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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

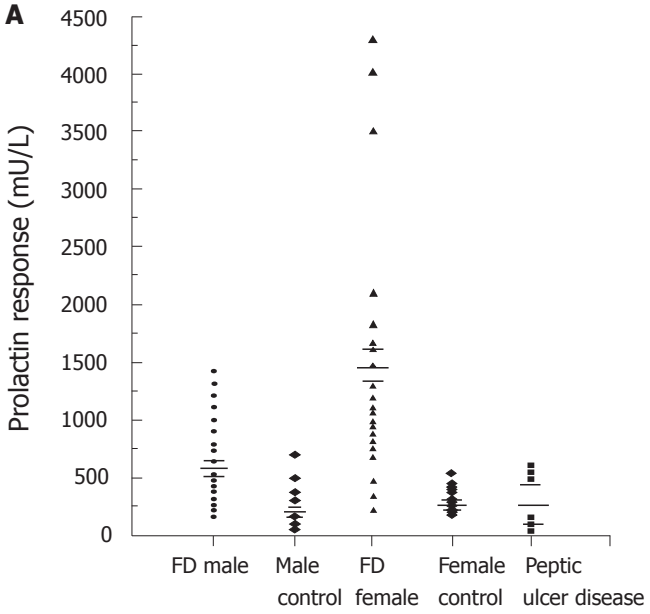

Figure 1A Prolactin response to buspirone challenge in healthy controls and patients with functional dyspepsia and peptic ulcer disease

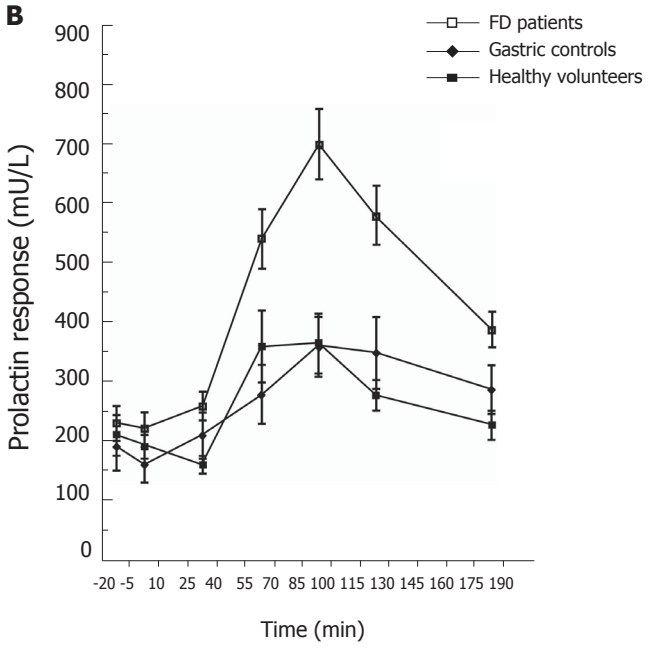

Figure 1B Prolactin response to buspirone 60 mg in male functional dyspeptics, peptic ulcer disease patients and healthy controls.

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-

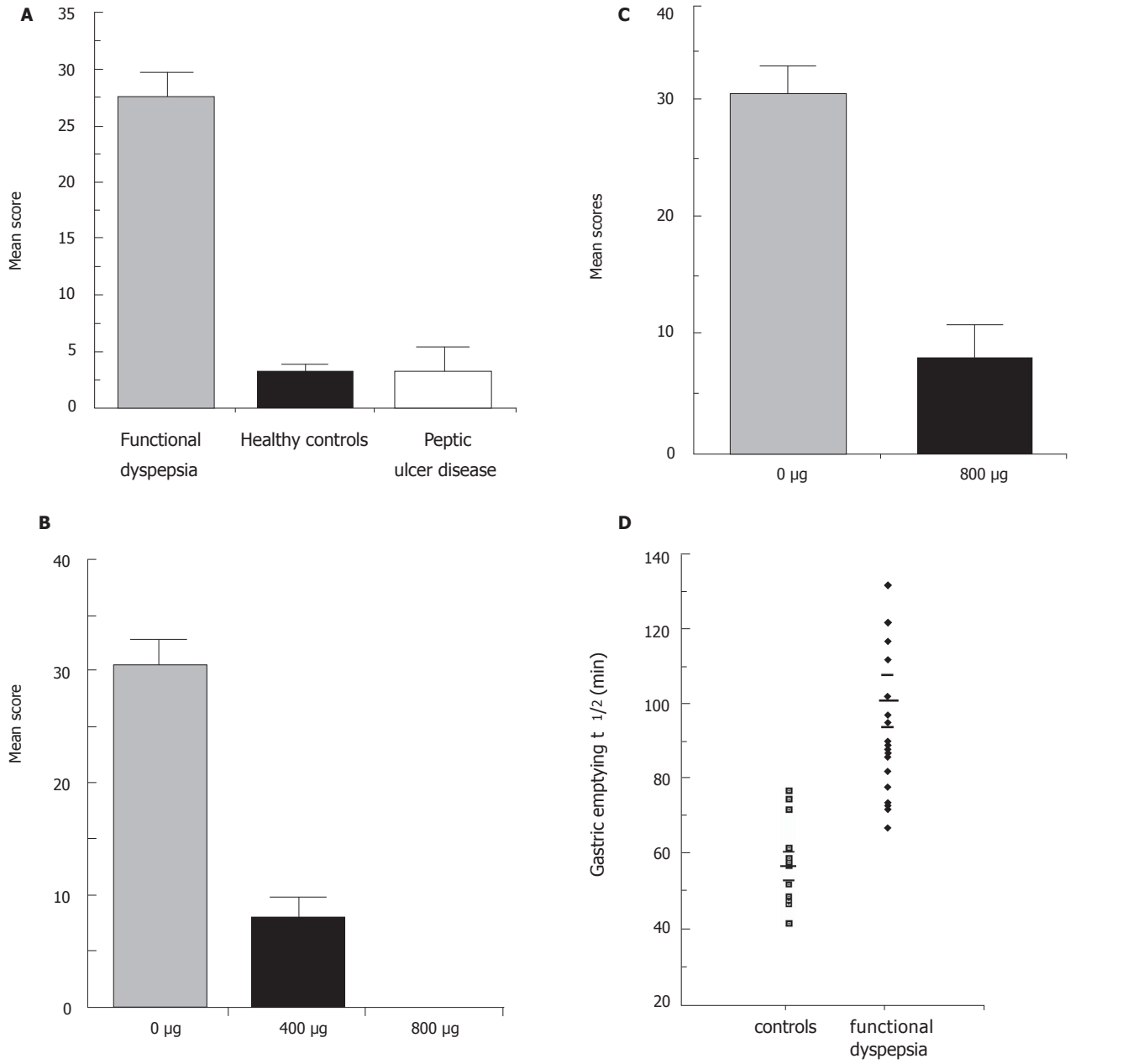

Figure 2 **A**: Response to CCK-8 challenge in healthy controls, patients with functional dyspcpsia and peptic ulcer disease; **B**: Effects of atropine on CCK-8 infusion in functional dyspeptic patients. **C**: Effects of loxiglumide 800mg on CCK-8 infusion in functional dyspeptic patients; **D**: Solid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

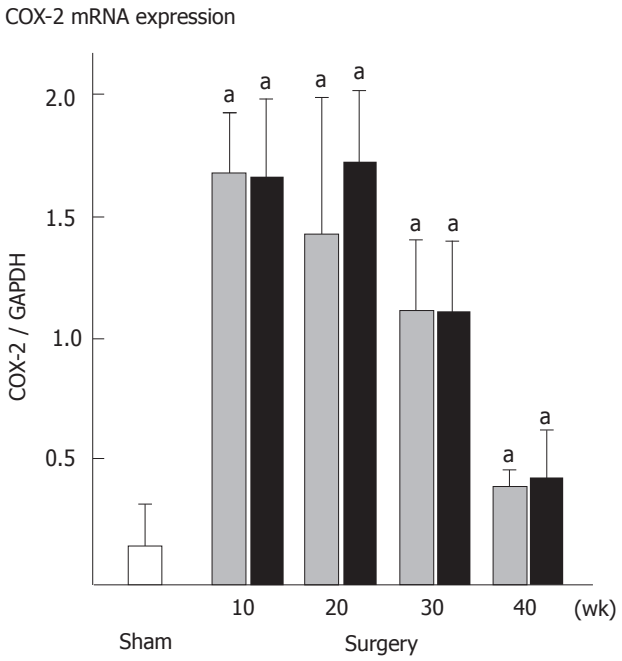
Our results showed surges of COX-2 and PGE_2 between the beginning and the 20th wk in the control group, thus suggesting that the COX-2 expression played an important role in the early phase of the esophageal carcinogenesis in the inflammation-metaplasia-adenocarcinoma sequence. The fact that the suppression 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 non-cardia 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



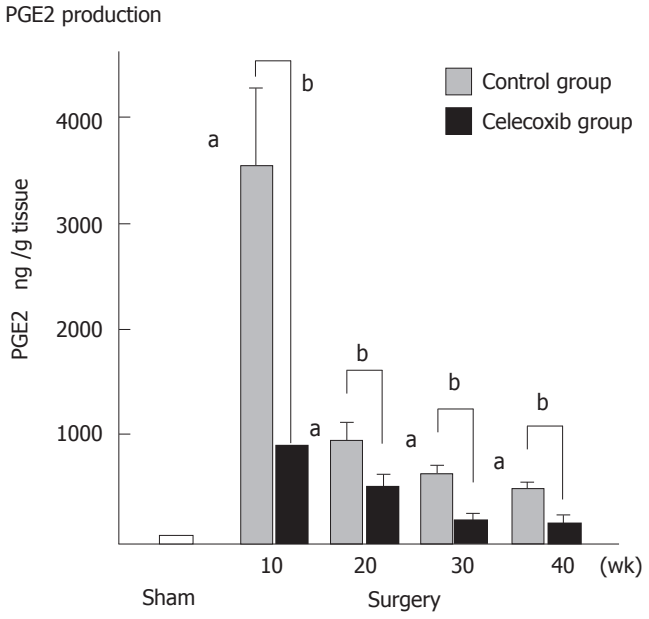
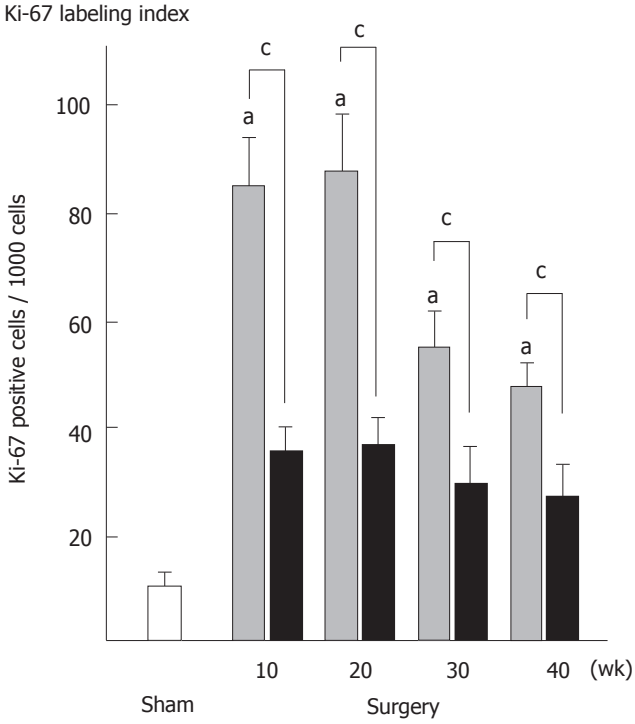


Figure 1 COX-2 mRNA expression and PGE2 production of esophageal mucosa. [a]$P < 0.005$ *vs* sham; [b]$P < 0.01$ between the two groups , Mann-Whitney U test



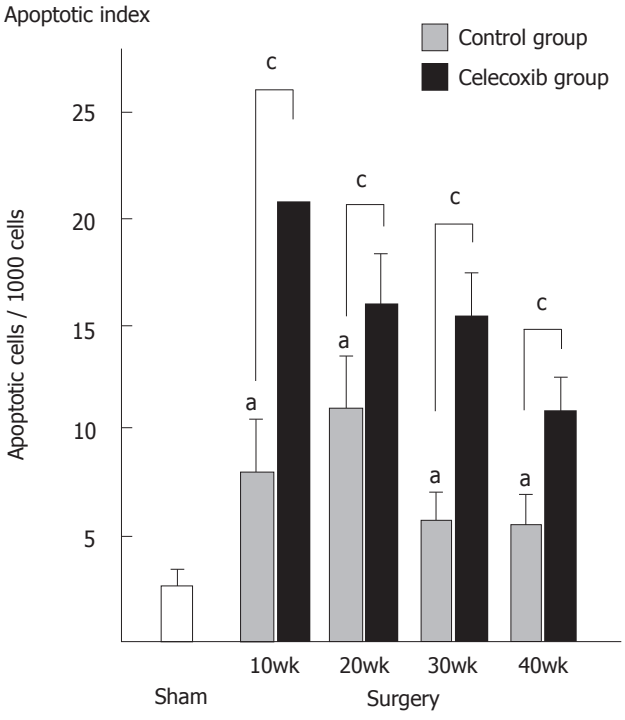


Figure 2 Cell kinetics of esophageal mucosa in a duodenoesophageal reflux model. [a]$P < 0.05$ *vs* sham; [c]$P < 0.05$ between the two groups , Mann-Whitney U test

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*

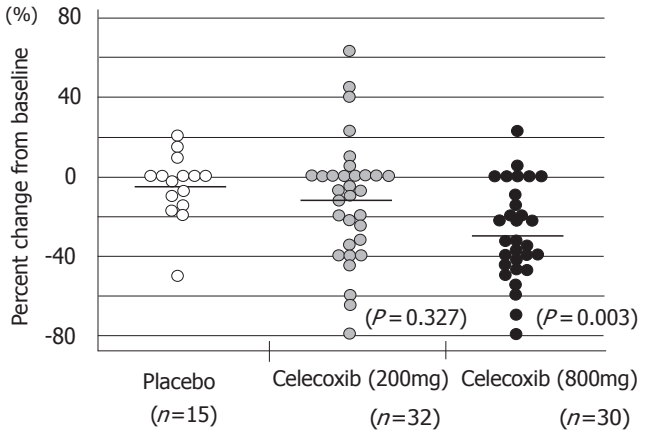


Figure 3 Percentage change from the baseline in the number of colorectal polyps in FAP patients

