 IU/L; LDH, 171 IU/L; and total bilirubin, 0.2 mg/dL. Human lymphocyte antigens were negative for B51, but positive for B52. All bacteriologic examinations of blood, urine, and cultured stool showed negative results. Upper gastrointestinal endoscopy demonstrated small oval and discrete ulcers with reddish margin scattered between the middle and lower parts of the esophagus (Figure 1A). Colonoscopy revealed multiple aphthas and erosions scattered in the terminal ileum (Figure 1B) as well as small aphthas on the ileocecal valve (data not shown). However, punched-out ulcers were not observed on ileocecal lesions. Multiple erosions and ulcers including longitudinal irregular-outlined ulcers were extant in the ascending and transverse colon (Figure 1C). Pathological examination of the endoscopic biopsy specimen showed nonspecific ulceration and no evidence of Crohn's disease could be found.

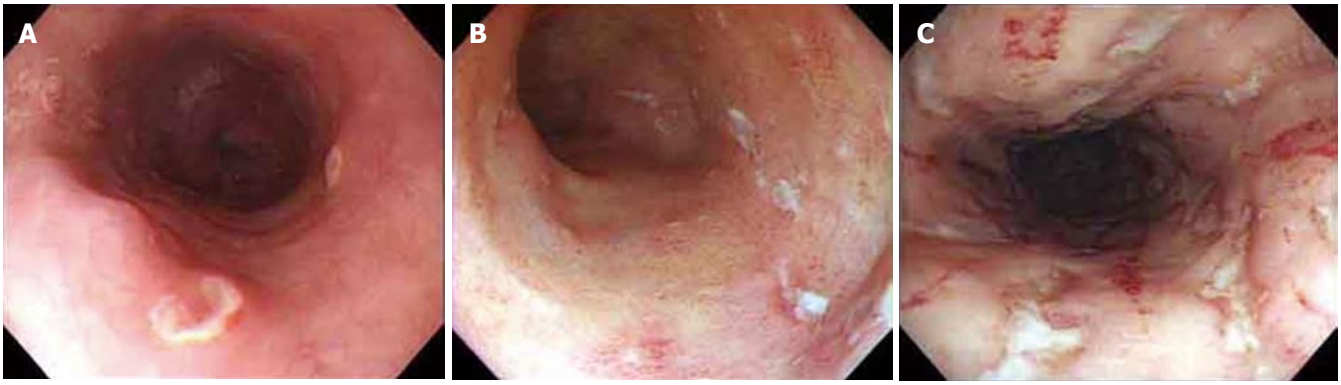

Figure 1 Endoscopic view of discrete ulcers with reddish margin in the middle part of the esophagus (**A**), multiple aphthas and erosions scattered in the terminal ileum (**B**), and multiple erosions and ulcers including longitudinal irregular-outlined ulcers extant in the transverse colon (**C**).

Table 1 Reported cases of Behcet's disease with colonic longitudinal ulcers

n	Age	Gender	Symptoms				Type	Colonic longitudinal ulcer		Other ulcers	Treatment	Outcome
			Oral	Eye	Skin	Genital		Location	Histology			
1	29	Female	+	+	+	+	Complete	Transverse Descending	Vasculitis		Operation Steroid	Healing
2	70	Female	+		+	+	Incomplete	Ascending Transverse Descending	Nonspecific colitis	Ileum	Colchicine Salazopyrin	Died
3	37	Female	+		+	+	Incomplete	Ascending Transverse	Granuloma	Ileum	Steroid 5-Aminosalicylate	Healing
4[1]	39	Female	+		+	+	Incomplete	Ascending Transverse	Nonspecific colitis	Ileum	Healing Mesalazine	Healing

[1]Case reported here.

In this patient, ulcers were detected in the esophagus, terminal ileum, and ascending and transverse colon, in addition to erythema nodosum-like eruptions in the bilateral inferior limbs and genital ulcers. Based on these findings and the history of recurrent oral aphthous ulcers, the patient was diagnosed as having incomplete type intestinal Behcet's disease.[1] The treatment for this patient was initiated by oral administration of prednisolone (50 g) and mesalazine (1 500 mg daily). Consequently, her symptoms disappeared rapidly, and the results of all clinical laboratory tests were normalized. Upper gastrointestinal endoscopy and colonoscopy performed 3 wk after admission revealed the disappearance of ulcers. Mesalazine was maintained at the same dose, but the dose of prednisolone was gradually decreased to 2.5 mg daily, which was maintained thereafter. However, remission was not obtained until 8 mo.

DISCUSSION

Behcet's disease is characterized by repeated eye, skin and visible mucosal lesions. The prevalence of this disease differs widely among races; the rate is 0.3/100 000 in the United States, but 1/10 000 in Japan[2]. Abdominal complaints also differ among races. Shimizu *et al* reported abdominal pain in 75% of patients with Behcet's disease[3], as noted in the present patient showing epigastralgia.

In Japan, diagnostic criteria for Behcet's disease have been established by the Behcet's Disease Research Committee[1]. Based on these criteria, a diagnosis was made in the present case of incomplete type intestinal Behcet' s disease manifests mainly in the terminal ileum, cecum, and ascending colon, although esophageal lesions are rare and colonic longitudinal ulcers are very rare rather than esophageal lesions. To the best of our knowledge, there are only three existing reports describing intestinal Behcet's disease with longitudinal ulcers (Table 1)[4-6]. Although Lee presumed the cause of the longitudinal ulcers to be multifocal vasculitis, in our case vasculitis was not significant. However, we cannot conclude that the patient had Crohn's disease, because microscopic characteristics of Crohn's disease - that is, chronic inflammation involving all layers of intestinal wall or granulomas - were absent.

