World Journal of Gastroenterology ®

Volume 12 Number 5
February 7, 2006

Supported by NSFC
2005-2006

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ISSN 1007-9327 CN 14-1219/R Local Post Offices Code No. 82-261

World Journal of Gastroenterology
www.wjgnet.com
Volume 12
Number 05
Feb 7
2006

ISSN 1007-9327
CN 14-1219/R

WJG

World Journal of Gastroenterology ®

Indexed and Abstracted in:
Index Medicus, MEDLINE, PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, Nature Clinical Practice Gastroenterology and Hepatology, CAB Abstracts and Global Health.

Volume 12 Number 5
February 7, 2006

World J Gastroenterol
2006 February 7; 12(5): 673-836
Online Submissions
www.wjgnet.com/wjg/index.jsp
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A Weekly Journal of Gastroenterology and Hepatology

National Journal Award
2005

World Journal of Gastroenterology®

Volume 12 Number 5
February 7, 2006

Supported by NSFC
2005-2006

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World Journal of Gastroenterology
Volume 12 **Number 5 February 7**, 2006

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World Journal of Gastroenterology (*World J Gastroenterol , WJG*), a leading international journal in gastroenterology and hepatology, has an established reputation for publishing first class research on esophageal cancer, gastric cancer, liver cancer, viral hepatitis, colorectal cancer, and *Helicobacter pylori* infection, providing a forum for both clinicians and scientists, and has been indexed and abstracted in *Index Medicus*, MEDLINE, PubMed, Chemical Abstracts, EMBASE, Abstracts Journals, Nature Clinical Practice Gastroenterology and Hepatology, CAB Abstracts and Global Health. *WJG* is a weekly journal published by The *WJG* Press. The publication date is on 7th, 14th, 21st, and 28th every month. The *WJG* is supported by The National Natural Science Foundation of China, No. 30224801 and No.30424812, which was founded with a name of *China National Journal of New Gastroenterology* on October 1,1995, and renamed as *WJG* on January 25, 1998.

PUBLISHED BY
The WJG Press

PRINTED BY
Printed in Beijing on acid-free paper by Beijing Kexin Printing House

Print ISSN 1007-9327 CN 14-1219/R.

EDITORIAL OFFICE
World Journal of Gastroenterology,
The WJG Press, Apartment 1066 Yishou Garden, 58 North Langxinzhuang Road, PO Box 2345, Beijing 100023, China
Telephone: +86-10-85381901
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

SUBSCRIOTION AND AUTHOR REPRINTS

Jing Wang
The WJG Press, Apartment 1066 Yishou Garden, 58 North Langxinzhuang Road, PO Box 2345, Beijing 100023, China
Telephone: +86-10-85381901
Fax: +86-10-85381893
E-mail: j.wang@wjgnet.com
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Personal Rates
2006 rates: USD 700.00

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World J Gastroenterol 2006 February 7; 12(5):673-677
World Journal of Gastroenterology ISSN 1007-9327

EDITORIAL

Novel mechanisms in functional dyspepsia

Rok Son Choung, Nicholas J Talley

Rok Son Choung, Nicholas J Talley, Clinical Enteric Neuroscience Translational and Epidemiological Research Program, Mayo Clinic, Rochester, MN, United States
Correspondence to: Nicholas J Talley, MD, PhD, Professor of Medicine, Mayo Clinic College of Medicine, Plummer 6-56, 200 First Street S.W., Rochester, MN 55905, United States. alley.nicholas@mayo.edu
Telephone: +1-507-266-1989 Fax: +1-507-538-7202
Received: 2005-08-05 Accepted: 2005-10-09

Abstract

Functional dyspepsia (FD) is a highly prevalent but heterogeneous disorder in which multiple pathogenetic mechanisms are involved. Although there are many studies that have investigated various pathophysiologic mechanisms, the underlying casual pathways associated with FD remain obscure. The currently proposed pathophysiologic mechanisms associated with FD include genetic susceptibility, delayed as well as accelerated gastric emptying, visceral hypersensitivity to acid or mechanical distention, impaired gastric accommodation, abnormal fundic phasic contractions, abnormal antroduodenal motility, acute and chronic infections, and psychosocial comorbidity. A greater understanding of the abnormalities underlying FD may lead to improved management. The aim of this editorial is to provide a critical overview of current pathophysiologic concepts in functional dyspepsia.

Key words: Functional dyspepsia; Gastric function; Pathophysiology

Choung RS, Talley NJ. Novel mechanisms in functional dyspepsia. *World J Gastroenterol* 2006; 12(5): 673-677

http://www.wjgnet.com/1007-9327/12/673.asp

INTRODUCTION

Dyspepsia, or pain or discomfort centered in the upper abdomen, is a common condition accounting for 2-5% of all primary care consults; the annual prevalence is 25%[1-3]. Dyspepsia is a major cause of morbidity and economic loss in the community[4]. A heterogeneous group of pathophysiologic mechanisms have been implicated in the etiology of functional dyspepsia (FD). Delayed gastric emptying[5], antral hypomotility and altered intestinal motility[6], diminished gastric accommodation[7], *Helicobacter pylori* infection[8], abnormal duodenal sensitivity to acid[9], enhanced visceral sensitivity[10], carbohydrate maldigestion[11], food intolerance[12], and psychological factors[13] have all been identified in subgroups of patients with functional dyspepsia, with much overlap.