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

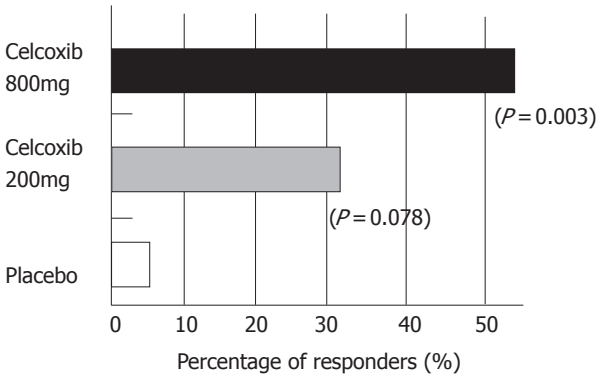


Figure 4 Percentage of responders who showed a 25% or more decrease in the mean number of colorectal polyps

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

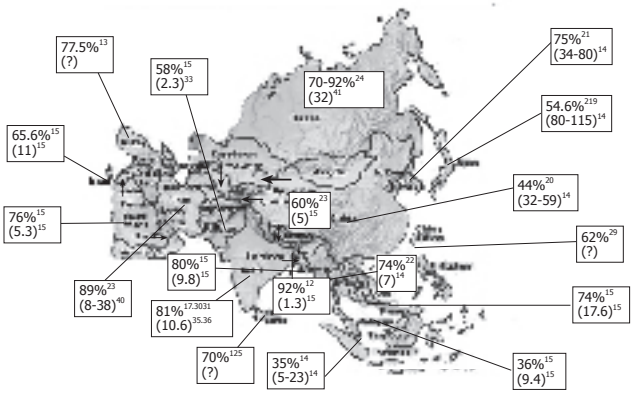

Figure 1 Map of Asia showing frequency of *Helicobacter pylori* infection in each country, crude annual incidence of gastric cancer per 100 000 populations (shown within parenthesis, where data are available). The reference from where data are obtained is shown in superscript. **A:** frequency in the 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

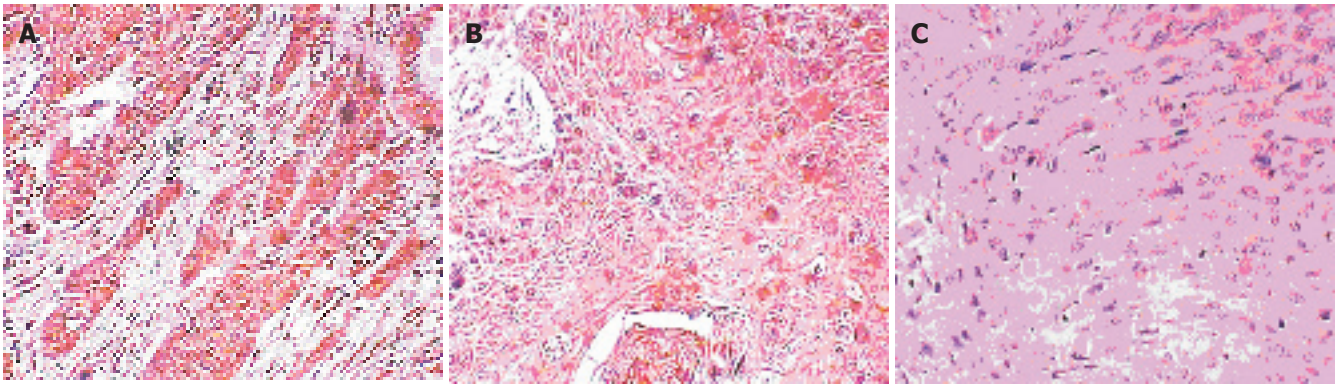

Figure 1 Positive expression of PV (**A**) and HPV(16/18-E6) (**B**) and negative expression of HPV (16/18-E6) (**C**) in esophageal carcinoma (IHC×100)

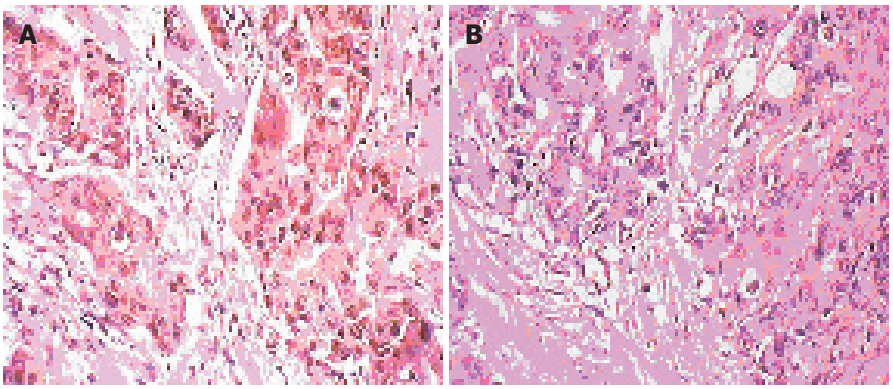

Figure 2 Positive (**A**) and negative (**B**) expression of HPV(16/18) DNA in esophageal squamous cell carcinomas (ISH×100)

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	

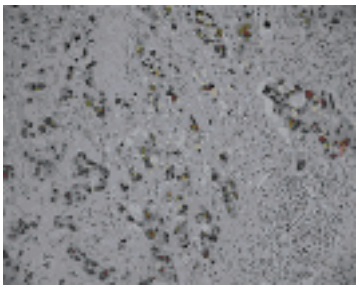

Figure 1 The expression of PCNA (*200).

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	

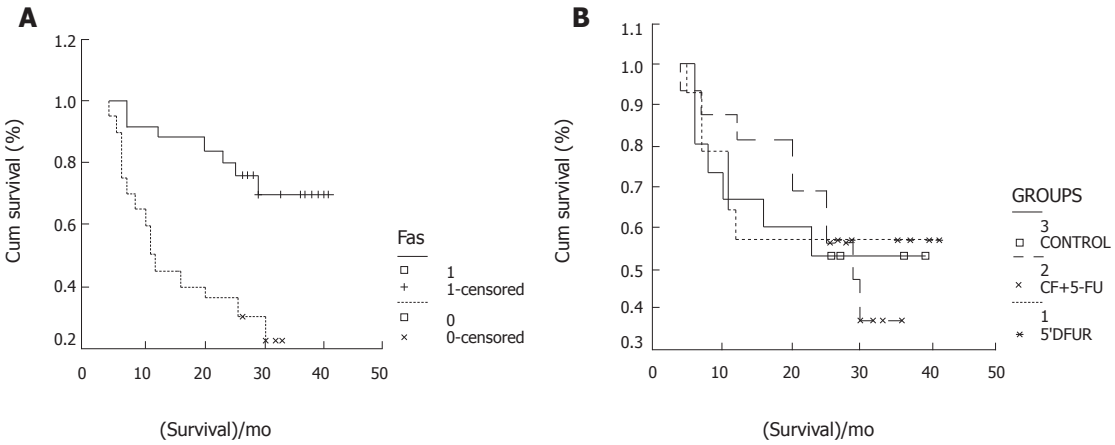

Figure 2 Fas expression (**A**) and survival rate (**B**) in three groups.

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

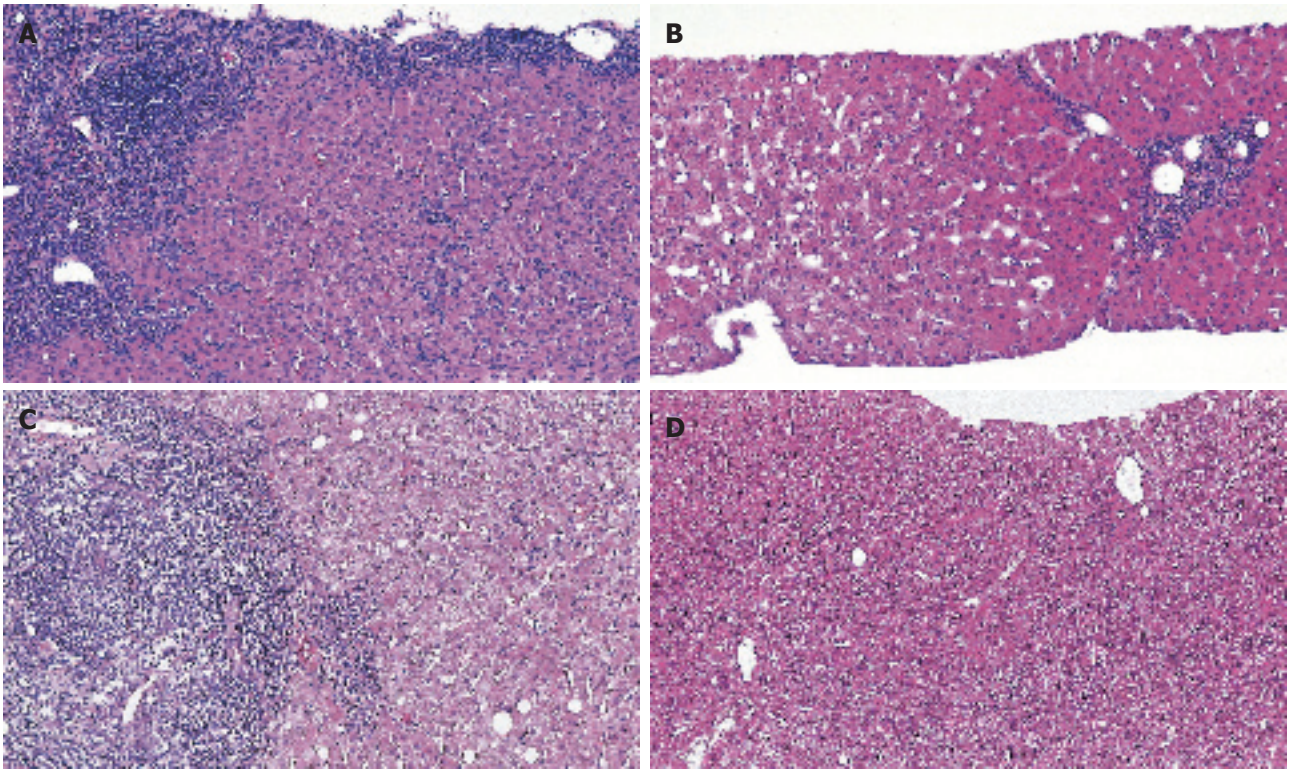

Figure 1 Histology of biopsy specimens obtained from a patient with AIH (**A**, **B**) and from a patient with AIH and PBC (overlap; **C**, **D**). Before treatment was started (**A**, **C**), liver biopsies showed a severe hepatitis with interface and lobular activity. Following administration of budesonide, 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

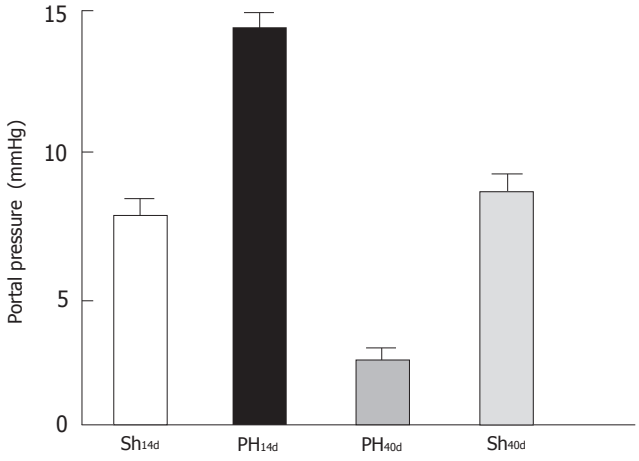

Figure 1 Portal pressure (mmHg). Group Sh14d *vs* Group PH_{14d} ($P<0.001$). Group PH14d *vs* Group PH_{40d} ($P<0.001$). Group Sh14d vs PH40d $P<0.01$. Sh14d *vs* Sh40d (n.s).

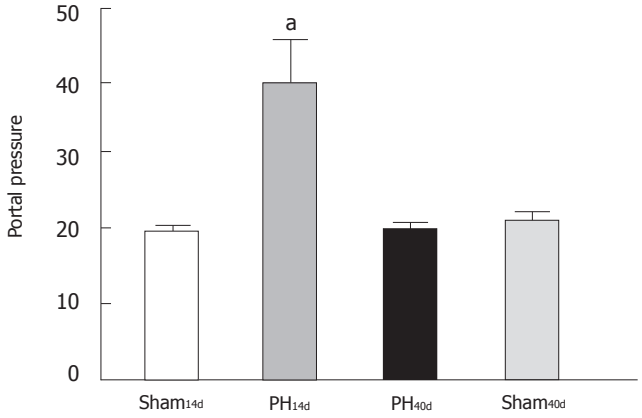

Figure 2 Protein content in cerebrospinal fluid. Hatched column corresponds to PH_{14d} ham shows an increased protein filtration through the BBB. This increment is not present, in black column,corresponding to PH_{40d} Results are expressed as mean ± SE.

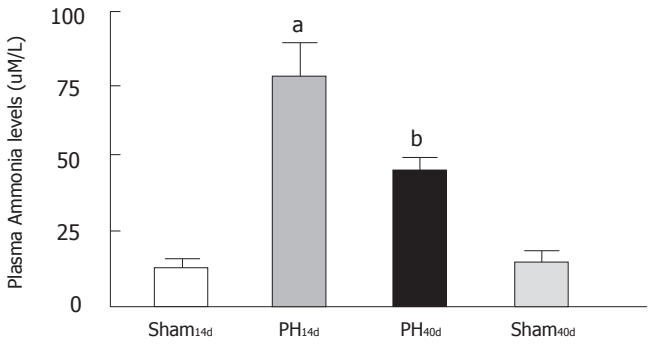

Figure 3 Plasma ammonia levels.significant elevation of plasma ammonia content in PH14d rats correspond to hatchet column. After 40 days of portal vein ligation, the portal pressure normalized, but plasma ammonia is still high (black column). $^{b}P<0.001$ and $^{a}P<0.05$, compared to Sham column.