In addition, only nine cases including our case of intestinal Behcet's disease with both esophageal and ileocolonic ulcers were reported in English literature[7-13]. One case was of the complete type; 6, the incomplete type; and 2, the suspected type. Three patients had strictures; and 2 had perforation. The treatment of intestinal Behcet's disease is controversial. Surgical treatment was not effective in 1 of the 9 patients (Table 2). However, the esophageal ulcers healed in 6 of 7 patients with medical treatment, including corticosteroids or acid suppressive drugs. Corticosteroids, the major therapeutic agent in this disease, were effective in 3 of the 4 patients. However, they can have serious adverse effects, and their use may be

Table 2 Reported cases of Behcet's disease with esophageal and ileocolonic ulcers

n	Age	Gender	Symptoms				Type	Esophageal ulcer		Other ulcers	Treatment	Outcome
			Oral	Eye	Skin	Genital		Number	Complication			
1	52	Female	+	+	+		Incomplete	1	Perforation	Stomach, Duodenum Jejunum, Ileum	?	?
2	21	Female	+			+	Suspected	3		Colon	Antacid Transfer factor	Healing
3	16	Female	+	+		+	Incomplete	1	Perforation	Colon	Steroid	Healing
4	12	Female	+	+	+	+	Complete	1	Stenosis	Ileum	Steroid Operaton	No healing
5	50	Male	+		+	+	Incomplete	1	Stenosis	Ileum, Colon	?	?
6	52	Male	+	+		+	Incomplete	Diffuse	Stenosis	Ileum	Healing	Healing
7	19	Female	+				Suspected	A few		Ileum	Healing Mesalazine	Healing
8	33	Male	+			+	Incomplete	1		Ileum	Healing	Healing
9[1]	39	Female	+		+	+	Incomplete	8		Ileum, Colon	Healing Mesalazine	Healing

[1]Case reported here.

associated with colonic perforation.

Sonta *et al* have suggested that mesalazine is effective for treatment of intestinal Behcet's disease[13]. It may decrease the total dose of a corticosteroid required to treat the disease. The esophageal and ileocolonic ulcers in the present case healed 3 wk of treatment with prednisolone and mesalazine without any adverse effect. However, it was reported that the recurrence rate even with medical treatment was 90% in patients with Behcet's disease[14]. The present case has not relapsed for 8 mo with treatment of 2.5 mg prednisolone and 1 500 mg mesalazine. Thus, the post-treatment course of this patient should be followed up carefully.

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CASE REPORT

Remission of primary low-grade gastric lymphomas of the mucosa-associated lymphoid tissue type in immunocompromised pediatric patients

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Abstract

We report the remission of primary gastric lymphoma of the mucosa-associated lymphoid tissue (MALT) type in two immunocompromised pediatric patients. Patient 1, a 14-year-old boy in an immunocompromised state of unknown cause, complained of repeated abdominal pain. Examinations revealed gastric MALT with local invasion and lymph node involvement. Serum anti-*Helicobacter pylori* (*H pylori*) antibody was positive. *H pylori* eradication was abandoned due to its adverse effects. The MALT lesion spontaneously regressed over the next 24 months without any treatment for lymphoma. Patient 2, a 6-year-old boy, underwent cord blood transplantation for the treatment of adrenoleukodystrophy. He was administered immunosuppressants for graft-versus-host disease after transplantation. Nausea and hematochezia appeared and further examinations revealed gastric MALT with *H pylori* gastritis. Treatment consisting of medication for the *H pylori* infection alone eradicated the *H pylori* and completely resolved the patient's MALT lesion, as well. Patients 1 and 2 were followed up over periods of 10 years and 3 years, respectively, without any signs of relapse. In conclusion, gastric lymphoma of the MALT type can be cured by conservative treatment even in immunocompromised pediatric patients.

Key words: Pediatric gastric lymphoma; Mucosa-associated lymphoid tissue; Immunocompromised states

Ohno Y, Kosaka T, Muraoka I, Kanematsu T, Tsuru A, Kinoshita E, Moriuchi H. Remission of primary low-grade gastric lymphomas of the mucosa-associated lymphoid tissue type in immunocompromised pediatric patients. *World J Gastroenterol* 2006; 12(16): 2625-2628

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INTRODUCTION

The stomach is one of the most common sites of extranodal malignant lymphomas. Since the first report by Isaacson and Wright in 1983, the concept of low-grade gastric B-cell lymphomas of the mucosa-associated lymphoid tissue (MALT) type has become widely accepted[1]. Studies from the 1990s have established that the development of low-grade gastric lymphoma of the MALT type is strongly associated with chronic gastritis caused by Helicobacter pylori[2-4]. Low-grade gastric lymphoma of the MALT type occurs commonly in middle and old age but rarely in children[5,6]. The first long-term follow-up of a pediatric patient with *H pylori*-associated gastric lymphoma of the MALT type was reported in 1995[7]. The present report describes the development of primary gastric lymphomas of the MALT type in two pediatric patients in immunocompromised states followed by tumor regression with conservative treatment.

CASE REPORT

Patient 1

A 14-year-old boy complained of repeated abdominal pain. Upper gastrointestinal endoscopy revealed a tumor-like lesion in the stomach and the patient was referred to our hospital. The patient had a history of autoimmune hemolytic anemia at the age of 1 year and measles infection at 2 years. Thereafter, he developed systemic lymphadenopathy and hyper-gamma-globulinemia. He had been administered oral prednine from the age of 5 years onward based on a putative diagnosis of immunoblastic lymphadenopathy.

A tender mass, hen-egg in size, was discovered in the epigastrium upon admission. Endoscopy revealed a polypoid lesion in the posterior wall of the lower corpus of the stomach. The mass was diagnosed as a low-grade gastric lymphoma of the MALT type based on examination of a biopsy specimen (Figure 1). The

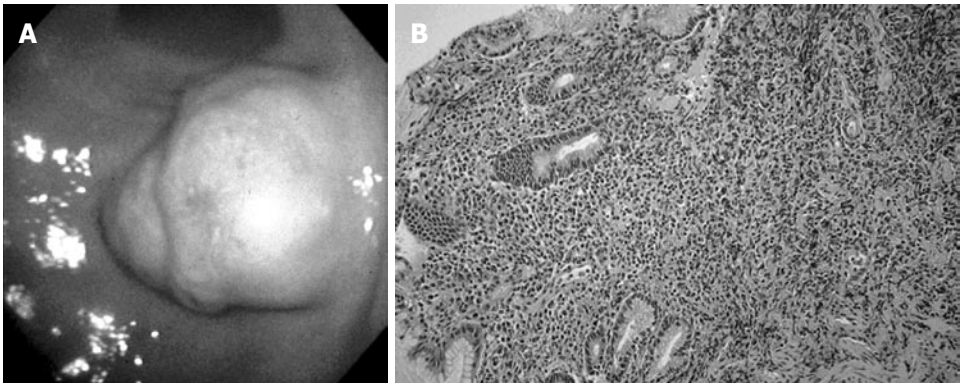

Figure 1 Mucosa-associated lymphoid tissue lymphoma in Patient 1. **A:** A polypoid lesion on the posterior wall of the lower corpus of the stomach, suggesting the existence of lymphoproliferative disease; **B:** The histological features show characteristic appearance of low-grade gastric lymphomas of the mucosa-associated lymphoid tissue type with a diffuse infiltrate of centrocyte-like cells and the formation of lymphoepithelial lesions (H & E, original magnification x10).