Despite the high prevalence of FD and its significant impact on social and health care costs, the underlying causal pathways are still obscure. Therefore, our objective here is to briefly provide an overview of the known pathophysiology of FD, and highlight recent advances in our knowledge.

GENETICS

Recent evidence supports the relevance of a genetic milieu in FD. A case-control study by Holtmann *et al*[14] suggested that there is a significant link between GNβ3(C825T) CC genotype and functional dyspepsia (*OR* = 2.2, 95% *CI* =1.4-3.3). Although their study[14] had some limitations including the high prevalence of the CC genotype among control subjects and the relatively modest excess of the genotype in FD subjects, their findings do represent the first identification of a putative genetic predisposition. The association has been independently confirmed[15]. Further studies of the relationship between genetic polymorphisms and other pathophysiologic factors in FD are now needed.

ABNORMALITIES IN UPPER GUT MOTOR FUNCTION

A prevalence of disturbances in gut motor function of about 20-40% in FD patients is now accepted[6,7,16]. The disturbances of gastric motor function most often reported in FD include delayed gastric emptying[16], abnormalities in antral contractility[6] and an impaired accommodation response (a vagally-mediated reflex relaxation of the fundus in response to a meal)[7].

Gastric emptying

Delayed gastric emptying has been described in 25-50% of patients with FD[16-19]. The majority of small sample sized investigations failed to identify association between delayed gastric emptying and any dyspeptic symptoms[20-22]. Stanghellini *et al* [16], in a large study with 343 patients with FD, showed that female sex, relevant and severe postprandial fullness, and severe vomiting were independently associated with delayed gastric emptying of solids. Other European studies have generally confirmed

these findings[17,18], but studies from the US have not[23,24]. Recently, accelerated gastric emptying in FD has also been associated with postprandial fullness, bloating, nausea, and pain, presumably through an early dumping syndrome[25]. These findings imply that administration of a prokinetic agent to accelerate gastric emptying may actually lead to worsening, rather than to improvement of symptoms, in at least a proportion of patients with FD.

Impaired gastric accommodation

Gastric accommodation is a vagally mediated reflex that occurs post-prandially, results in reduction of tone, and provides a reservoir for the meal; it enables the stomach to handle increases in intragastric volume without proportional increases of intragastric pressures[7,26,27].

Studies by the barostat, scintigraphy, ultrasonography, single photon emission computed tomography (SPECT), and non-imaging assessments (satiation drinking test) have all suggested abnormal accommodation in up to 40% of patients with functional dyspepsia[7,26-30]. The mechanism of impaired gastric accommodation is, however, not clearly understood. Tack *et al* [26] proposed that impaired proximal stomach accommodation of an ingested meal was associated with early satiety and weight loss in FD, but other studies have not confirmed these symptom associati ons[24,28].

Relaxation of the proximal stomach can be activated by duodenal distension or nutrient infusion, or via a vago-vagal reflex pathway, and it requires activation of intrinsic nitrergic neurons in the stomach. Recent reports[31,32] have suggested that a defect at the level of the gastric intrinsic nitrergic neurons may be relevant. Abnormal vagal reflexes may also play a role, because postvagotomy patients have a similar impairment of gastric accommodation, and abnormal vagal function is observed in patients with FD[33,34].

Abnormal phasic contractility of the proximal stomach

Phasic fundic contractions induce transient increases in gastric wall tension and can be perceived in FD[8,35]. In the recent study using a gastric barostat, unsuppressed postprandial phasic activity in the proximal stomach was present in a small subgroup of patients with FD[36]. This was associated with symptoms of severe postprandial bloating. However, the mechanisms underlying unsuppressed phasic contractility are unclear and further studies will be required.

Abnormalities of gastric myoelectrical rhythm

Electrogastrography obtains a cutaneous recording of gastric myoelectrical activity from abdominal surface electrodes. Some studies[37-39] have reported gastric myoelectrical abnormalities are found in up to two-thirds of patients with FD. A close relationship between the presence of delayed gastric emptying and abnormalities of gastric electrical rhythm has been reported[37-39]. However, their relevance to symptom generation is still uncertain.

Antro-duodenal dysmotility

Manometric studies have demonstrated antral hypomotility in a large proportion of those with FD[27,40,41]. Antral hypomotility could be associated with gastric stasis manifested by a slower emptying rate of meals[40], but its correlation with specific symptoms is poor.