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.

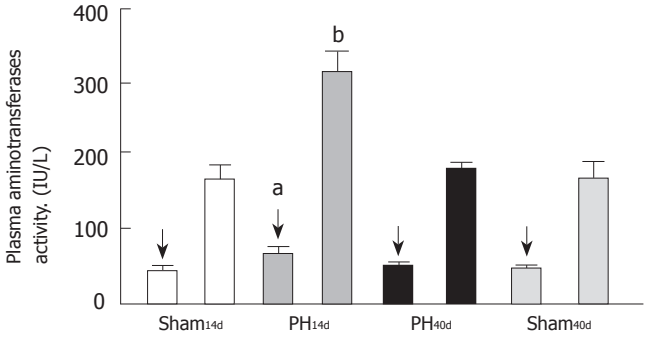

Figure 4 Plasma aminotransferases. The first column of each group corresponds to ALT activity (arrows) and the 2nd to AST activity. Both enzymes activities appear elevated in PH_{14d} (hatched columns) when compared to $Sham_{14d}$ ($^{b}P<0.001$; $^{a}P<0.05$).

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

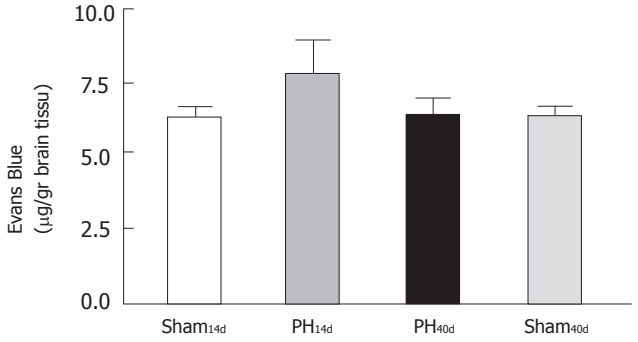

Figure 5 Evans blue test.The systemic injection of Evans blue showed a statistically significant increase of the dye presence in the hippocampal region in PH_{14d} presence in the indicate $p < 0.01$ when compared with the other 3 groups.

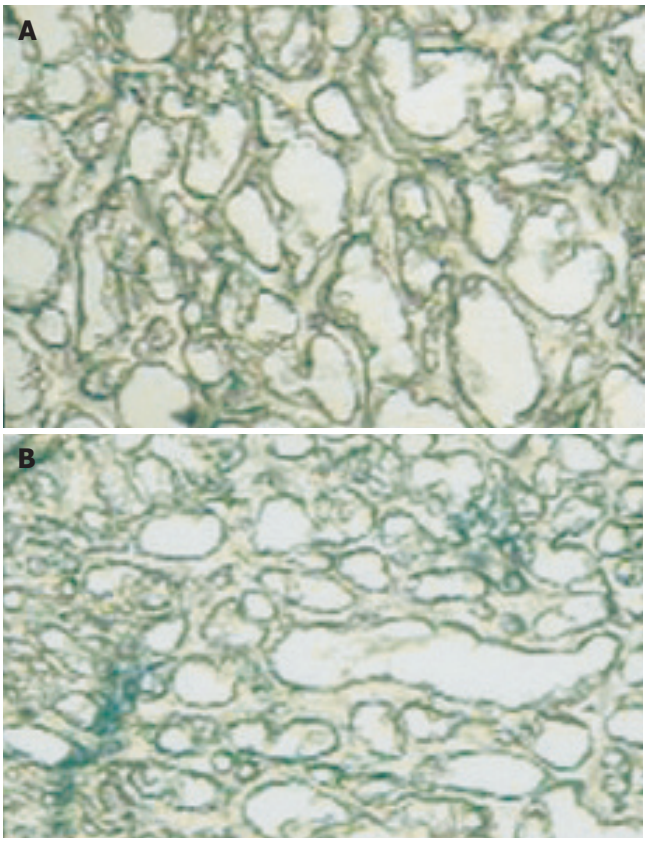

Figure 6 **A:** In the rest of the groups, including that were portal pressure normalized 40 days after portal vein stricture and Sham groups, the image is negative for Trypan blue dye (100 x); **B**: Light microscopy showed a marked Trypan blue dye diffusion in brain hippocampal of PH rats of 14 d (100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

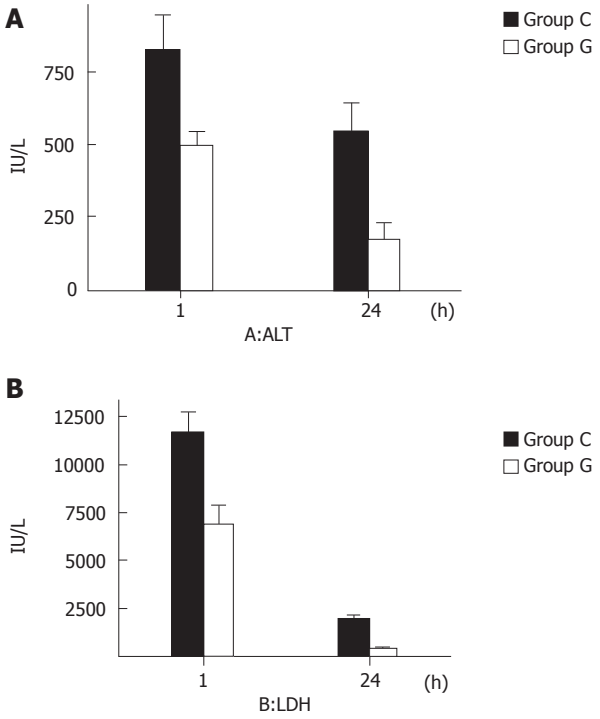

Figure 1 Liver function 1 and 24 h after reperfusion of ALT (**A**) and LDH (**B**) in groups C and G.

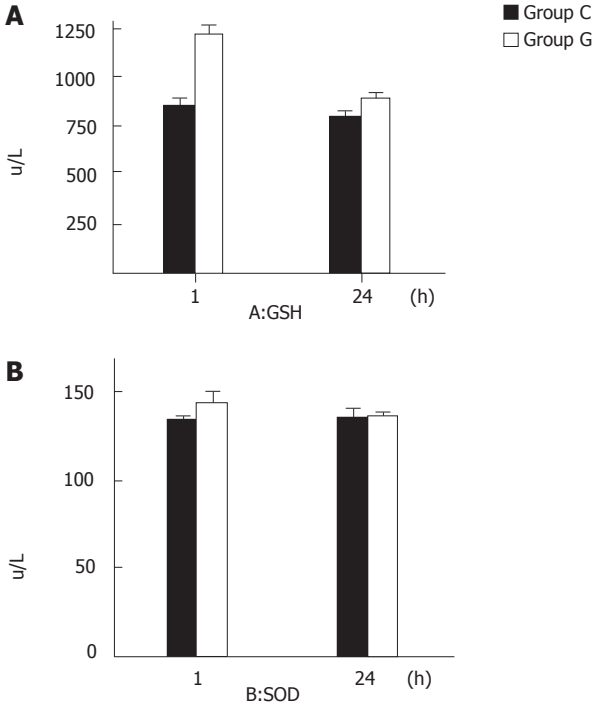

Figure 2 GSH content (**A**) and SOD activity (**B**) 1 and 24 h after reperfusion in groups C and G.

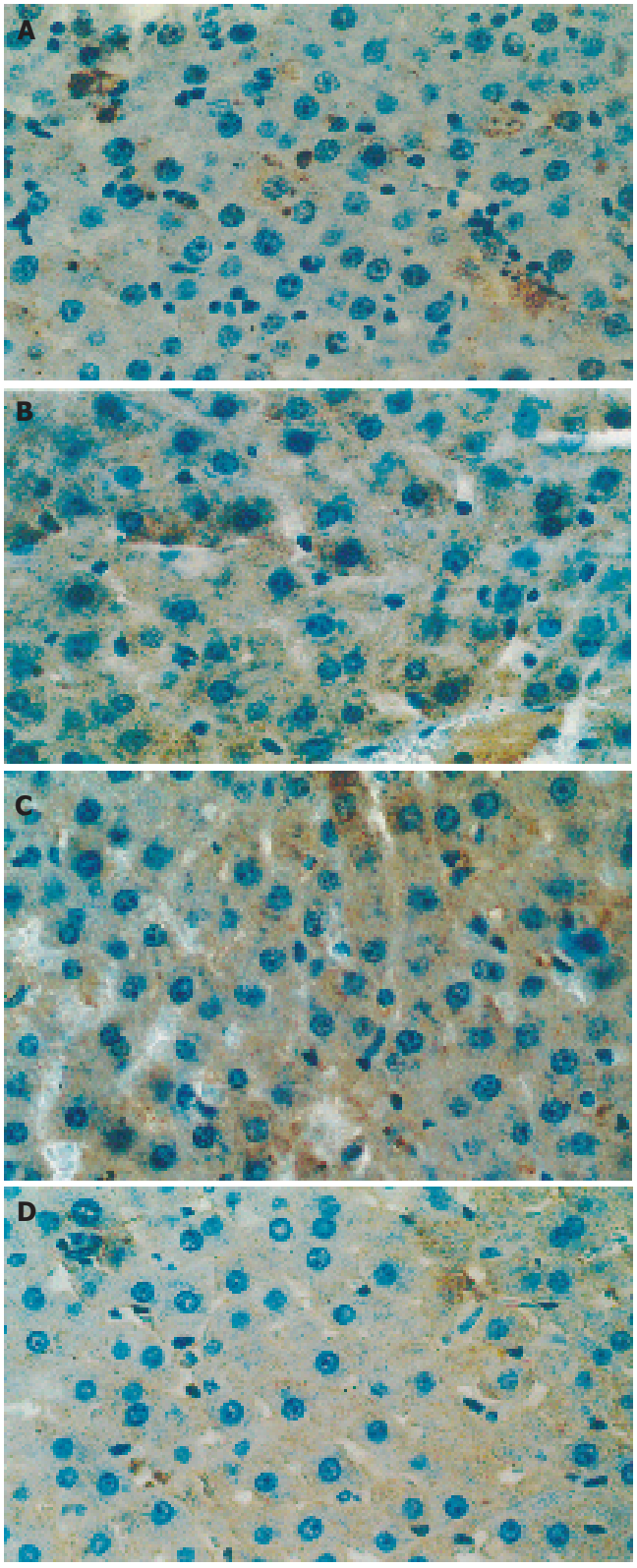

Figure 3 Bcl-2 expression in group C (**A**) and group G (**B**) (post-reperfusion 1 h) (× 400), and Bax expression in group C (**C**) and group G (**D**).

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.

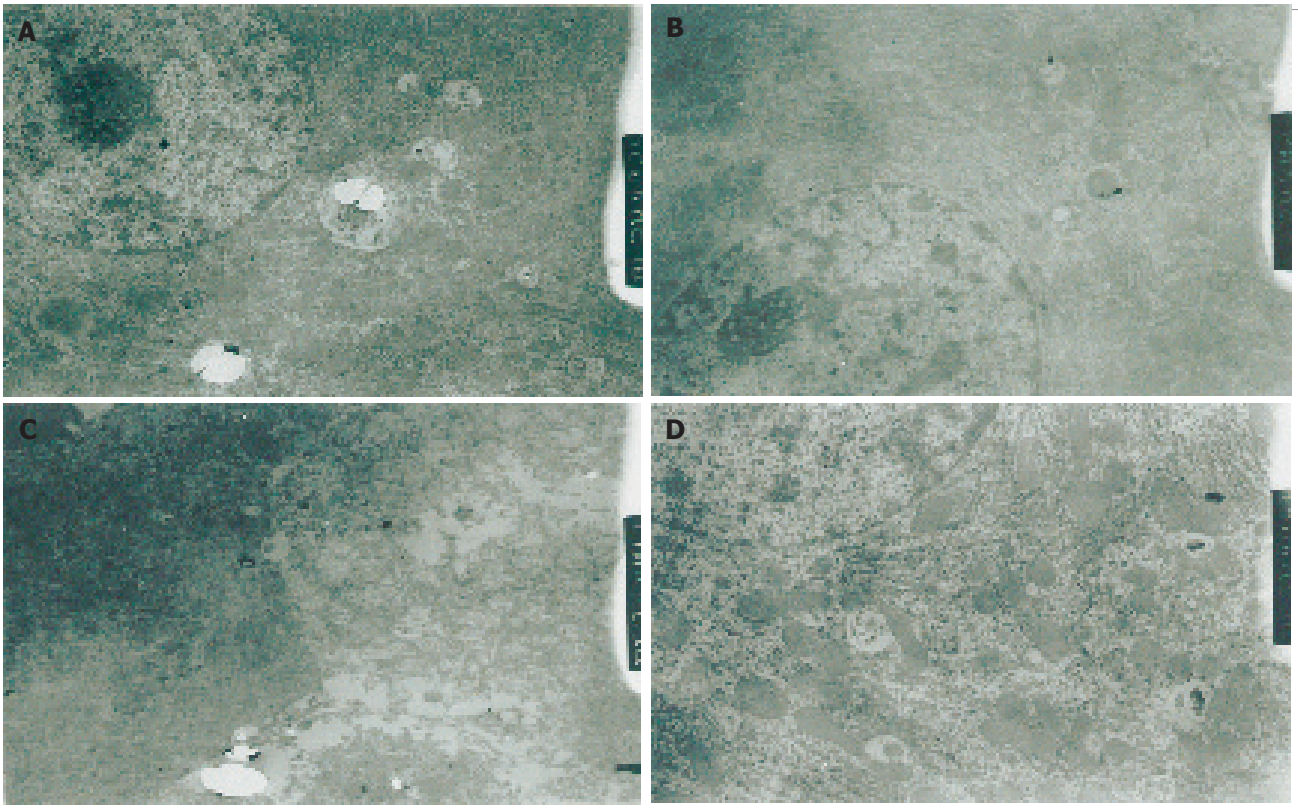

Figure 4 Hepatic ultrastructure 1 h after reperfusion (×1500) in group C (**A**) and group G (**B**), 24 h after reperfusion in group C (**C**) and group G (**D**).