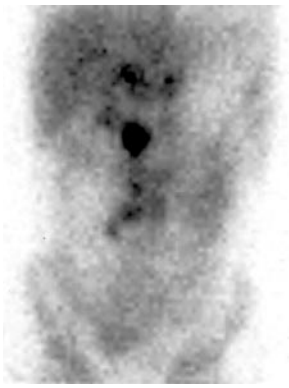

Figure 2 Gallium scintigraphy in Patient 1 shows an abnormal accumulation of the nuclide in the para-aortic lymph nodes.

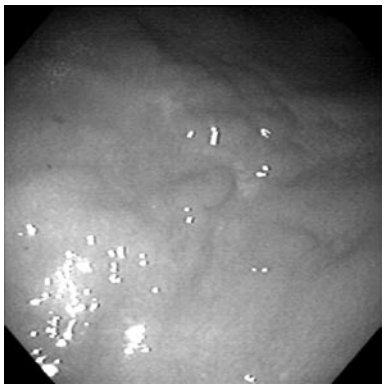

Figure 3 Twenty-four months later, an area of partly whitish rugged mucosa appeared at the site previously occupied by the mucosa-associated lymphoid tissue lymphoma in Patient 1.

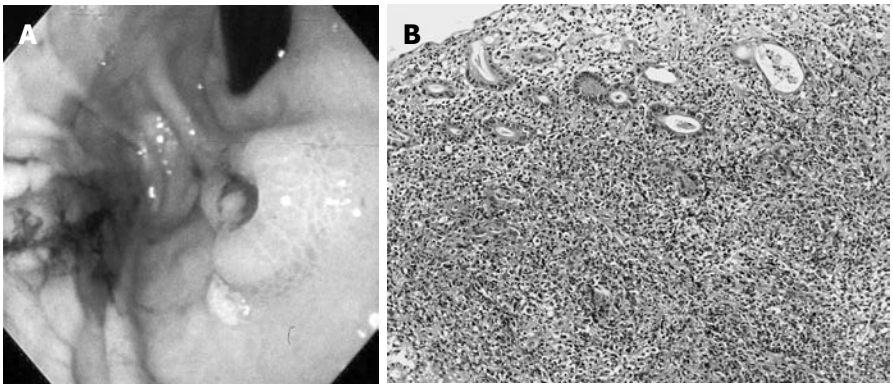

Figure 4 Mucosa-associated lymphoid tissue lymphoma in Patient 2. **A:** An ulcerated lesion on the anterior wall of the upper corpus before eradication; **B:** The histological features show the appearance of low-grade gastric lymphomas of the mucosa-associated lymphoid tissue type similar to that found in Patient 1 (H & E, original magnification x10).

biopsy also confirmed an invasion to the duodenum. Both computed tomography and Gallium scintigraphy strongly suggested metastases to the para-aortic lymph nodes (Figure 2).

Fearing that a complete, en bloc removal of such an advanced tumor would be too invasive in an immunocompromised patient, the authors opted for a watch-and-wait strategy. The patient's serum was positive for anti-*H pylori* antibody, but *H pylori* eradication had to be abandoned due to its adverse effects. Thankfully, the MALT lesion spontaneously regressed over the next 24 mo without any treatment for lymphoma (Figure 3). As the MALT lesion grew less serious, an abdominal pain gradually disappeared. The patient has been followed up for 10 years and continues to do well with no signs of relapse as of this writing.

Patient 2

A 6-year-old boy was referred to our department with a complaint of nausea with hematochezia. He had been diagnosed with adrenoleukodystrophy at the age of 5 years and underwent cord blood transplantation for the treatment of the condition at that time. After the transplantation he was administered oral immunosuppressants, including methylprednisolone and FK506, for graft-versus-host disease.

Upper gastrointestinal endoscopy revealed an ulcerated lesion in the anterior wall of the upper corpus of the stomach. The mass was diagnosed as a low-grade gastric lymphoma of the MALT type associated with *H pylori* gastritis, based on an examination of a biopsy specimen (Figure 4). Neither local invasion nor lymph node involvement was present on diagnostic modalities. When the dosages of the immunosuppressants were tapered he was additionally treated for the *H pylori* infection using a proton pump inhibitor combined with clarithromycin and amoxicillin according to international guidelines[8]. The treatment not only eradicated the *H pylori*, but brought

about a complete remission of the MALT lesion as well (Figure 5). The patient has been followed up for 3 years and continues to do well without any signs of relapse as of this writing.

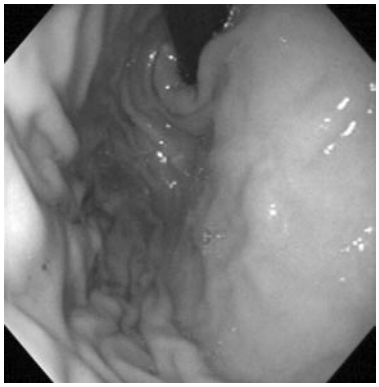

Figure 5 Two months after eradication therapy in Patient 2, an area of glossy whitish mucosa appeared at the site previously occupied by the low-grade gastric lymphoma of the mucosa-associated lymphoid tissue type.

DISCUSSION

Primary low-grade gastric B-cell lymphoma of the MALT type is generally defined as an extranodal lymphoma characterized by an infiltration of the mucosa by centrocyte-like B-cells with formation of lymphoepithelial lesions[9]. Gastric lymphoma of the MALT type is thought to be derived from chronic gastritis caused by *H pylori*[2-4]. *H pylori* infection has been documented in up to 90% of patients with low-grade gastric lymphoma of the MALT type. At first, it has been believed that the MALT acquired in response to *H pylori* infection provides the background on which unidentified factors act and then lead to the development of lymphoma. However, the immune response of B-cell lymphomas of MALT type to *H pylori* antigen is proved to occur only with the presence of T cells on the culture[10,11]. Furthermore, the cellular proliferation of low-grade gastric B-cell lymphomas of MALT type to *H pylori* seems to be dependent on *H pylori*-specific T cells and their products, rather than the bacteria themselves[11]. The inhibitive action against such a mechanism would break down in immunocompromized state so that B-cell lymphomas of MALT type may develop even in *H pylori* infected children.