DISTURBANCES IN VISCERAL SENSITIVITY

The gut wall contains three kinds of neural receptors: chemoreceptors in the mucosa, which respond to chemical stimuli; mechanoreceptors in the smooth muscle layer, which respond to stretch or compression; and nociceptors, the most numerous receptors in all layers, which are commonly silent, but can be recruited by any stimulus strong enough to induce pain[42].

Visceral hypersensitivity to mechanical distension

Increased perception to visceral physiological or minor noxious stimuli, i.e., a lower threshold for sensation, has been considered one of major pathophysiologic mechanisms in the functional gastrointestinal disorders[28,43-44]. The level of abnormality where visceral hypersensitivity is located is not clear, but several observations suggest that alterations exist at the level of the central nervous system or in addition to hyperexcitability of visceral afferents[43-46].

Several studies have found dyspeptic patients have lower thresholds for first perception and discomfort or pain during isobaric gastric distension or jejunal distension[29,47,48]. Mertz *et al*[44] reported that hypersensitivity to gastric balloon distension was specific for FD, and Holtmann *et al*[49, 50] have shown a failure of sensory thresholds to increase in patients with FD. While Tack *et al*[43] have shown hypersensitivity to gastric distention was associated with a higher prevalence of post-prandial pain, belching and weight loss. The symptom correlations remain to be confirmed.

Abnormal central nervous system processing of afferent information during gastric stimuli has been suggested to be one of the mechanisms of visceral hypersensitivity[46]. Ladabaum *et al*[51] performed PET scanning during distal gastric distension in healthy volunteers, and found increases in activation of somatic and visceral pain processing areas that were proportional to the distension-evoking stimuli. Vandenbergh *et al*[52] also performed PET scanning after proximal gastric distension in healthy volunteers, and found a neuronal network processing distention stimuli from the proximal stomach. Further study is needed to assess whether abnormalities in central processing of visceral stimuli occur in FD.

Visceral hypersensitivity to chemical stimuli

Several studies[53-55] have been conducted to evaluate chemical sensitivity in FD, but its mechanism is not clear. In both healthy subjects and in patients with FD, perception of gastric distension was enhanced by nutrient lipids but not by glucose[55]. Feinle *et al*[56,57] studied the effects of lipid on gastrointestinal sensation in healthy and dyspeptic subjects and suggested that cholecystokinin A (CCK-A) and serotonergic (5-HT_3) receptors mediate, at least in part, gastrointestinal sensation. They reported that intravenous administration of a CCK-A receptor antagonist reduced the effects of duodenal lipid on gastric

relaxation and gastrointestinal sensations during gastric distension. Although they studied only small numbers of patients, these findings support changes in chemical sensitivity in FD; this needs to be validated in larger studies.

In a subset of FD patients, but not in healthy controls, intraduodenal infusion of hydrochloric acid (HCl) was found to induce nausea[9], suggesting duodenal hypersensitivity to acid. Some patients had impaired clearance of acid from the duodenum and impaired duodenal motor response to acid. Recently, Tack *et al* [58] observed that duodenal acidification increased proximal gastric mechanosensitivity, induced proximal gastric relaxation, and inhibited proximal gastric accommodation to a meal. However, the underlying mechanisms remain to be elucidated in future studies.

INFECTIOUS AGENTS

Acute and chronic infections may be involved in the pathogenesis of the functional bowel disorders[31,59-61].

Helicobacter pylori infection

Many studies and several meta-analysis have tried to establish a relationship between *Helicobacter pylori* (*H pylori*) infection and FD[59-63]. The most recently published meta-analysis[64] suggests that *H pylori* eradication at 12 months has a small but statistically significant benefit in the treatment of FD (relative risk of remaining dyspeptic with *H pylori* eradication therapy = 0.91; 95% *CI* = 0.87-0.99). While statistically significant, the clinical significance of this finding is less clear because the effect is small, that is, 15 *H pylori-positive* dyspeptic patients will need to be treated to achieve just one cure.

The association between *H pylori* infection and pathophysiologic mechanisms has been investigated; however, no consistent differences in the prevalence and severity of individual symptoms, gastric emptying rate, or gastric relaxation after a meal were found between *H pylori*-positive and *H pylori*-negative subjects[60,61].

Post-infectious dyspepsia

It has now been well established that irritable bowel syndrome may follow an acute intestinal infection. Post-infectious dyspepsia has been recognized as a possible clinical entity by Tack *et al* [31]. In a large retrospective, tertiary referral center study, they showed that a subset of dyspeptic patients has a history suggestive of post-infectious dyspepsia. Compared with patients with unspecified-onset dyspepsia, patients with presumed post-infectious dyspepsia have more prevalent symptoms of early satiety, weight loss, nausea, and vomiting and had a significantly higher prevalence of impaired accommodation of the proximal stomach, whereas both groups did not differ in the prevalence of delayed gastric emptying, or hypersensitivity to gastric distention[31]. They suggested that impaired accommodation in patients with presumed post-infectious dyspepsia is attributable to a dysfunction at the level of gastric nitrergic neurons. In this study, the lack of prospective design limited the validity of the results.