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

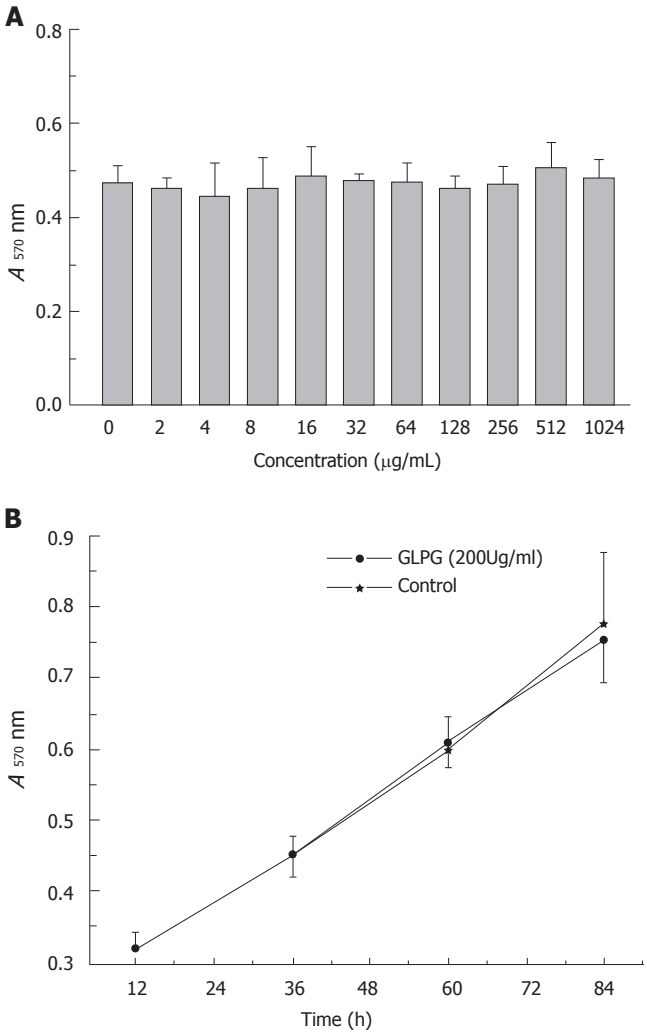

Figure 1 Effects of GLPG on proliferation of L-02 cells treated with GLPG at the concentration of 2-1024 μg/mL for 2 d (A) and at the concentration of 200 μg/mL for 3 d (B). Viable cells were detected every 24 h. The cells untreated with GLPG were used as controls in experiment. Results shown represent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

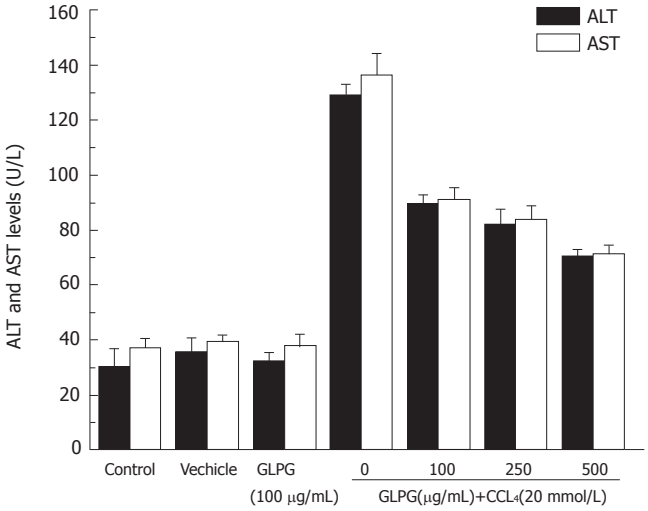

Figure 2 Effects of GLPG on ALT and AST activities in CCl4-induced L-02 cell (n = 3) injury. Compared to control group, GLPG suppressed the activities of ALT and AST in the other groups. Results shown represent the mean ± SD from three separate experiments.

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

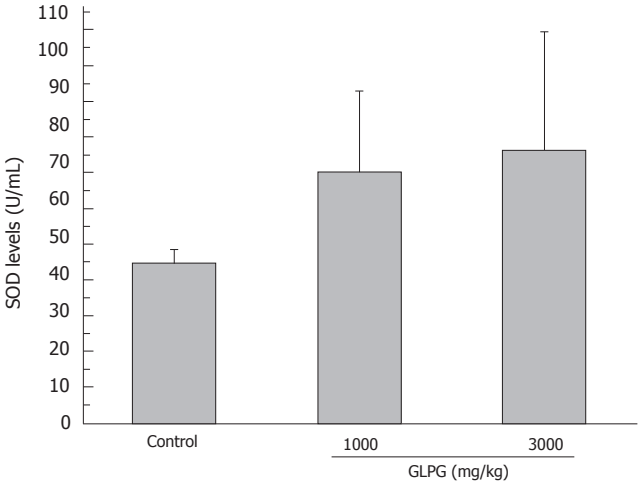

Figure 3 Effect of GLPG on SOD activity in serum of rats. The rats were administered orally with or without GLPG at 1000 mg/kg and 3000 mg/kg. The increment of SOD activities was 57.2 % and 70.6 %, respectively.

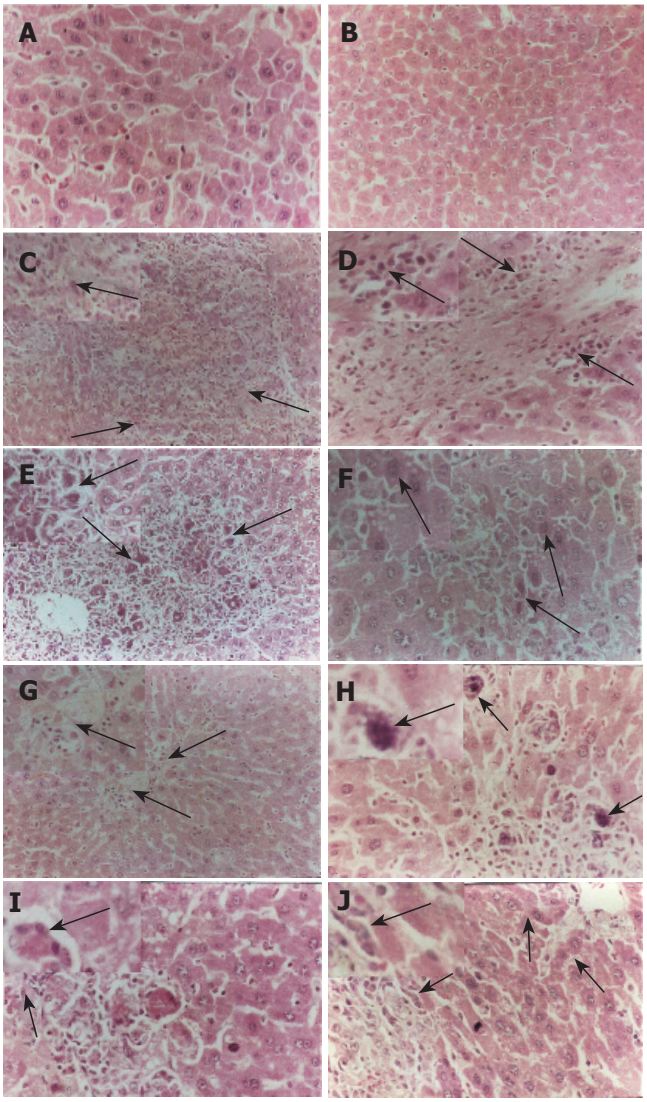

Figure 4 Histological changes (marked by arrows) of CCl_4-induced hepatic injury in the presence or absence of GLPG in mice by hematoxylin-eosin (**H&E**) staining in control group (**A**), GLPG group (**B**), and CCl_4-induced liver injury group (**C-J**) 24 and 72 h after GLPG pretreatment at different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

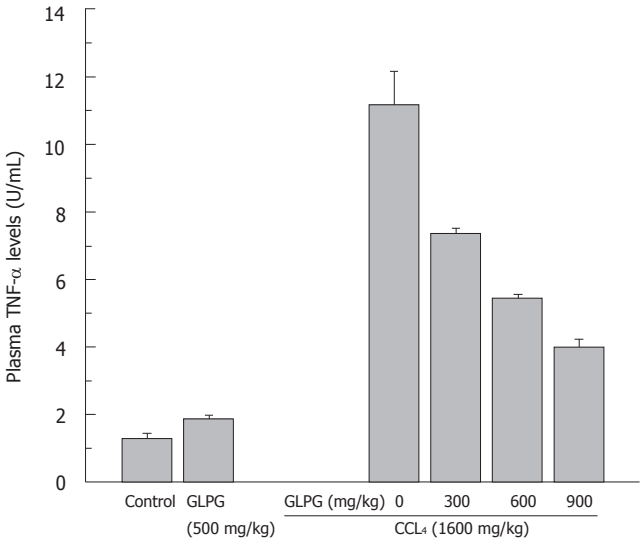

Figure 5 Effect of GLPG on the levels of TNF-α in the plasma of mice, as determined by TNF-α radioimmunoassay kit. Compared with control group, administration of GLPG at various doses could significantly suppress the level of TNF-α in a dose-dependent manner after 48 h. Data are the mean ± SD from 4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

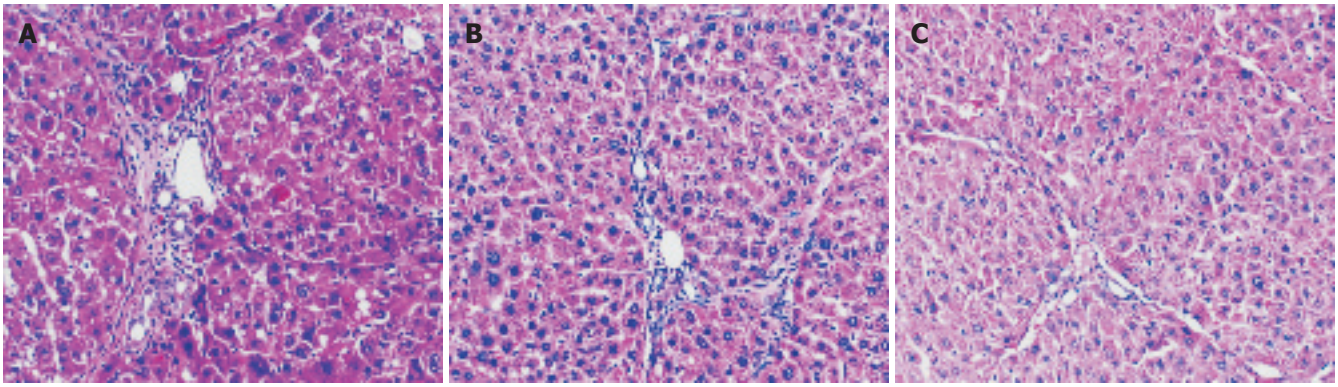

Figure 1 Effects of IL-10 on histology of CC4-induced fibrotic rat liver after treated with CCL4 for 9 wk (**A**), IL-10 for 3 wk (**B**) and spontaneous recovery for 3 wk (**C**).

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.

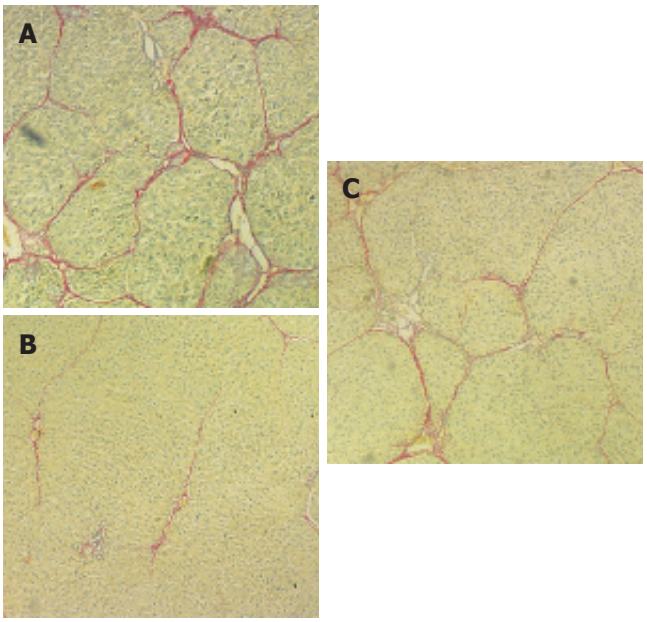

Figure 2 Photomicrographs of liver tissue from rats after treatment with with CCL_4 (**A**), IL-10 for 3 wk (**B**) and spontaneous recovery for 3 wk(**C**) by Picrosirius staining (40x).

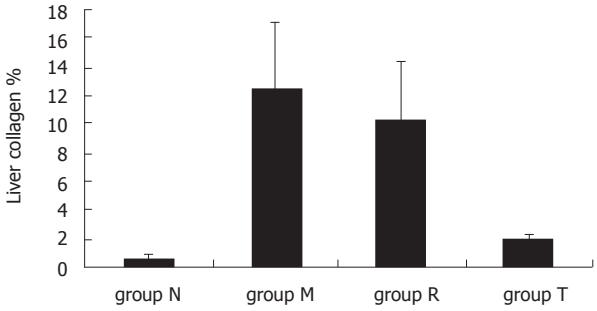

Figure 3 Percentage of collagen types I and III in fibrotic rats.