H pylori eradication is now established to be effective in reducing primary gastric lymphomas of the MALT type and is well accepted as an initial therapy for patients with localized low-grade gastric lymphomas. Remission rates in the recent literature range from 60% to 80%, though recurrence can be expected in some 5% of cases[12-15]. As described above, authors addressed the contributions of the persistent exposure to *H pylori* antigen and the presence of *H pylori*-specific T cells in the pathogenesis of lymphomas of MALT type. With the eradication of *H pylori*, chronic inflammation decreases and the density of submucosal lymphocytes dramatically declines[7]. Thus, the cessation of exposure to *H pylori* antigen due to either the spontaneous cure of *H pylori* or the result of the eradication might progress the tumor regression through the immunologic response.

The major negative predictive factor of tumor response to anti-*H pylori* treatment in patients with primary low-grade gastric lymphoma of the MALT type is involvement of the regional lymph nodes. The depth of the tumor infiltration in the gastric wall correlates with the involvement of the regional lymph nodes and with the degree of tumor malignancy[16-18].

Primary gastric lymphoma of the MALT type has been documented in liver, heart, and kidney transplant recipients[19-22]. The development of these post-transplantation lymphomas of the MALT type appears to be closely related to the abnormal response of the dysregulated host immune system. Post-transplantation lymphoma of the MALT type is also associated with *H pylori* infection and is usually of an Epstein-Barr Virus-negative, B-cell origin. A combination of treatments to minimize immunosuppression and eradicate *H pylori* frees most patients from disease[21-23].

Primary gastric malignant tumors are very rare in children, as are most lymphomas. The majority of primary gastric lymphomas are high-grade non-Hodgkin's lymphomas of B-cell origin. To our knowledge, the literature has not reported significant numbers of primary gastric lymphomas of the MALT type in children[7,24-26]. The clinicopathological features in pediatric cases are considered to be similar to those observed in adults.

In this report we have described the complete remission of primary gastric lymphomas of the MALT type in two pediatric patients under an immunocompromised state. In patient 1, an advanced gastric lymphoma of the MALT type with duodenal invasion and lymph node involvement regressed spontaneously without any treatment. This experience prompts us to question whether regional lymph node involvement is a negative predictive factor of the tumor response, as described above. Anti-*H pylori* treatment with minimization of immunosuppression was satisfactory in our second patient. To the best of our knowledge, patient 2 is the first reported case of primary gastric lymphoma of the MALT type occurring in a post-cord blood transplantation setting.

In conclusion, the authors propose that low-grade gastric lymphoma of the MALT type can be cured by conservative treatment even in immunocompromised pediatric patients. A watch-and-wait strategy combined with *H pylori* eradication and close follow-up should be an option before attempting more aggressive treatments, even in cases with advanced disease.

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CASE REPORT

A case of idiopathic colonic varices: A rare cause of hematochezia misconceived as tumor

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Abstract

Colonic varices are a very rare cause of lower gastrointestinal bleeding. Fewer than 100 cases of colonic varices, and 30 cases of idiopathic colonic varices (ICV) have been reported in the English literature. Among these 30 cases of ICV, 19 cases were diagnosed by angiography, and 7 operated cases were diagnosed later as ileocecal vein deficit, hemangioma, and idiopathic in 1, 1, 5 cases, respectively. We report the case of a 24-year-old man who suffered from multiple episodes of hematochezia of varying degree at the age of 11 years. He had severe anemia with hemoglobin of 21 g/L. On colonoscopy, tortuously dilated submucosal vein and friable ulceration covered with dark necrotic tissues especially at the rectosigmoid region were seen from the rectum up to the distal descending colon. It initially appeared to be carcinoma with varices. Mesenteric angiographic study suggested a colonic hemangioma. Low anterior resection was done due to medically intractable and recurrent hematochezia. Other bowel and mesenteric vascular structures appeared normal. Microscopic examination revealed normal colonic mucosa with dilated veins throughout the submucosa and serosa without representing new vessel growth. Taken all of these findings together, the patient was diagnosed as ICV. His postoperative course was uneventful.

Key words: Idiopathic colonic varices; Hematochezia; Colon cancer; Hemangioma

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INTRODUCTION

Colonic varices, a very rare cause of lower gastrointestinal bleeding, are usually secondary to portal hypertension. It was reported that the incidence of colonic varices is 0.07% (2 of 2912 cases)[1]. One case had liver failure, and the other case had chronic pancreatitis. There are no autopsy data about idiopathic colonic varices. Fewer than 100 cases of colonic varices have been reported[1], and 23 reports containing 30 patients with idiopathic colonic varices (ICV) have been reported in the English literature[2-23]. Diagnosis of idiopathic colonic varices is most accurately achieved by mesenteric angiography[3-19]. But in some cases, operation could reveal secondary causes or changed pathologic diagnosis[18,19,24]. We report a patient whose diagnosis of idiopathic colonic varices was confirmed by the segmental colonic resection.

CASE REPORT

A 24-year-old man was admitted to hospital with exertional dyspnea and weakness. He had a 13-year history of multiple episodes of hematochezia of varying degree. At age 22, he visited our hospital due to dizziness. He denied alcohol consumption, or any family history of gastrointestinal bleeding. He had severe anemia with hemoglobin of 27 g/L. After 2-unit blood transfusion, he refused further investigations or management.

On admission, he was pale with mild tachycardia but had no abnormality on physical examination. Laboratory studies showed 4.93×10^9/WBC, 21 g/L hemoglobin, 0.09% hematocrit, 256×10^9/L platelets, and 22.2 μmol/L iron. Coagulation studies, liver function tests, and viral hepatitis serology were normal. Colonoscopy revealed considerable bluish and tortuously dilated submucosal veins which were seen from the rectum up to the distal descending colon, and friable ulceration covered with dark necrotic tissues

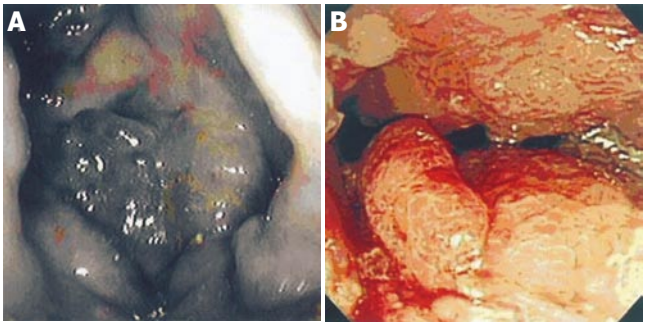

Figure 1 Colonoscopy view of colonic varices at the rectosigmoid region.