In a prospective cohort study, Mearin *et al* [65] found that development of dyspepsia was increased 5-fold at 1 year after acute *Salmonella* gastroenteritis as compared with control subjects without baseline infection. There were some limitations because of the low response rate in the 1-year follow-up survey and lack of information on psychological status and stressful events[65]. Further studies are needed to identify the underlying pathophysiology and risk factors, and define the long-term prognosis.

PSYCHOSOCIAL ABNORMALITIES

In FD patients, a number of psychological and personality factors have been observed, including somatization, depression, and anxiety as well as health seeking behavior and alterations in illness behavior and coping styles[66-68]. These psychosocial factors are influenced by and influence GI symptoms; this bidirectional flow is mediated through the brain-gut axis[43]. The value of psychological therapies in FD, to date, is promising, but further studies of psychological treatment of FD are needed[69].

CONCLUSIONS

FD is a highly heterogeneous disorder. Traditional pathophysiological paradigms are still inadequate to explain the variation in the symptoms observed. Current symptom subclassifications have also largely failed to identify responders to specific therapies. A new classification based on mechanisms and specific symptoms needs to be considered to further advance the field. The contributors to this heterogeneity need to be better defined, and be environmental, pathological, psychological, or genetic. This could lead to better targeting of treatment in FD patients with unexplained epigastric pain or discomfort.

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S- Editor Wang J and Kumar M **L- Editor** Elsevier HK **E- Editor** Li HY

PO Box 2345, Beijing 100023, China
www.wjgnet.com
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World J Gastroenterol 2006 February 7; 12(5):678-685
World Journal of Gastroenterology ISSN 1007-9327

REVIEW

Liver cirrhosis and arterial hypertension

Jens H Henriksen, Soren Moller

Jens H Henriksen, Soren Moller, Department of Clinical Physiology, 239, University of Copenhagen, H:S Hvidovre Hospital, Copenhagen, Denmark
Correspondence to: Jens H Henriksen, MD, Professor of Clinical Physiology, Department of Clinical Physiology, 239 Hvidovre University Hospital, DK-2650 Hvidovre, Denmark. jens.h.henriksen@hh.hosp.dk
Telephone: +45-3632-2203 Fax:+45-3632-3750
Received: 2005-08-09 Accepted: 2005-08-22

Abstract

Characteristic findings in patients with cirrhosis are vasodilatation with low overall systemic vascular resistance, high arterial compliance, increased cardiac output, secondary activation of counter-regulatory systems (renin-angiotensin-aldosterone system, sympathetic nervous system, release of vasopressin), and resistance to vasopressors. The vasodilatory state is mediated through adrenomedullin, calcitonin gene-related peptide, nitric oxide, and other vasodilators, and is most pronounced in the splanchnic area. This constitutes an effective (although relative) counterbalance to increased arterial blood pressure. This review considers the alterations in systemic hemodynamics in patients with cirrhosis in relation to essential hypertension and arterial hypertension of the renal origin. Subjects with arterial hypertension (essential, secondary) may become normotensive during the development of cirrhosis, and arterial hypertension is rarely manifested in patients with cirrhosis, even in cases with renovascular disease and high circulating renin activity. There is much dispute as to the understanding of homoeostatic regulation in cirrhotic patients with manifest arterial hypertension. This most likely includes the combination of vasodilatation and vasoconstriction in parallel.

Keywords: Arterial compliance; Central vascular filling; Chyperdynamic circulation; Kidney function, Nitric oxide; Blood pressure regulation; Renin–angiotensin–aldosterone system; Sympathetic nervous system; Vasodilatation; Vasoconstriction

Henriksen JH, Moller S. Liver cirrhosis and arterial hypertension. *World J Gastroenterol* 2006; 12(5): 678-685

http://www.wjgnet.com/1007-9327/12/678.asp

INTRODUCTION

Arterial hypertension is seldom found in patients with liver disease, but patients with alcoholic fatty liver quite often present with raised arterial blood pressure. Renal involvement is seen in hepatitis B and in certain cases this may be accompanied by arterial hypertension. Otherwise, the clinical course of most liver diseases is characterized by low arterial blood pressure. This paper focuses on the circulatory dysfunction in cirrhosis, which is the most common characteristic of the chronic liver diseases.

Abnormalities in the splanchnic circulation are well described in patients with cirrhosis[5-7]. These include increased inflow to the splanchnic system and portal venous hypertension. Moreover, such patients are often hyperkinetic with increased heart rate and cardiac output. The hyperkinetic systemic circulation is related to the severity of cirrhosis, sodium-water retention, and presence of ascites[8]. The overall systemic vascular resistance is decreased, the blood volume is abnormally distributed with relative and absolute central hypovolemia, and cardiovascular, renal, and other organ dysfunction is common, including abnormal neurohormonal regulation[8-10]. Essential hypertension is frequently reported in both developed and developing countries, and the prevalence of essential hypertension in patients with cirrhosis would be expected to be around 15%[11,12]. However, most reports put the coexistence of cirrhosis and arterial hypertension much lower. As in patients with essential hypertension, most circulatory changes in cirrhosis are dysfunctional, rather than structural.