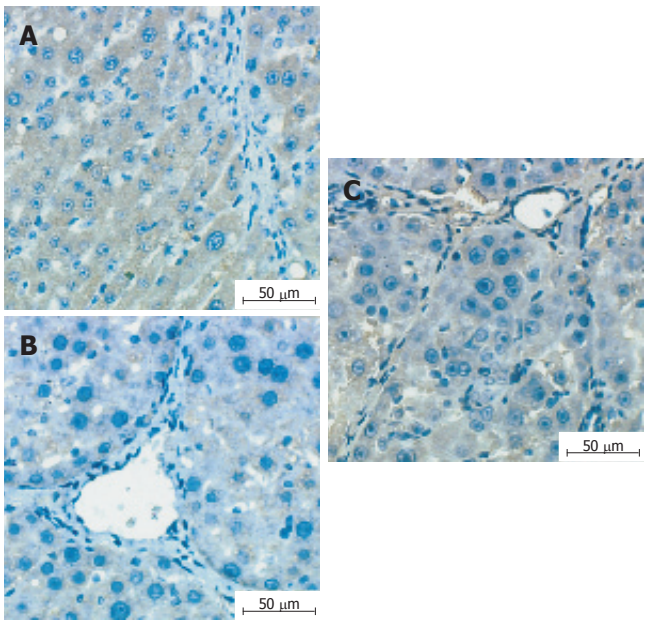

Figure 4 Expression of MMP-2 protein in liver tissue of rats treated with CCL_4 for 9 wk (**A**), IL-10 for 3 wk (**B**) and spontaneous recovery for 3 wk (**C**).

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

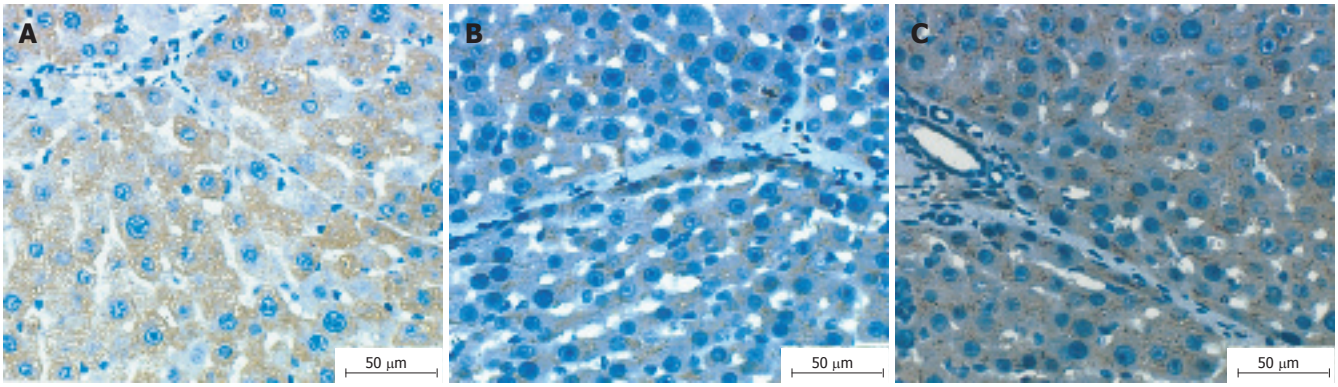

Figure 5 Expression of TIMP-1 protein in liver tissue of rats treated with CCL_4 for 9 wk (**A**), IL-10 for 3 wk (**B**) and spontaneous recovery for 3 wk (**C**).

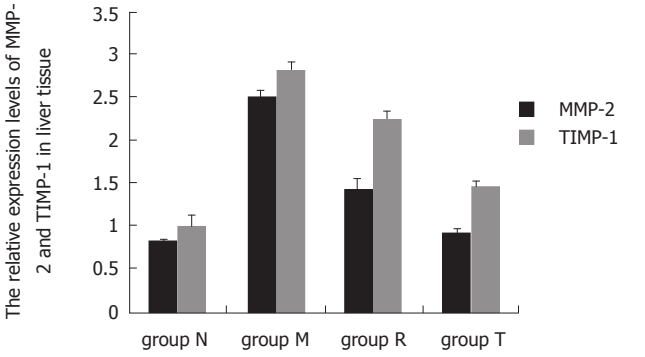

Figure 6 Relative expression levels of MMP-2 and TIMP-1 in liver of different groups.

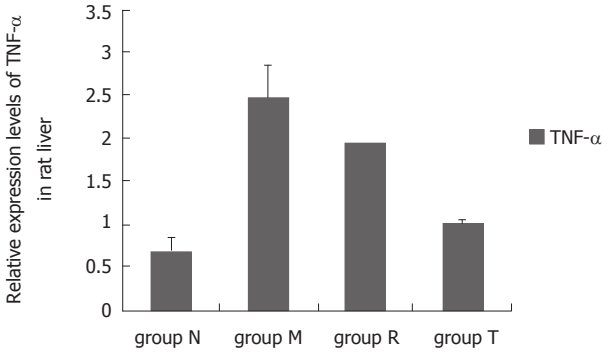

Figure 8 Relative expression of TNF-α in rat liver of different groups.

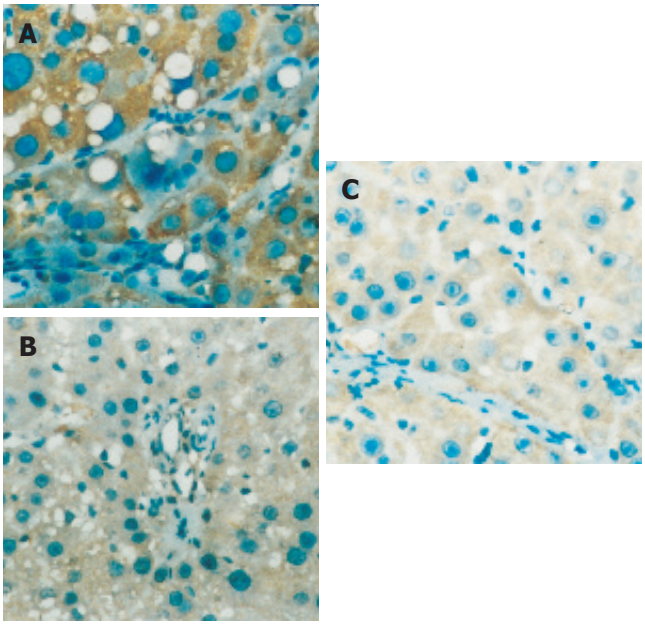

Figure 7 Expression of TNF-a protein in liver tissue of rats treated with CCL4 for 9 wk (**A**), IL-10 for 3 wk (**B**) and spontaneous recovery for 3 wk (**C**).

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

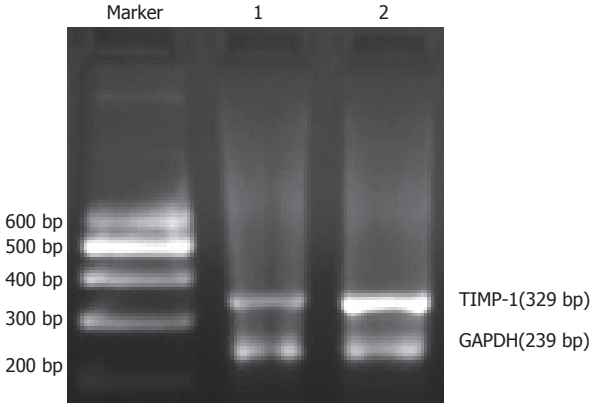

Figure 1 Expression of TIMMP-1 mRNA in HSC detected by RT-PCR. Marker: 200-600 DNA marker; lane 1: control group (without IL-1beta treatment); lane 2: IL-1β group (with IL-1β treatment)

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).

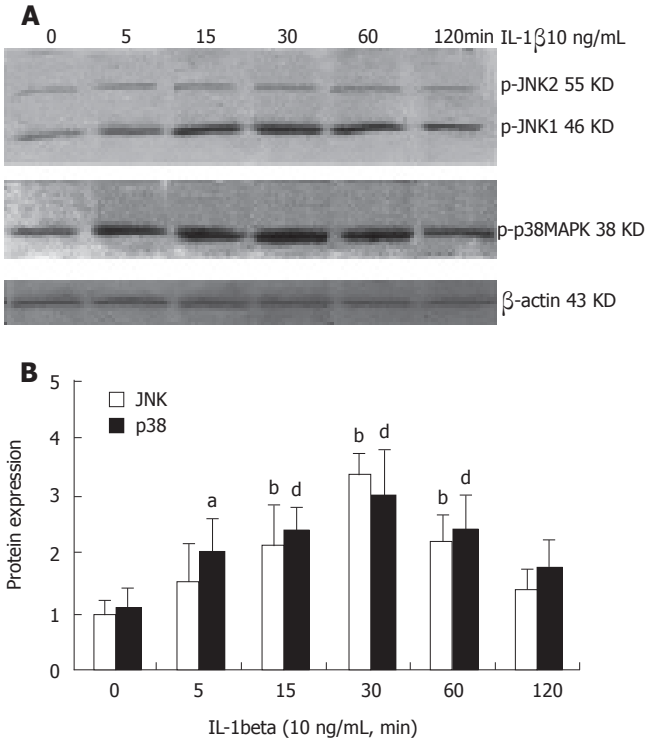

Figure 2 IL-1β activates JNK and p38 in a time-dependent manner in rat HSC. **A:** Representative Western blot results of JNK and p38; **B:** Densitometry of Western blot analyzed by Gel-Pro software. *n*=6. $^bP < 0.01$ *vs* 0 h of JNK, $^aP < 0.05$ *vs* 0 h of p38, $^dP < 0.01$ *vs* 0 h of p38.

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

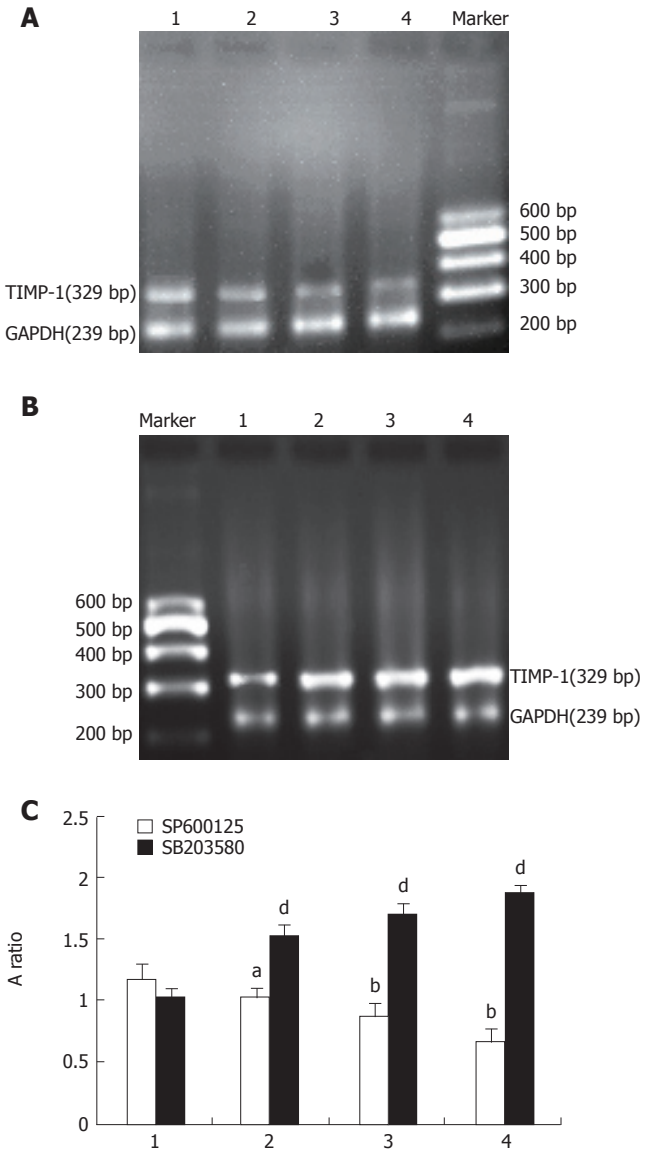

Figure 3 Effect of SP600125 and SB203580 on IL-1β-induced expression of TIMMP-1 mRNA in rat HSC. **A:** Representative photos of different concentrations of SP600125 of RT-PCR; **B:** Representative photos of different concentrations of SB203580 of RT-PCR. **C:** TIMMP-1 mRNA expression in **A** and **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

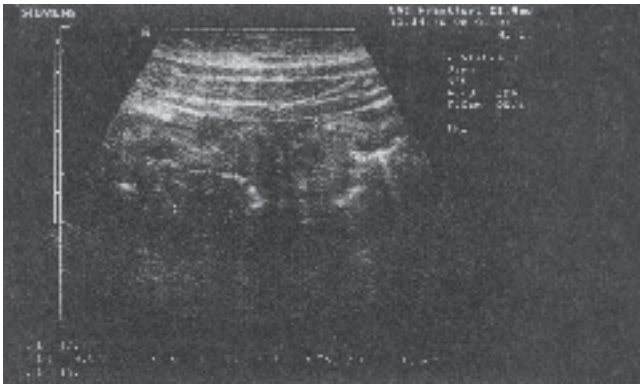

Figure 1 Characteristic sonographic findings in neutropenic enterocolitis showing asymmetric, echogenic (between markers) and inhomogenous bowel wall thickening with areas of hypoechogenity (between markers).

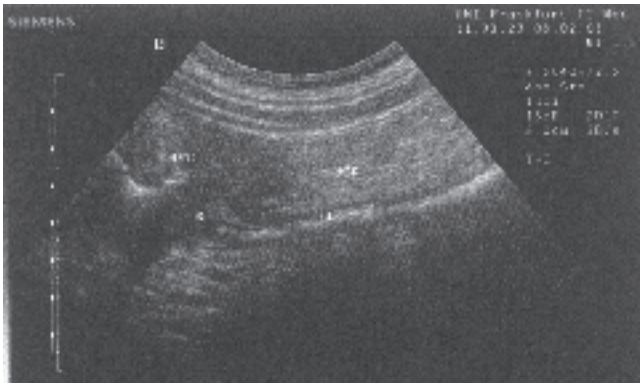

Figure 2 Asymmetric and inhomogenous wall thickening, transmural inflammatory reaction in a patient with neutropenic enterocolitis (NPC). The ileocoecal valve of Bauhin (**B**) and the terminal ileum (not affected, TI) are also indicated. Typically, the surrounding omentum shows echogenic reaction (UGR).

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.