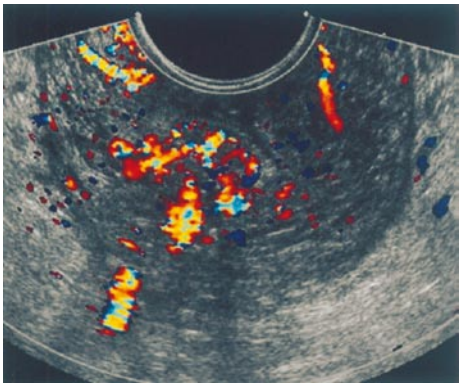

Figure 3 Trans-rectal Doppler sonography of colonic varices.

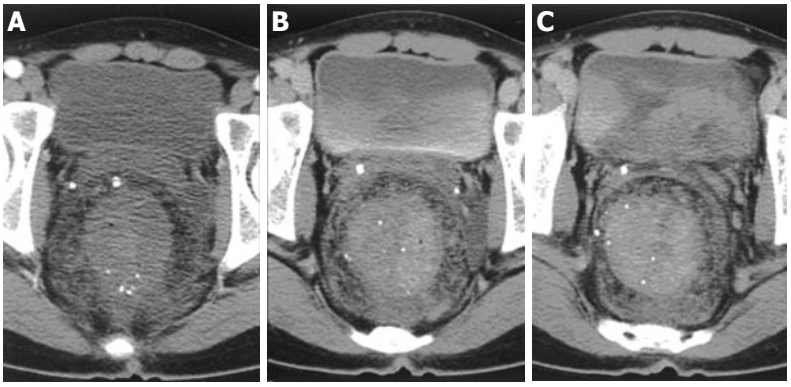

Figure 2 Abdomen-pelvis triphasic computed tomography showing an arterial phase image of mucosal enhacement (**A**) venous (**B**) and delayed(**C**) phase images of delayed diffuse wall enhancement.

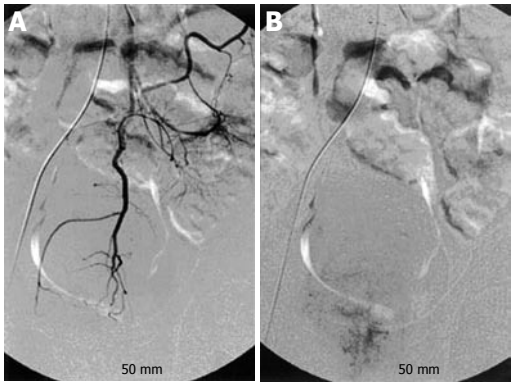

Figure 4 Selective angiography of superior and inferior mesenteric artery showing normal arterial phase (**A**) and venous pooling in rectum in the delayed phase (**B**).

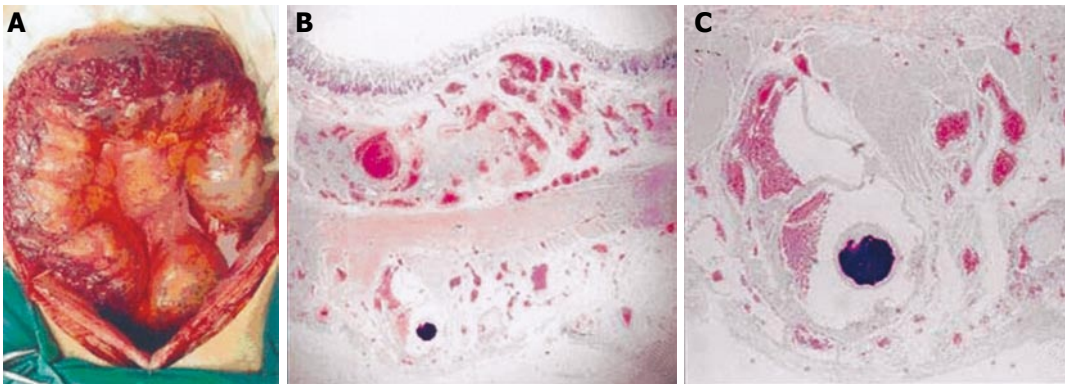

Figure 5 Dilated subserosal veins seen in operation (**A**), great enlarged vessels observed in submucosal and serosal layer at low power, HE×10 (**B**), and distortion of vascular wall found in enlarged vessels at high power, HE×40 (**C**).

especially at the rectosigmoid region (Figure 1). It seemed to be carcinoma with varices. Gastroduodenoscopic examination showed unremarkable finding, without evidence of varices or arteriovenous malformations. Abdominal ultrasonography also revealed no abnormal finding. An abdomen-pelvis triphasic computed tomography showed thickened bowel wall, intramural calcification and delayed enhancement from the rectum up to the descending colon (Figure 2). Transrectal Doppler sonography also demonstrated diffuse wall thickening with internal hypervascular structure (Figure 3). Selective angiography of the superior and inferior mesenteric artery showed delayed venous pooling in rectum but no other abnormal vascular structure (Figure 4). The angiographic diagnosis was a colonic hemangioma.

Low anterior resection was done due to medically intractable and recurrent hematochezia. During surgery, tortuously dilated subserosal and mesenteric vessels were seen at the rectum, sigmoid colon and distal descending colon (Figure 5A). Other bowel and mesenteric vascular structures appeared to be normal. Microscopic examination revealed normal colonic mucosa with dilated veins throughout the submucosa and serosa without new vessel formation (Figures 5B and 5C). In addition, thrombi were frequently observed in the endovascular lumen, and dystrophic calcification was also often found in the wall of vessels. He remained well showing no further signs of gastrointestinal hemorrhage at the time when he was reported in this paper.