This review considers the cardiovascular system, neurohormonal regulatory systems, kidney function, and sodium-water retention with respect to arterial blood pressure and its regulation in patients with cirrhosis with the purpose of analyzing the background of the profound circulatory changes, their development, and complications. These elements are considered with respect to essential hypertension and arterial hypertension of renal origin.

HEPATOSPLANCHNIC CIRCULATION

The mechanisms behind the circulatory changes in the hepatosplanchnic system are both functional and structural[13]. Thus, a reduced hepatic vascular cross-sectional area, owing to the formation of fibrosis and noduli, is well known[13]. Swelling of hepatocytes and contraction of myofibrillary cells also contribute to hinder the hepatic outflow. Low content in endothelial cells of the vasodilator, nitric oxide (NO), may contribute to

Table 1 Haemodynamics in central and peripheral vascular beds in patients with cirrhosis

Heart
Heart rate ↑
Cardiac output ↑
Left atrial volume ↑
Left ventricular volume → (↑)
Left ventricular end-diastolic pressure →
Right atrial volume →↑↓
Right atrial pressure →↑
Right ventricular volume →↑↓
Right ventricular end-diastolic pressure →
Systemic circulation
Plasma volume ↑
Total blood volume ↑
Central and arterial blood volume ↓ (→)
Noncentral blood volume ↑
Arterial blood pressure ↓ (→)
Systemic vascular resistance ↓
Arterial compliance ↑
Total vascular compliance ↑
Pulmonary circulation
Pulmonary blood flow ↑
Pulmonary artery pressure →↑
Pulmonary vascular resistance ↓ (↑)
Skeletal and cutaneous muscle circulation
Skeletal muscular blood flow ↑ → ↓
Cutaneous blood flow ↑ → ↓
Hepatic and splanchnic circulation
Hepatic blood flow ↑ → (↑)
Hepatic venous pressure gradient ↑
Renal circulation
Renal blood flow ↓
Glomerular filtration rate ↓ →

↑, increase; →, no change; ↓, decrease. Parentheses denote minor changes.

hepatic vasoconstriction[14]. In addition, vasodilators are produced in excess and escape degradation by the diseased liver, which may play a role in splanchnic and systemic vasodilatation in concert with local factors, especially shear stress[6,15,16]. The result is an increased splanchnic inflow that aggravates and prolongs the portal hypertension, which is an essential element in cirrhosis[7]. The formation of portosystemic collaterals with a highly increased flow through the azygos venous system contributes to the decreased overall vascular resistance[5,7].

SYSTEMIC CIRCULATION

Overall systemic vascular resistance

Overall systemic vascular resistance is decreased in most patients with cirrhosis. Recent studies have shown that the circulation in most vascular territories is disturbed. A close look at the individual organs and tissues reveals vascular beds with decreased perfusion, normal perfusion, and increased perfusion. This indicates areas with high resistance (such as the kidneys), normal resistance (such as the brain), and low resistance (such as the splanchnic system)[6,7] (Table 1). The pathogenesis of hyporeactivity of the vascular system in cirrhosis is multifactorial[17]. Clinical and experimental observations favor the presence of a surplus of circulating vasodilators. Combined with a decreased sensitivity to pressor substances, this leads to vasodilatation and reduced vascular resistance[7,17]. Vasodilatation and the resulting activation of counter-regulatory mechanisms are closely related to the circulatory dysfunction in chronic liver disease[18,19]. The reduced vascular responsiveness may be generated by a decrease in the number of receptors, a change in receptor affinity, and several postreceptor defects[20]. Recent evidence suggests that all of these mechanisms are working in concert in cirrhosis[20].

The coexistence of decreased splanchnic resistance (as in cirrhosis) and increased peripheral resistance (as in essential arterial hypertension) is likely and may depend on the altered circulating vasoactive substances, reactivity of vasomotor and regulatory systems, etc[21,22] The result may be increased, normal, or decreased systemic vascular resistance. However, most evidence indicates that a decreased overall systemic vascular resistance with a low arterial blood pressure is most often the outcome, even in patients with primary essential hypertension[23-25]. Research on vascular hyporeactivity has primarily focused on calcitonin gene-related peptide (CGRP), NO, tumor necrosis factor-α (TNF-α), adrenomedullin, and endocannabinoids[17,18]. In addition, there is substantial evidence of autonomic defects in patients with cirrhosis. Thus, the sympathetic nervous system is both dysfunctional and overactivated The reason for vasoconstriction in some vascular areas is mainly overwhelming activity of pressor systems, but dysfunctional elements are also present[5,19,26].