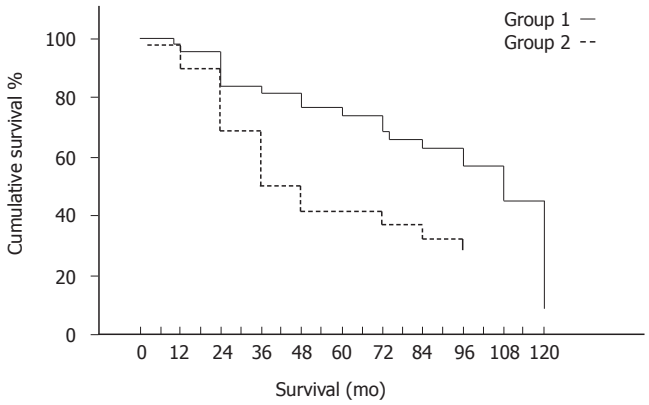

Figure 1 Cumulative survival rate in group 1 (albumin) and group 2 patients. Breslow test = 7.05; *P* = 0.0079.

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.

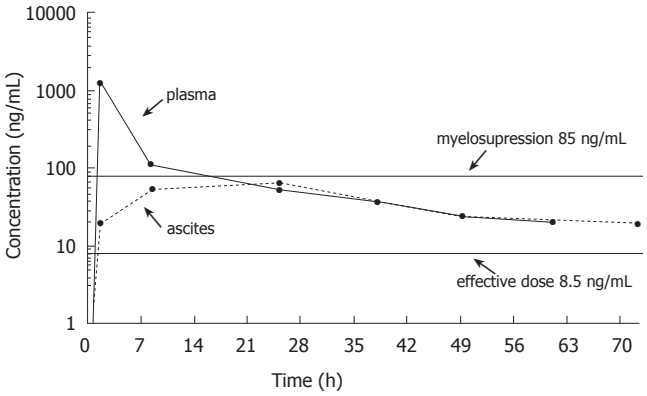

Figure 1 Concentrations of PTX in plasma and ascites in case 1 administrated with 60 mg/m2 PTX intravenously for 1.5 h.

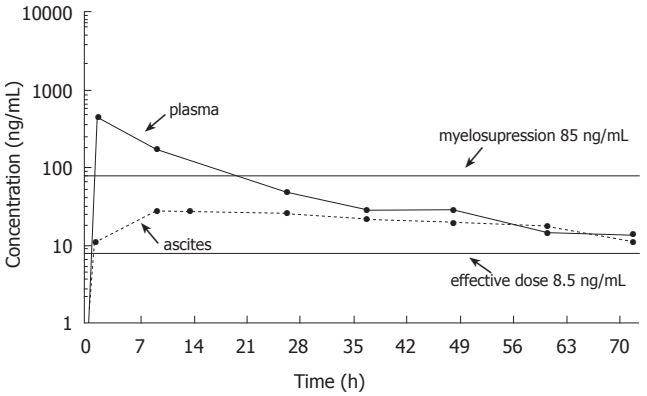

Figure 2 Concentrations of PTX in plasma and ascites in case 2 administrated with 80 mg/m2 PTX intravenously for 1.5 h.

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

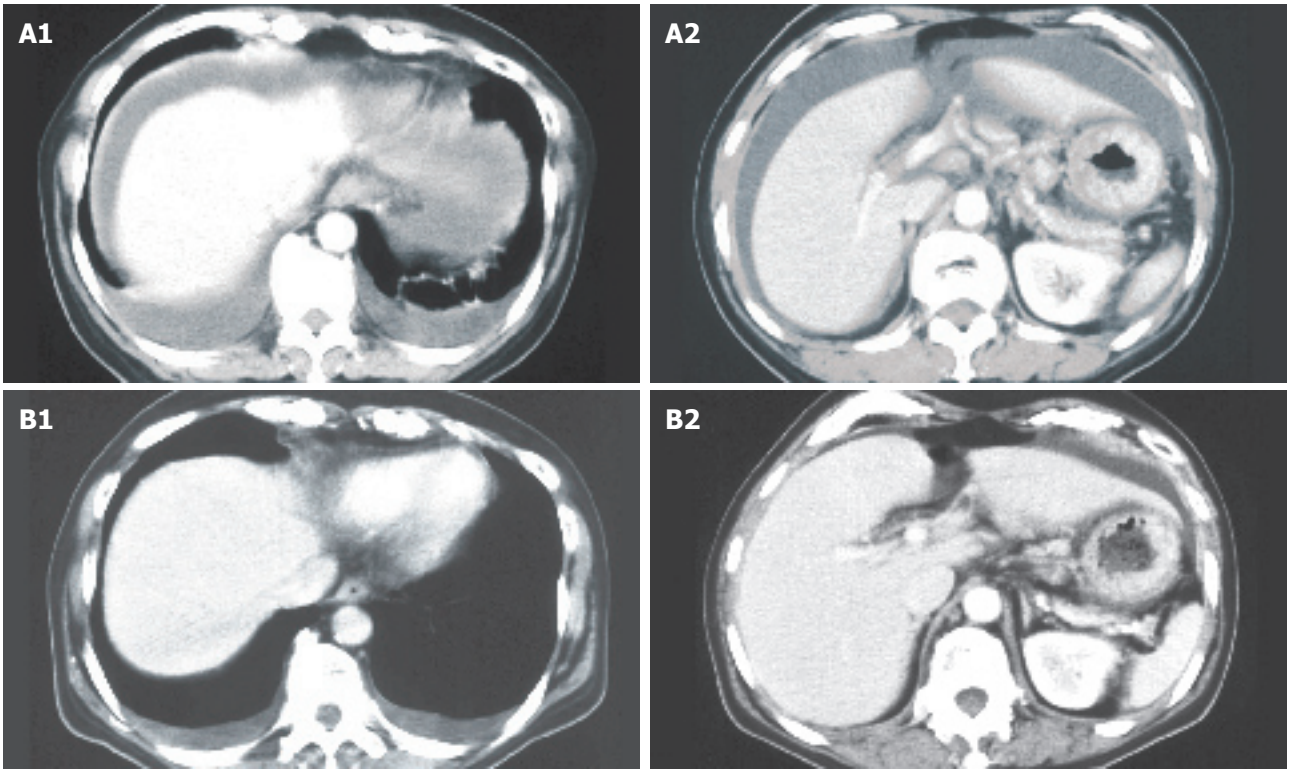

Figure 3 CT scanning of case 1 showing markedly decreased amount of ascites after 2 courses of the combined chemotherapy of 5-fluorouracil and PTX. **A1, A2:** before chemotherapy; **B1, B2:** after chemotherapy.

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

Kato S, Fu KI, Sano Y, Fujii T, Saito Y, Matsuda T, Koba I, Yoshida S, Fujimori T. Magnifying colonoscopy as a non-biopsy technique for differential diagnosis of non-neoplastic and neoplastic lesions. *World J Gastroenterol* 2006; 12(9):1416-1420

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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

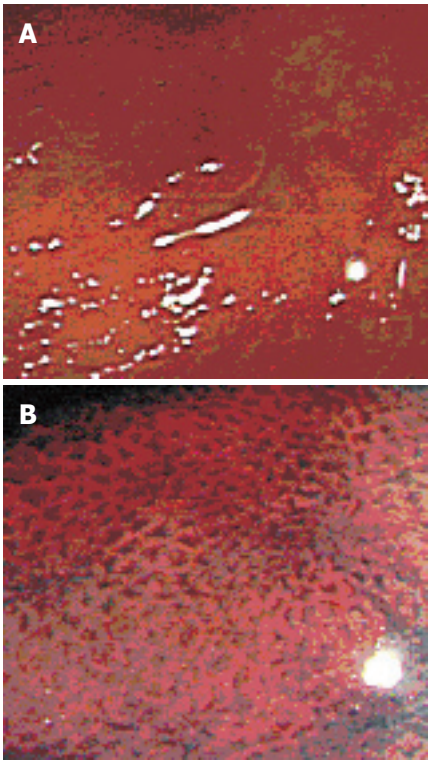

Figure 1 A: Colonoscopy revealed a small reddish polypoid lesion 3 mm in diameter. **B:** Chromoendoscopy with magnification disclosed a type II pit, and the lesion was diagnosed as non-neoplastic and examined at biopsy. The histologic diagnosis was hyperplastic polyp.

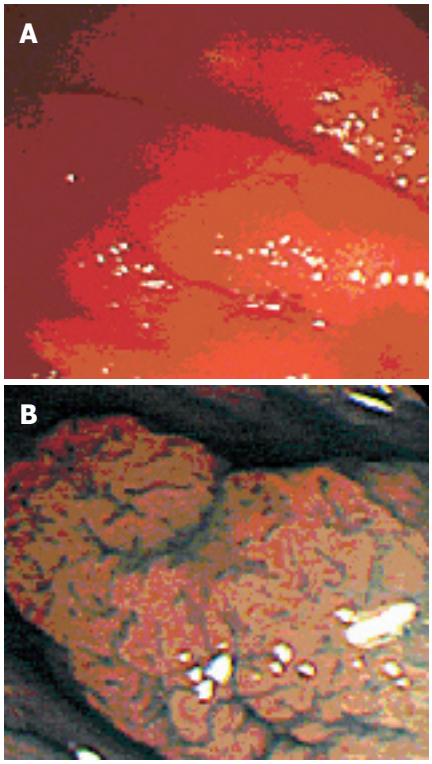

Figure 2 A: Colonoscopy revealed a small flat lesion 5 mm in diameter. **B:** Chromoendoscopy with magnification disclosed a type IIIL pit, and the lesion was diagnosed as neoplastic and resected by snare polypectomy. The histologic diagnosis was adenoma with moderate atypia.

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

Guzel S, Sunamak O, AS A, Celik V, Ferahman M, Nuri MMK, Gazioglu E, Atukeren P, Mutlu O. Effects of hyperbaric oxygen and Pgg-glucan on ischemic colon anastomosis. *World J Gastroenterol* 2006; 12(9): 1421-1425

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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.

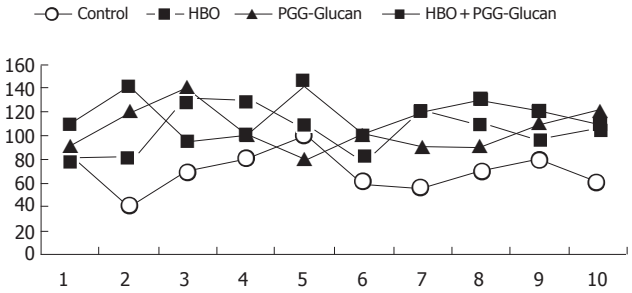

Figure 1 The burst pressure curves of rats for each group (mmHg).

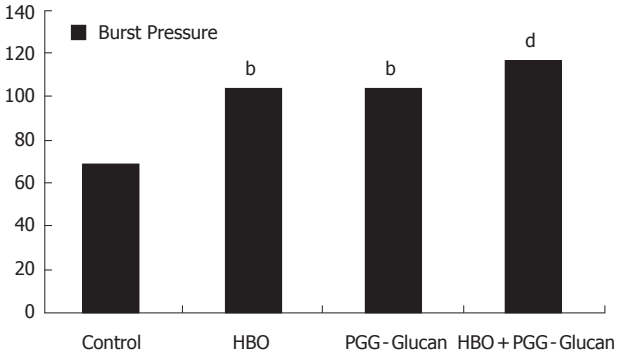

Figure 2 Burst pressure compartment between groups (mm/Hg). [b]$p<0.01$ significant compared to the control group. [d]$p<0.001$ very significant compared to the control group.

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).

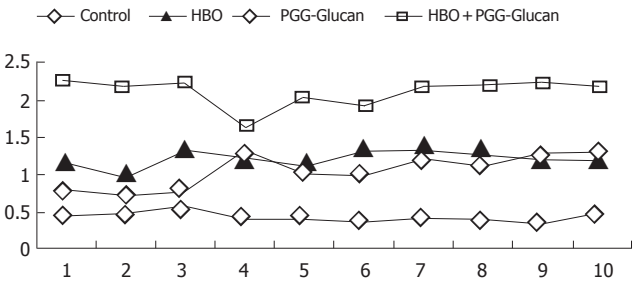

Figure 3 Hydroxyproline level curves of rats for each group (mcg/mg wet tissue).

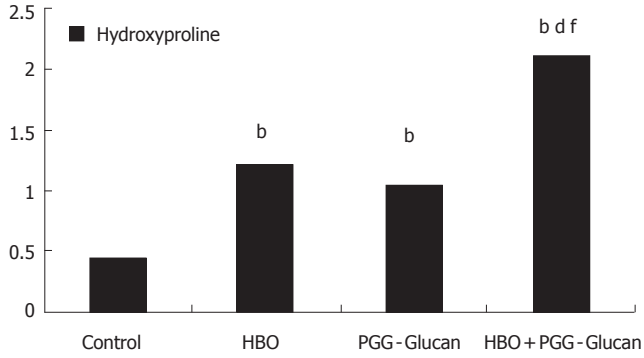

Figure 4 Comparison of Hydroxyproline levels (mcg/mg wet tissue). [b]$P<0.001$ very significant compared to the control group. [d]$P<0.001$ very significant compared to HBO group. [f]$P<0.001$ very significant compared to HBO+PGG-Glucan group.

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.

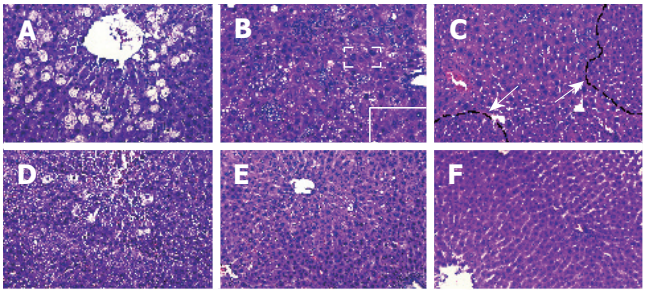

Figure 1 Pathological analysis of rat liver. **A**: Much more severe hepatocyte balloon degeneration and necrosis in perivenous areas of retrorsine-treated group compared with non-treated group (**D**). **B**: Mild bile duct proliferation and megalocytosis (the insert showed the area enclosed in the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

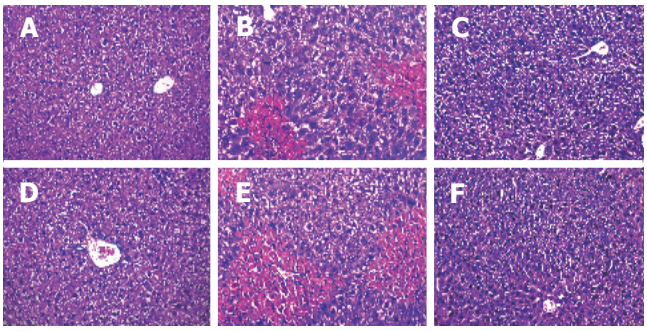

Figure 2 Pathological changes of mouse liver. **A** and **D**: No obvious morphological abnormity. **B** and **E**: Necrosis in perivenous areas, arrow indicates the mitotic figure of hepatocytes. **C** and **F**: Hepatic parenchyma in both groups became normal.