DISCUSSION

Colonic varices are a very rare cause of lower gastrointestinal bleeding. Portal hypertension is the most common cause of colonic varices, which are usually located in the rectosigmoid region and the cecum[16]. Other less common causes of colonic varices are congestive heart failure, mesenteric vein thrombosis, pancreatitis with splenic vein thrombosis and adhesions, and mesenteric vein compression[1, 2].

The varix is a descriptive term of enlarged and convoluted vein, artery or lymphatic vessel, which does not indicate the etiology of them. Establishing the diagnosis of ICV amid various factors remains difficult. It needs to exclude secondary causes by laboratory and imaging studies, such as liver function test, hepatitis serology, and liver sonography or computed tomography. Diagnosis of ICV usually requires angiography with visualization of the enlarged vessels showing prolonged observation of the venous phase but no portal/mesenteric vein obstruction[3, 5]. Gross examination and biopsy under laparoscope or exploration can definitely confirm ICV.

We fully reviewed 30 cases reported as ICV. Hemangioma and vascular malformation are described as ICV in some of these reports[2,3,18,19]. But hemangioma has obviously different pathologic findings, and vascular malformation has plainable etiology. So, in our opinion, hemangioma and vascular malformation can be ruled out from ICV. According to these, 21 reports containing 25 patients remain to be idiopathic[4-17,20-23]. In more than half of the cases, the total colon is affected[6-9,14-16,20-22], and in segment-involved cases, the lesions are distributed equally between right and left colon. About 30% of these have familial tendency[8-11,14-16,20,21] and more than half of these manifest the onset of hematochezia before the third decade[9-13,15-17,22], suggesting that ICV may be congenital in origin. But the number of the cases is too small to conjecture the inheritance pattern of ICV. Angiography has been performed in only 19 cases[3-19]. Before angiography, two of these cases, suspected to be ICV, were diagnosed as congenital anomaly of the portocaval system[2] and hemangioma[3]. 7 cases which were surgically confirmed, were diagnosed as congenital failure of ileocecal vein[18], hemangioma[19], and ICV[15-17,19,20] in 1, 1, 5 cases, respectively.

ICV is usually accompanied with recurrent and massive rectal bleeding. It is presumed that bleeding from colonic varices is the result either of abrasion from hard stool in the distal colon, or of pressure ischemia and sloughing of the overlying mucosa in the cecum[16].

The barium contrast enema may be helpful, but is often misinterpreted as air bubble, fecal material, polyposis, carcinoma, or as normal[12]. Therefore, it is unreliable. Colonoscopy is more sensitive, but collapse of varices by excessive air inflation can make the physician to mistake ICV as normal condition. ICV can mimic polyp, cancer, and ulcerative colitis. Biopsy may induce massive hemorrhage[15].

Scintigraphic studies have not been found to be adequate in localizing the segment of colon associated with bleeding varices but can detect hemorrhage with greater sensitivity than angiography[25].

Mesenteric angiography is a most accurate radiologic diagnostic tool, but secondary colonic varices could be misinterpreted as an ICV on angiography. Defreyne *et al*[24] have explained that angiographical diagnosis is in disagreement with the histopathological diagnosis of an arteriovenous malformation probably because the blood passage through the small fistulas is too slow. The same principle can be applied to our case. In about 30% cases, operation revealed secondary causes or changed histopathologic diagnosis as previously described[15-21], suggesting that angiography is an incomplete tool to confirm ICV. Laparoscopy may be a more accurate and definitive diagnostic tool than histology, because histological tissue sampling is very dangerous if not in operation.

The prognosis of ICV seems to be good at all ages compared with cirrhotic varices, which may be related to low pressure in the varices as well as the absence of significant hepatocellular disease[5]. Conservative therapy is sufficient for most ICV patients. Patients with intractable and persistent bleeding may require partial colectomy for involved area.

In conclusion, it is very difficult to diagnose ICV as in our case. The most important thing is to role out secondary causes of varices. In the suspected case, the physician should consider the clinical, radiologic, and surgical information collectively for the proper diagnosis. This case report describes a life-threatening bleeding episode of ICV, mimicking carcinoma in colonoscopy and hemangioma in angiography. ICV can be confirmed by surgical histopathogy, and successfully treated with partial colectomy on involved area.

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CASE REPORT

A rare case: Spontaneous cutaneous fistula of infected splenic hydatid cyst

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Abstract

Hydatid disease is caused by the larval stage of the genus *Echinococcus*. Live hydatid cysts can rupture into physiologic channels, free body cavities or adjacent organs. Although hydatid disease can develop anywhere in the human body, the liver is the most frequently involved organ, followed by the lungs. Cysts of the spleen are unusual. There are only five case reports of spontaneous cutaneous fistulization of liver hydatid cysts in the literature. But there isn't any report about cutaneous fistula caused by splenic hydatid cyst. We report a first case of spontaneous cutaneous fistula of infected splenic hydatid cyst.

A 43-year-old man was admitted to our Emergency Service with abdominal pain and fluid drainage from the abdominal wall. He has been suffering from a reddish swelling on the abdominal wall skin for four months. After a white membrane had been protruded out from his abdominal wall, he was admitted to our Emergency Service. On physical examination, a white membrane was seen to protrude out from the 2 cm x 1 cm skin defect on the left superolateral site of the umblicus. Large, complex, cystic and solid mass of 9.5 cm-diameter was located in the spleen on ultrasonographic examination. At operation, partial cystectomy and drainage was performed. After the operation, he was given a dosage of 10 mg/kg per day of albendazole, divided into three doses. He was discharged on the postoperative 10th d. It should be kept in mind that splenic hydatid cysts can cause such a rare complication.

Key words: Hydatid cyst; Cutaneous fistula; Spleen

INTRODUCTION

Hydatid disease is caused by the larval stage of the genus *Echinococcus*. Echinococcosis has a worldwide distribution because of increasing migration and a growing incidence of world travel. It is endemic in many Mediterranean countries, the middle and Far East, South America, Australia, New Zealand and East Africa[1].

The most common form, cystic hydatid disease, is caused by *Echinococcus granulosus*, whereas the alveolar type is caused by *E. multiocularis*. Approximately 70 percent of hydatid cysts are located in the liver and there are multiple cysts in one-quarter to one-third of these cases[2].