Arterial blood pressure

The arterial blood pressure depends on the cardiac output and the systemic vascular resistance. The former is primarily determined by heart rate, venous return, and myocardial contractility. The latter is determined by the tone of smooth muscle cells in the small arteries and arterioles, which is governed by complex local and central neurohumoral factors. Arterial blood pressure has a circadian rhythm, but is kept within its habitual range by negative feedback baroreceptor reflexes, volume regulation, and other regulatory systems[12].

According to standardized recommendations, arterial blood pressure in patients with cirrhosis is often measured in the morning. However, 24-h determinations of blood pressure show that, during the day, the arterial blood pressure is substantially reduced compared with that of the controls, but at night it is normal[27]. The shifted and flat blood pressure-heart rate relation in patients with cirrhosis suggests that the regulation of their blood pressure is abnormal[27]. Moreover, the hemodynamic dysregulation is more pronounced with increasing severity of the liver disease[9]. The abnormal diurnal variation of the arterial blood pressure and the drastic activation of the neurohormonal systems probably contribute to the sodium-water retention.

Studies of patients with cirrhosis and arterial hypertension show that the prevalence is substantially reduced with raised arterial blood pressure in only 3-7% of such patients[21,24,28,29]. In addition, a few longitudinal studies have indicated that in patients with essential arterial hypertension, the raised arterial blood pressure is

Table 2 Haemodynamics and neuroendocrine mechanisms in patients with cirrhosis and different types of arterial hypertension

	Cirrhosis	Cirrhosis with hypertension	Essential hypertension	Renovascular hypertension
Plasma and blood volume	↑	↑	→	→
Central blood volume	→↓	→↓	→(↑)	→(↑)
Systemic vascular resistance	↓	→	↑	↑
Arterial compliance	↑	→	↓	↓
Renal blood flow	↓	↓→	↓	↓
Sodium retention	↑	↑	→↑	↑
Sympathetic nervous activity	↑↑	↑	→	→↑
Renin–angiotensin–aldosterone system	↑↑	↑→	→↓	↑
Vasopressin	↑		→	→

→ ↑ ↓, denote normal, increased, and decreased values, respectively; parentheses denote minor changes.

reduced to normal values after the onset of cirrhosis[23]. Thus, patients with essential hypertension may become normotensive during the development of chronic liver disease. In patients with cirrhosis, the liver disease may protect against the development of arterial hypertension, and in patients with advanced cirrhosis raised arterial pressure is very uncommon[24,30].

Gentilini *et al.* have investigated patients with early non-alcoholic cirrhosis and arterial hypertension[21]. They found that the arterial hypertensive cirrhotic patients were normodynamic with a normal heart rate and cardiac output. By contrast, their normotensive cirrhotic counterparts were hyperdynamic in the supine position and normodynamic in the upright position. They concluded that hypertensive cirrhotic patients had an impaired cardiovascular response to postural changes, but a lesser degree of renal dysfunction while standing than that of their normotensive counterparts[21]. No difference in the plasma renin activity of the normotensive and hypertensive cirrhotics was present[21]. By contrast, Veglio *et al* had previously found that hypertensive cirrhotic patients had a lower plasma renin activity, both supine and upright[29]. They concluded that the peripheral pressor effect of vasoactive hormones was increased and the effective blood volume was enhanced in arterial hypertensive cirrhotic patients[29]. The authors of the present review have been unable to confirm Gentilini *et al's* result of a normodynamic circulation in early hypertensive cirrhotic patients and Veglio *et al's* hypothesis of an enhanced effective blood volume in these patients[31]. However, Henriksen *et al's* study showed that hypertensive cirrhotic patients are less vasodilated than their normotensive counterparts[31], (Figure 1).Essential differences in arterial hypertension and cirrhosis are summarized in Table 2.

Arterial compliance

Arterial compliance (i.e., an increase in intra-arterial volume relative to an increase in transmural arterial blood pressure) is raised in patients with decompensated cirrhosis[9,21]. Recent investigations have shown that the altered static and dynamic characteristics of the wall of large arteries in patients with cirrhosis are closely associated with the circulatory and homoeostatic derangement[9,21,32-35]. The changes in arterial mechanics are, at least in part, reversible[32]. Arterial compliance is an important determinant of the coupling between the heart and the arterial system and of the dynamics of intravascular volume relocation[36]. Reduced arterial blood volume and blood pressure are the most likely elements in the elevated arterial compliance in advanced cirrhosis[9,13]. The increased arterial compliance in patients with cirrhosis is directly related to the severity of the liver disease and the circulating level of the vasodilator CGRP[32]. It is inversely related to circulating adrenaline, but is not significantly related to the indicators of the potent vasoconstrictor systems (sympathetic nervous system (SNS) and endothelin-1)[32-35]. In addition, the abnormal arterial compliance is related to blood volume abnormalities, hypoxia, and to the abnormalities in the C-type natriuretic peptide (CNP), but not to the atrial natriuretic peptide (ANP)[33,35]. Arterial compliance is not affected by beta-adrenergic blockade, but infusion of the vasopressin analog terlipressin almost normalizes it[37]. Both types of drugs are used in the treatment of portal hypertension[38]. In arterial hypertensive cirrhotic patients, arterial compliance is normal or almost normal[31].