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).

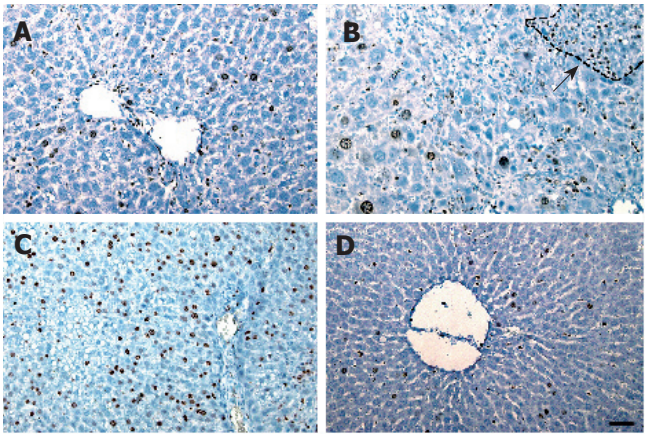

Figure 3 Ki-67 immunohistochemical analysis of rat liver. **A**: A few hepatocytes were Ki-67 positive. **B**: Ki-67 positive hepatocytes were mainly found in small hepatocyte nodules as the arrow indicated. **C**: Abundant Ki-67 positive cells. **D**: Only a few hepatocytes were Ki-67 positive in rat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

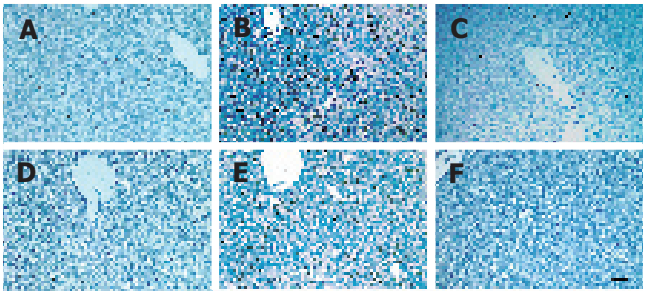

Figure 4 Ki-67 immunohistochemical analysis of mouse liver. **A** and **C**: Some Ki-67 positive cells appeared in retrorsine-treated mice on days 0 and 15. **B** and **D**: The maximum number of Ki-67 positive cells in retrorsine-treated mice on day 4. **E** and **F**: The maximum number of Ki-67 positive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

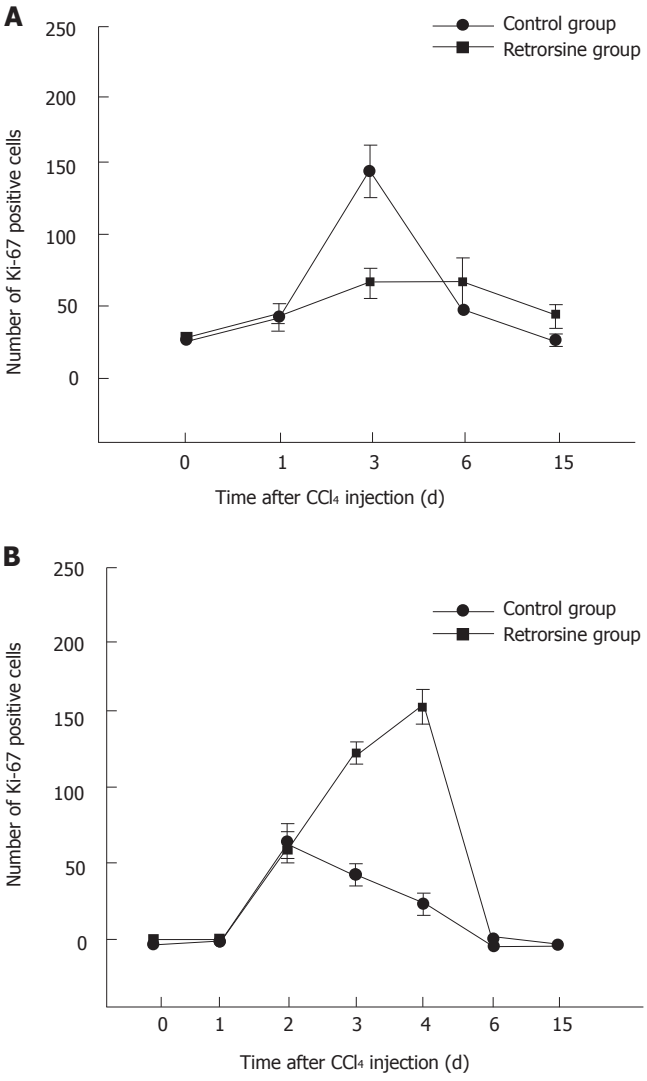

Figure 5 Kinetics of Ki-67 expression in rats (A) and mice (B) after CCl4 injection. **A**: The maximum number of Ki-67 positive hepatocytes on day 6 after injection of CCl4 in retrorsine-treated rats and on day 3 in non-treated group. **B**: The number of Ki-67 positive hepatocytes in retrorsine-treated 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

Chen YK, Zhao XX, Li JG, Lang S, Wang YM. Ductular proliferation in liver tissues with severe chronic hepatitis B: An immunohistochemical study. *World J Gastroenterol* 2006; 12(9): 1443-1446

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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).

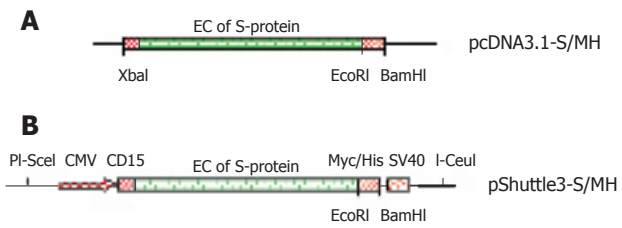

Figure 1 Construct of codon-optimized SARS S-protein genes. The full-length of SARS codon-optimized S-protein gene was modified by inserting Myc/His gene immediately downstream extracellular domain of S-protein gene to generate pcDNA3.1-S/MH (**A**) and the S-protein fused with Myc/His gene was transferred 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.

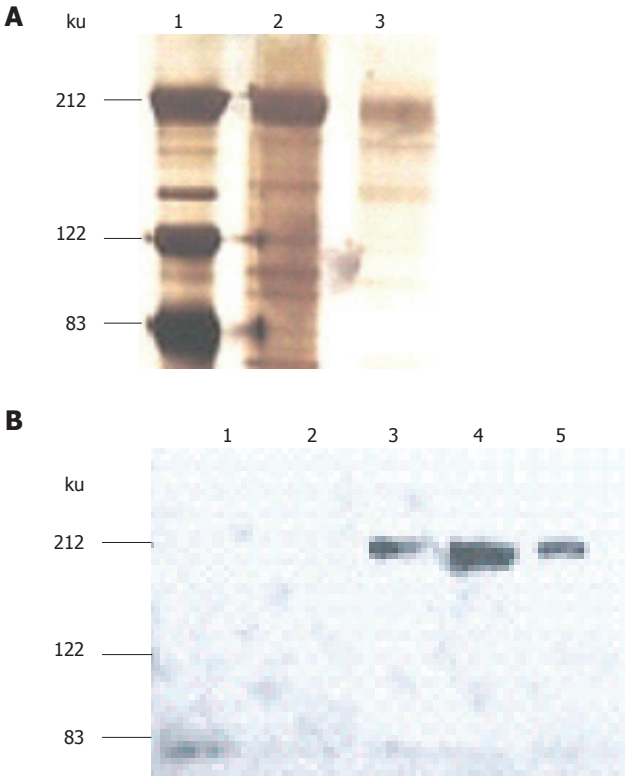

Figure 2 Expression and purification of fused S-protein soluble. S-protein fused with Myc/His was expressed in HEK293 cells mediated by adenovirus and purified on Ni-NTA-agarose column and eluted with imidazole at 6 fractions. The eluted proteins were separated by 7% SDS-PAGE gels and identified by both silver staining (**A**) for elutions 4, 5 and Western-blotting using anti-Myc antibody (**B**) for elutions 1-5.

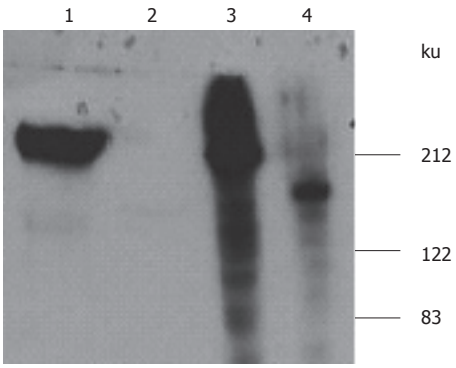

Figure 3 Effects of tunicamycin on expression of S-protein. HEK293 cells were infected with Ad-S/MS virus for 48h in the presence or absence of tunicamycin (7μg/mL). The media were mixed with Ni-NTA-agarose beads and bound protein was eluted with imidazole elution buffer. The pelleted cells were lysed. 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

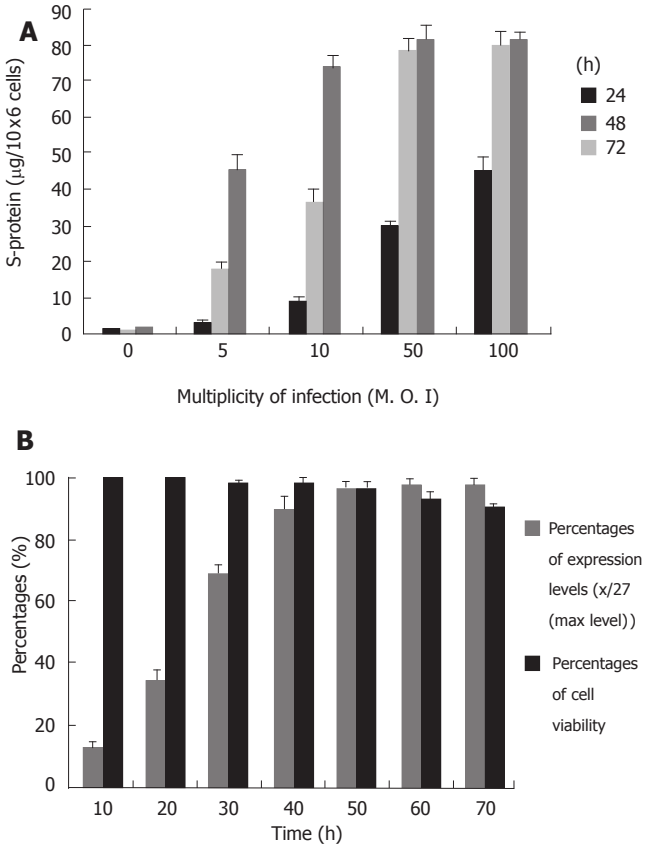

Figure 4 Analysis of expression conditions. **A:** HEK293 cells were infected with Ad-S/MH viruses at different MOI of 5-100. The culture media were harvested at 24, 48 and 72 h after infection. **B:** Cells were infected with same viruses at MOI of 50. The media were harvested at 10, 20, 30, 40, 50, 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

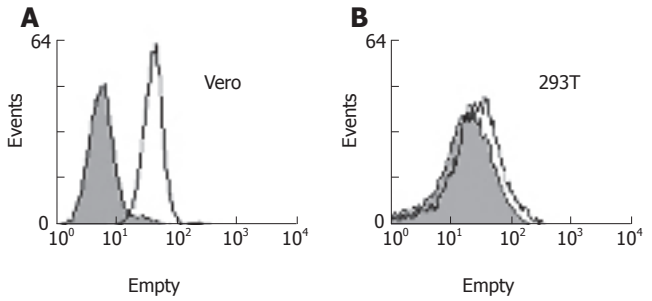

Figure 5 Binding of S-protein to Vero and 293T cells. Vero and 293T cells were detached from culture flask with 0.5 mmol/L EDTA/ PBS, washed and incubated with purified S-protein in 0.5% BSA/ PBS. Binding was measured by flow cytometry using anti-Myc antibody and goat anti-mouse IgG antibody conjugated 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

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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.

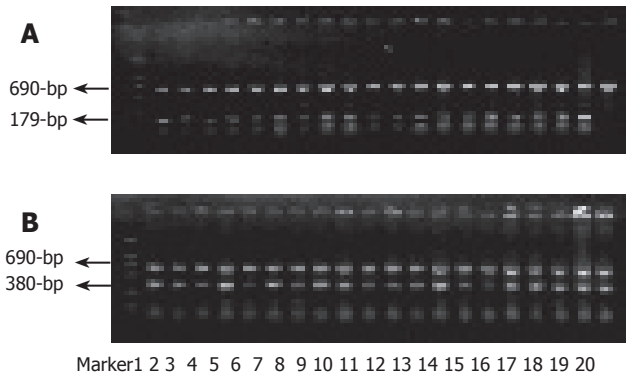

Figure 1 Expression of $VPCAP_1$-R (**A**) and $VPCAP_2$-R (**B**) in gallbladder tissues of patients with gallstones or gallbladder polyps. Lanes 1-15: gallbladder stone group; lanes 16-17: control group; lanes 18-20: gallbladder polyp group.