Live hydatid cysts can rupture into physiologic channels, free body cavities or adjacent organs[1]. There are only five case reports of spontaneous cutaneous fistulization of liver hydatid cysts in the literature[3-7]. But there isn't any report about cutaneous fistula caused by splenic hydatid cyst.

We report a first case of spontaneous cutaneous fistula of infected splenic hydatid cyst.

CASE REPORT

A 43-year-old man was admitted to our Emergency Service with abdominal pain and fluid drainage from the abdominal wall. He has been suffering from a reddish swelling on the abdominal wall skin for four months. He stated that some hemopurulent drainage was occured from this swelling two months before. He didn't go to any doctor during this period. The drainage was stopped spontaneously in two days. He was no other complaints after that. Two days before, hemopurulent drainage and pain were started again. After a white membrane had been protruded out from his abdominal wall, he was admitted to our Emergency Service. On physical examination, there was a 2 cm x 1 cm skin defect on the left superolateral site of the umblicus (Figure 1). This defect was at a distance of 3 cm to the umblicus. A white membrane was protruded out from this defect. This membrane looked like the germinative memb-

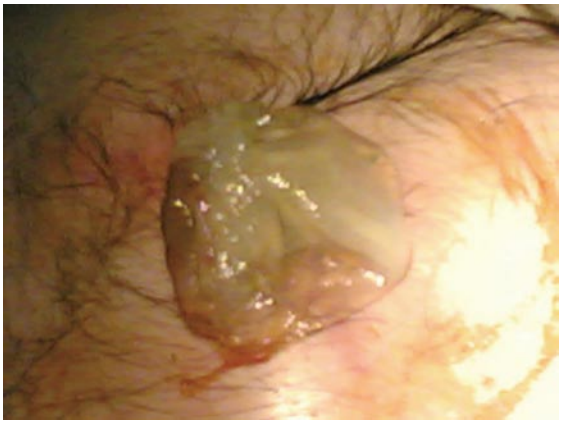

Figure 1 Germinative membrane protruded out from the 2 cm x 1 cm skin defect on the left superolateral site of the umblicus.

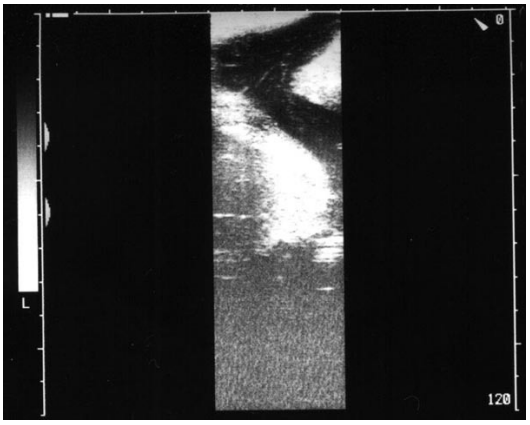

Figure 2 Fistula tract on the anterior abdominal wall.

rane of hydatid cyst. Laboratory findings were as follows; hemoglobin: 11.7 g/dL, white blood count: 18 800/mm^3, platelet: 632 000/mm^3, alkaline phosphatase: 270 U/L, gamma glutamyl trans-peptidase: 51 U/L. Other laboratory findings were normal. Ultrasonography (USG) was the modality of choice for this patient. Large, complex, cystic and solid mass of 9.5 cm-diameter was located in the spleen. Mass showed a complex internal character with multiple floating membranes, echogenic foci due to hydatid sand and heterogenous-echogenic matrix which are characteristic imaging findings for hydatid disease. Mass passed through the anterior abdominal wall from 5 cm-diameter peritoneal defect. Fistula tract on the anterior abdominal wall and cutaneous orifice were also seen (Figures 2 and 3).

At operation, 300 mL purulent material was drained from the subcutaneous tissue after skin incision. The germinative membrane in the fascial defect was taken in the abdomen. Approximately 1.5 liters of infected hydatid cyst material was aspirated from the cyst cavity. On exploration, the cyst was found to be localized in the splenic parenchyma. There were multiple adhesions between the cyst and spleen, liver, stomach and abdominal wall. There was a 5 cm-diameter fascial defect on the lateral side of the left rectus muscle. This was the defect from where the germinative membrane was protruded out of the abdomen. Adhesions were dissected and partial cystectomy was performed. There were splenic fragments in the cyst cavity. The cyst cavity was cleaned and 10% povidone iodine was applied to the cyst wall. Two drains were left in the abdomen; one in the cyst cavity and the other in the left paracolic region. The perforated skin area was debridated but not sutured. After the operation, he was given a dosage of 10 mg/kg/d of albendazole, divided into three doses. He was discharged on the postoperative 10th d. He came to first control three mo after the operation. USG and computerized tomography (CT) examinations together with serologic tests were normal.

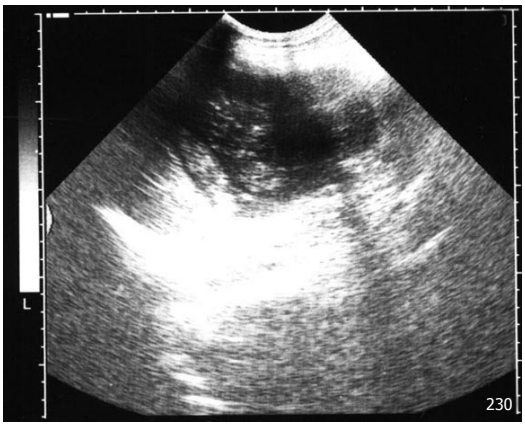

Figure 3 Large, complex, cystic and solid mass located in the spleen.

DISCUSSION

We present the first case of spontaneous cutaneous fistula of infected splenic hydatid disease. The clinical features of hydatid disease depend on the site, size, stage of development, whether the cyst is dead or alive and whether there is a complication or not[1]. Patients with simple or uncomplicated multivesicular cysts are usually asymptomatic. The clinical symptoms are related to pressure on adjacent organs or the presence of complications. Abdominal pain and tenderness are the most common complaints[2].

The hydatid disease can remain symptom-free for years or cause serious complications resulting in death. The main complications are rupture into the peritoneal cavity, infection, compression of the biliary tree, intrabiliary rupture, anaphylaxis, and secondary hydatosis[8].