Blood volume distribution

Patients with cirrhosis have increased blood and plasma volumes[8,10]. Distribution of blood to the different vascular beds is abnormal and more so in severe liver disease[39-42]. Moreover, dynamic, as well as static, methods have revealed that the central (thoracic) blood volume is most often decreased, (Figure 1c), whereas the non-central (especially splanchnic) blood volume is increased in patients with cirrhosis[10,39]. The effective arterial blood volume is decreased, and the abnormal volume distribution contributes to the systemic circulatory derangement[42]. Thus, the central circulation time (that is, central blood volume relative to the cardiac output) in cirrhosis has a significant relation to the survival and is substantially reduced also in cirrhotic patients with arterial hypertension[31]. During volume expansion (either by blood transfusion, or infusion of albumin or osmotic material) most cirrhotic patients respond with a further reduction in systemic vascular resistance rather than an increase in arterial blood pressure[33,42]. Future studies may disclose whether raised arterial blood pressure is indicative of normal arterial filling or the result of arterioconstrictive adaptation to a low arterial blood volume . Moreover, the balance between vascular areas with vasodilatation and vasoconstriction may be substantially altered in arterial hypertensive patients with cirrhosis[31].

Cardiac dysfunction

In patients with advanced cirrhosis increased heart rate and stroke volume led to a high cardiac output[18,36]. A left ventricular failure may be latent, because of the decreased afterload (as reflected by reduced systemic vascular resistance and increased arterial compliance)[20,36]. Cardiac dysfunction may become manifest under stran or treatment with vasoconstrictors. Cirrhotic cardiomyopathy includes