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

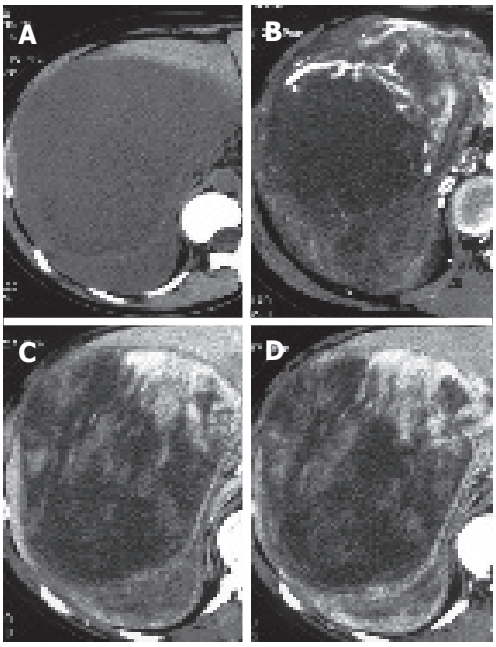

Figure 1 Preoperative dynamic CT images. **A**: plain; **B**: early phase; **C**: delayed phase; **D**: late phase. The tumor detected as a low demsity area and opputied the right hepatic lobe and the peritoneal cavity (**A**). Dynamic study revealed that the tumor enhanced irregularly at any phase (**B**-**D**).

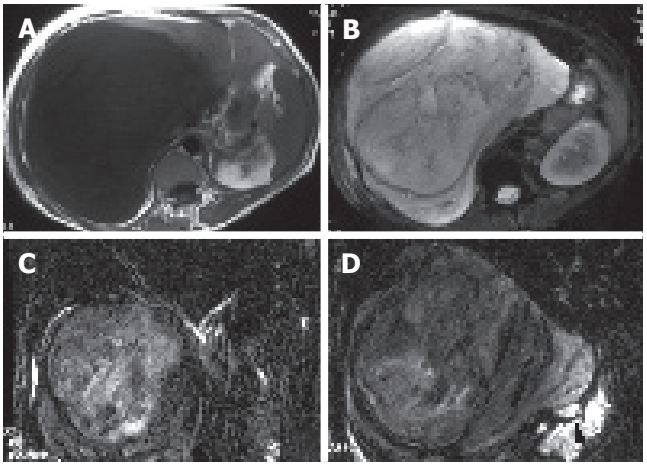

Figure 2 Preoperative MRI images. A: T1-weighted;B:fat-supressed T2-weighted;B and C: MR Cholangiography. The tumor appeared hypointense on T1-weighted images (**A**) and hyperintense on fat-suppressed T2-weighted images (**B**). MR cholangiography revealed that the tumor compressed the right lobe of 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

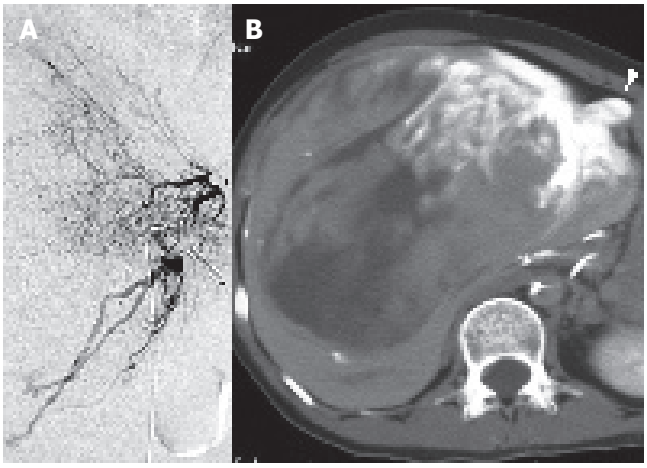

Figure 3 Preoperative angiography. **A**: Hepatic arteriography; **B**: CT during cystic arteriography. Many arteries (i.e., right, middle, left hepatic, and subphrenic arteries fed the tumor (**A**) (arrow head:the root of cystic artery). However, CT during the selective cystic arteriography revealed stronger enhancement of the tumor than during any other arteriographies (**B**)(arrow head:gallbladder).

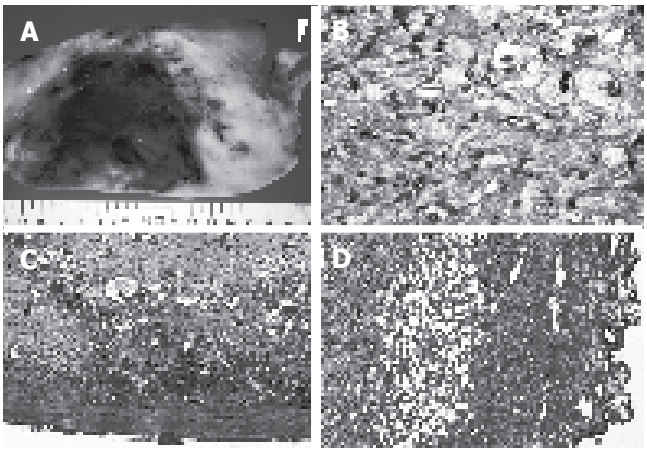

Figure 4 Resected specimen and histological findings. **A**: Cutting section of the tumor; **B**: The main tumor; **C**: The edge of the tumor(hepatic side); **D**: The posterior wall of the gallbladder. The cutting section of the tumor appeared whitish solid and included parts of hemorrhage and necrosis 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-

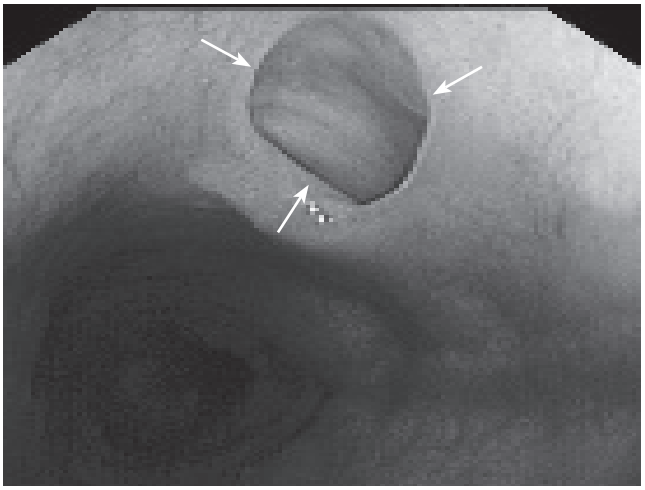

Figure 1 Esophagoscopy reveals a depressed lesion (arrows) that looked as if it was punched at a distance of 23 cm from the incisors in the middle intrathoracic esophagus (E).

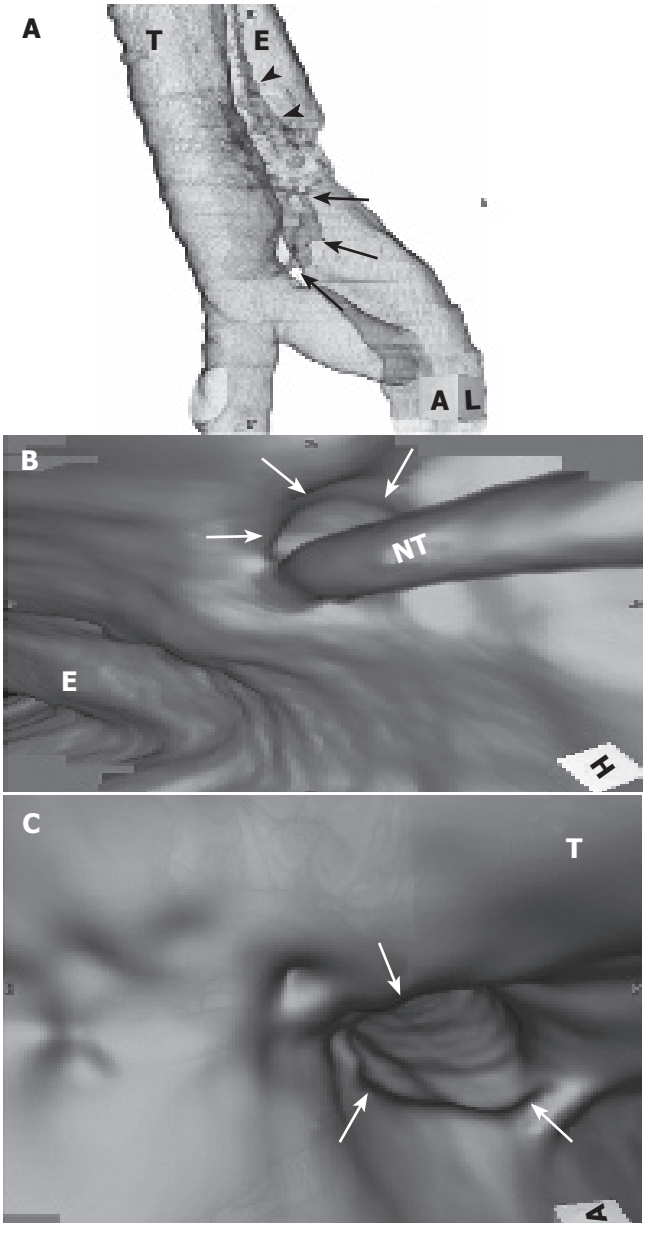

Figure 2 **A:** CT esophagography demonstrates communication between the middle intrathoracic esophagus and the distal trachea just proximal to the carina (arrows). Note a distortion caused by tube inserted into the esophagus (arrowheads). T, trachea; E, esophagus. **B:** Virtual esophagoscopy shows 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.

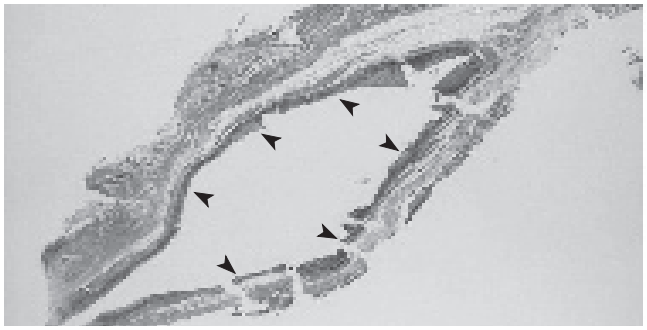

Figure 3 Pathological examination of the resected specimen revealed that the fistula was lined by benign squamous epithelium with the muscularis mucosa (arrowheads).

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.

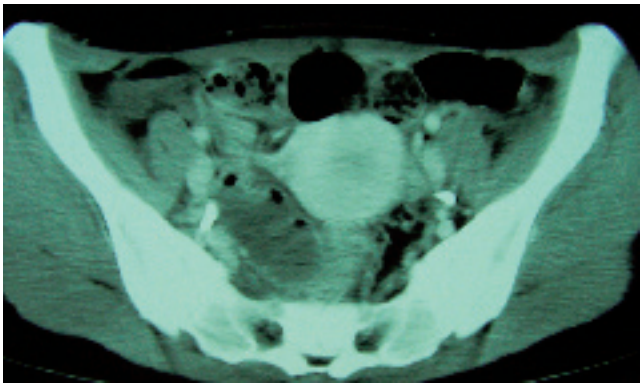

Figure 1 Contrast-enhanced computed tomography demonstrating a dilated small bowel loop in the Douglas's fossa.

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

Tsekouras DK, Katsaragakis S, Theodorou D, Kafri G, Archontovasilis F, Giannopoulos P, Drimousis P, Bramis J. Rectal carcinosarcoma: A case report and review of literature. *World J Gastroenterol* 2006; 12(9): 1481-1484

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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

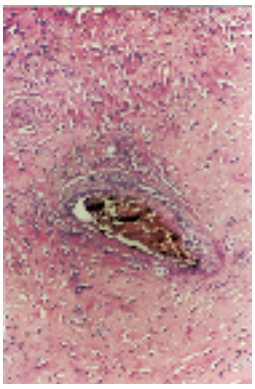

Figure 1 Rectal carcinosarcoma. Infiltration of a vessel wall by carcinomatous cells. The vessel is surrounded by sarcomatous element of carcinosarcoma. (Hematoxylin-eosin, original magnification X100).

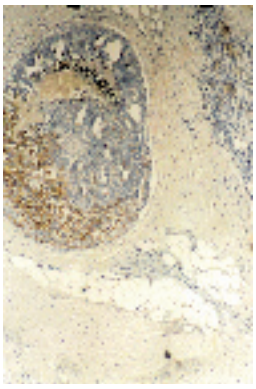

Figure 2 Rectal carcinosarcoma. Carcinomatous elements are positive for cytokeratins (CAM 5.2, original magnification X100).

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

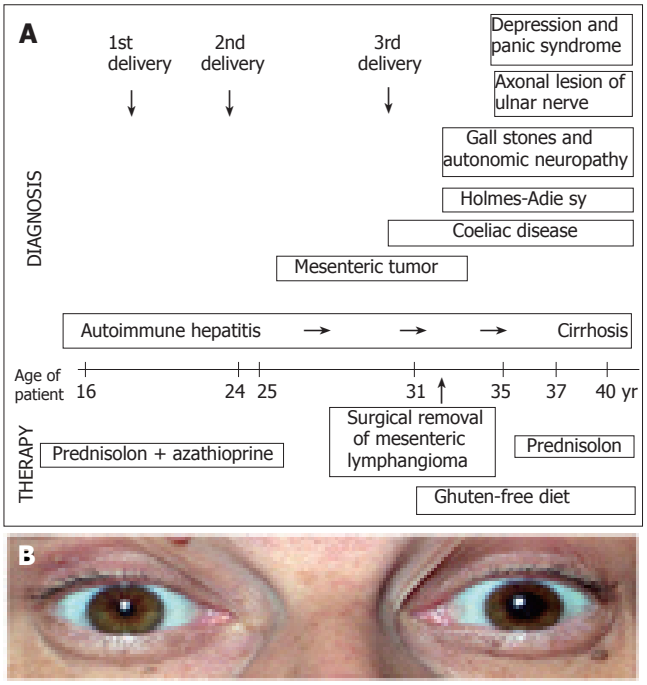

Figure 1 A: Sequence of events, appearance of symptoms and applied treatment. B: Dilated and light insensitive left pupil as symptom of Holmes-Adie syndrome.

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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39	umol	μmol	
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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
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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
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68	Mean±SEM	mean±SE	In figures, tables or text narration
69	*im*	im	intramuscular injection
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75	Iga	*Iga*	writing form of genes
76	*igA*	IgA	writing form of proteins
77	-70 kDa	~70 ku	