Intrabiliary rupture represents the most common complication and occurs in 5 to 10 % of cases[2]. T-tube drainage, choledochoduodenostomy and transduodenal sphincteroplasty are effective procedures in the management of intrabiliary ruptured hydatid disease[9-11]. Suppuration, the second most common complication, is caused by bacteria from the biliary tract. Intraperitoneal rupture results in the showering of hydatid fluid, brood capsules, and scolices into the peritoneum; leading to systemic anaphylactic reactions or the development of new hydatid cyst[2,12,13].

Spontaneous cutaneous fistulization is a very rare complication of liver hydatid cysts. There are only five case reports in the literature[3-7]. Two of them are cysto-hepato-bronchial fistula[3,5]. In these cases, liver hydatid cysts were

fistulized simultaneously and spontaneously to the skin and in the bronchia. The other cases were cutaneous fistula of liver hydatid cysts[4,6,7].

A viable hydatid cyst is a space-occupying lesion with a tendency to grow. In confined areas, such as the central nervous system, small cysts cause serious symptoms. In less restricted areas the symptoms depend on the site and size of the cyst. Symptoms may result from direct pressure or distortion of neighboring structures or viscera. The cyst grows in the direction of the least resistance. Another consequence of cyst enlargement is that it can rupture. Live hydatid cysts can rupture into physiologic channels, free body cavites or adjacent organs. The other factor responsible for fistulization of hydatid disease is inflammation. Infection and continued expansion of the cyst causes pressure erosion and adhesion to the adjacent structures. In time, with increasing intracystic pressure, the cyst ruptures. Inflammation leads to necrosis and causes fistulization[1]. In our case, inflammation is probably the main factor of cutaneous fistulization.

Although hydatid disease can develop anywhere in the human body, the liver is the most frequently involved organ (52%-77%), followed by the lungs (10%-40%)[8]. Cysts of the spleen are unusual. Parasitic cysts are usually due to echinococcal involvement, while non-parasitic cysts can be categorized as dermoid, epidermoid, epithelial, and pseudocysts[2]. The spleen is infrequently involved in hydatid disease. Ozdogan *et al*[14] reported that the spleen was involved in 2.5% of all abdominal hydatidosis cases. They suggested that although splenectomy was the conventional treatment, partial cystectomy and omentopexy could be another choice for the treatment of splenic hydatosis.

Splenic abscess is an uncommon cause of splenic abdominal sepsis. Splenectomy is the operation of choice, but some patients have been treated with splenectomy and drainage when there were gross adhesions or the condition of the patient did not permit splenectomy[2]. In our case, we performed partial cystectomy and drainage. Because of dense adhesions and abscess formation, we didn't perform splenectomy. There was no recurrence in abdominal cavity, including spleen, at USG and CT imaging performed 3 mo after the operation. If recurrence occur, we will perform total splenectomy as a second operation.

Benzimidazole carbamates (mebendazole and albendazole) are antihelmintic drugs that kill the parasite by impairing its glucose uptake. Albendazole is the drug of choice because of its better absorption and better clinical results in comparison with mebendazole. Continuous daily treatment for a 3-mo period has better results[13]. We proposed our patient a dosage of 10 mg/kg per day albendazole for 3 mo and a 3-mo period controls.

We reported a first case of spontaneous cutaneous fistula of infected splenic hydatid cyst. It should be kept in mind that splenic hydatid cysts can cause such a rare complication.

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Meetings

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Digestive Disease Week
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20–25 May 2006
Loas Angeles Converntion Center, California

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

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3–8 September 2006
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Easl 2006 - the 41st annual
26–30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
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Prague
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www.czech-hepatology.cz/phm2006

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Dakar
hepatitis2006@mangosee.com
mangosee.com/mangosteen/hepatitis2006/hepatitis2006.htm

World Congress on Gastrointestinal Cancer
28 June 2006–1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6–8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

5th International Congress of The African Middle East Association of Gastroenterology
24–26 February 2006
Sharjah
InfoMed Events
infoevent@infomedweb.com
www.infomedweb.com

7th World Congress of the International Hepato-Pancreato-Biliary Association
3–7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

13th International Symposium on Pancreatic & Biliary Endoscopy
20–23 January 2006
Los Angeles - CA
laner@cshs.org

2006 Gastrointestinal Cancers Symposium
26–28 January 2006
San Francisco - CA
Gastrointestinal Cancers Symposium Registration Center
giregistration@jspargo.com

Society of American Gastrointestinal Endoscopic Surgeons
26–29 April 2006
Dallas – TX
www.sages.org

Digestive Disease Week 2006
20–25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25–26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3–7 June 2006
Seatlle - Washington
www.fascrs.org

71st ACG Annual Scientific and Postgraduate Course
20–25 October 2006
Venetian Hotel, Las Vegas, Neveda
The American College of Gastroenterology

AASLD 57th Annual – The Liver Meeting™
27–31 October 2006
Boston, MA
AASLD

New York Society for Gastrointestinal Endoscopy
13–16 December 2006
New York
www.nysge.org

EVENTS AND MEETINGS IN 2007

9th World Congress on Gastrointestinal Cancer
20–23 June 2007
Barcelona
Imedex
meetings@imedex.com

Gastro 2009, World Congress of Gastroenterology and Endoscopy London, United Kingdom 2009

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4 **Sherlock S,** Dooley J. Diseases of the liver and billiary 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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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_2SO_4	0.5 mol/L H_2SO_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_r18 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 $\cdot L^{-1}$	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	A_{260} nm	*A* should be in italic.
	$^bP<0.05$	$^aP<0.05$	In Table, no note is needed if there is no significance instatistics: $^aP<0.05$, $^bP<0.01$ (no note if $P>0.05$). If there is a second set of *P* value in the same table, $^cP<0.05$ and $^dP<0.01$ are used for a third set: $^eP<0.05$, $^fP<0.01$.
45	**F*=9.87, $^§F$=25.9, $^\#F$=67.4	^1F=9.87,^2F=25.9, ^3F=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	$10\times10\times10cm^3$	10 cm×10 cm×10 cm	
66	N·km	KN·m	moment
67	$x\pm s$	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 *et al.*	
72	EcoRI	*Eco*RI	*Eco* in italic and RI in positive. Restriction endonuclease has its prescript form of writing.
73	Ecoli	*E.coli*	Bacteria and other biologic terms have their specific expression.
74	Hp	*H pylori*	
75	Iga	*Iga*	writing form of genes
76	*igA*	IgA	writing form of proteins
77	-70 kDa	~70 ku	