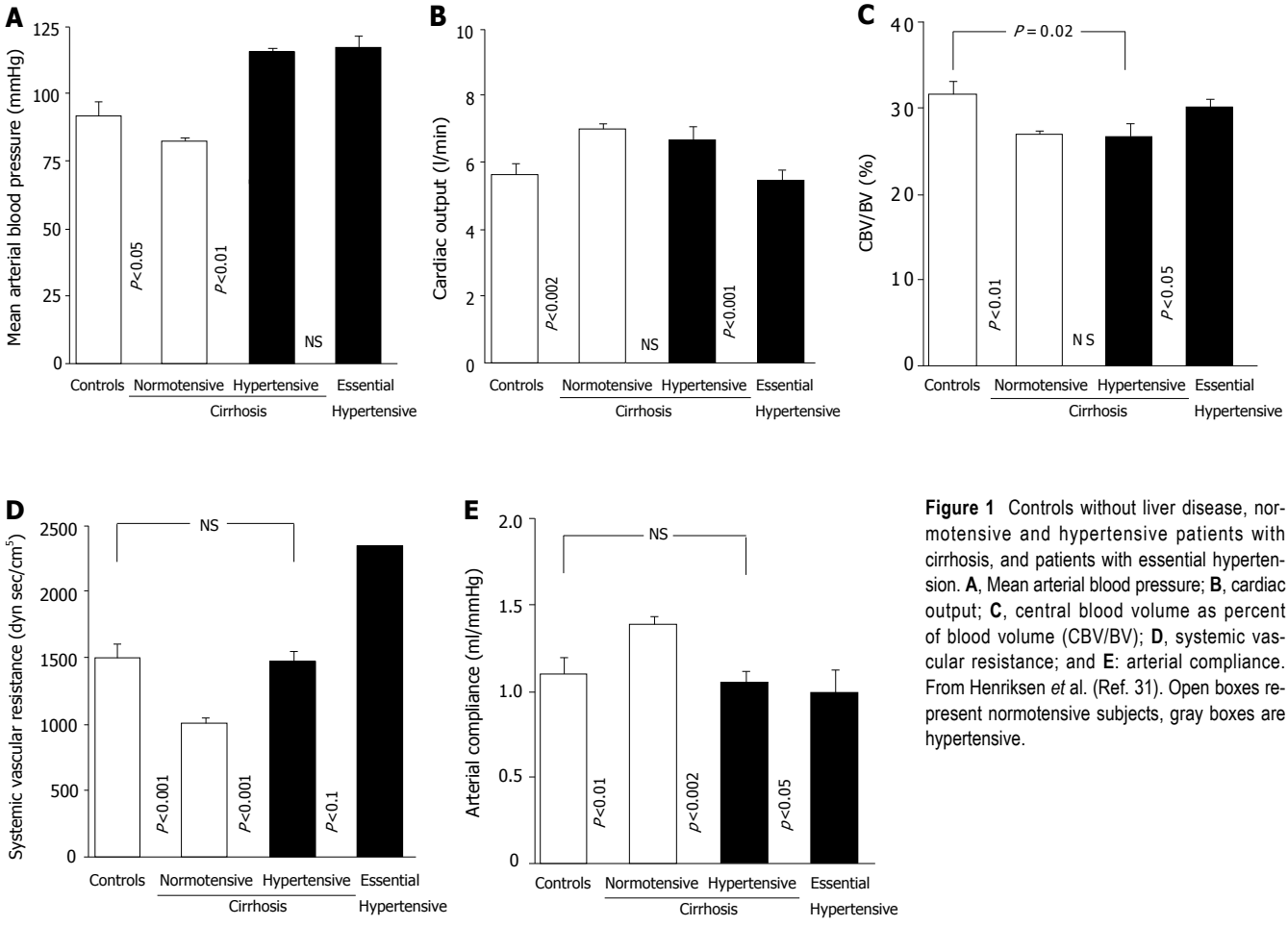

Figure 1 Controls without liver disease, normotensive and hypertensive patients with cirrhosis, and patients with essential hypertension. **A**, Mean arterial blood pressure; **B**, cardiac output; **C**, central blood volume as percent of blood volume (CBV/BV); **D**, systemic vascular resistance; and **E**: arterial compliance. From Henriksen *et* al. (Ref. 31). Open boxes represent normotensive subjects, gray boxes are hypertensive.

impaired cardiac contractility (systolic dysfunction), diastolic dysfunction, prolonged *Q-T* interval, reduced beta-adrenoceptor density, postreceptor signal defects, abnormal excitation-contraction coupling, and molecular abnormalities[20,36,43]. Recently, it has been substantiated that the elevated levels of circulating pro-brain natriuretic peptide (pro-BNP) and brain natriuretic peptide (BNP) found in patients with cirrhosis are not related to the decreased disposal of these peptides, but to the increased production in the cirrhotic heart, thus indicating cardiac dysfunction in cirrhosis[44]. In cirrhotic patients with arterial hypertension signs of myocardial involvement may be especially pronounced[21,30].

Cirrhotic cardiomyopathy must be differentiated from alcoholic heart muscle disease, which is caused by impaired contractile protein synthesis and formation of immunogenic cardiac protein-acetaldehyde adducts. The presence of antibodies to these adducts may be a marker for alcoholic heart muscle disease and possibly for its pathogenesis[36].

NEUROHUMORAL REGULATION OF ARTERIAL BLOOD PRESSURE AND VOLUME DISTRIBUTION IN CIRRHOSIS

Splanchnic arteriolar vasodilatation may lead to abnormal distribution of the circulating medium with a decrease in the effective blood volume and low arterial blood volume and pressure, and to an expansion of the non-central blood volume, including the splanchnic bed[14,26,42]. Most patients with advanced decompensated cirrhosis have a highly increased activity of vasopressor systems with a highly elevated plasma renin activity, circulating noradrenaline, and plasma vasopressin[5,6,26]. In most cases there is a progressive increase from normal values to moderately increased values in preportal hypertensive patients to a further increase in portal hypertensive patients to highly increased values in ascitic patients, especially in those with functional renal failure[5,45]. Recent studies indicate that infusion of albumin may suppress the renin–angiotensin–aldosterone system (RAAS), especially in patients with advanced cirrhosis, and this may prevent further circulatory dysfunction and even reduce it[33].

In addition to the activation of the SNS, RAAS, and vasopressin, several studies have shown increased circulating endothelin-1, which indicates that endothelin systems are also activated in advanced cirrhosis[46]. Systemic vasodilatation is a key feature in the activation of vasoconstrictive and sodium-water retaining systems[5,16,22]. Systemic hemodynamic alterations are also important for the low renal blood flow and renal dysfunction in cirrhosis. The pressor systems most often counter-regulate the otherwise low arterial blood pressure in cirrhosis to a level almost within the normal range. Whereas a significant negative correlation of endothelin-1 to arterial blood pressure has been described, most studies have focused on the very low arterial blood pressure after the blockade

of the SNS (for instance, by clonidine, prazosin), RAAS (for instance by ACE-inhibitors, A II blockers), and the vasopressin effect (V_1 antagonists)[16,45-48]. The activity in the pressor systems in advanced cirrhosis is among the most extreme known to pathophysiology. The presence of autonomic defects in patients with cirrhosis is evident from various studies on hemodynamic response to standardized cardiovascular reflex tests, such as heart rate variability, the Valsalva ratio, and isometric exercise[17]. Most studies on these issues have found a high prevalence of autonomic dysfunction in cirrhosis directly associated with liver dysfunction and survival. The results point to receptor and postreceptor defects as important elements of the hyporeactive response in cirrhosis. Other studies suggest that the autonomic dysfunction secondary to liver dysfunction is temporary, and potentially reversible after liver transplantation[48]. Arterial hypertensive cirrhotic patients may have less impaired blood pressure regulation and cardiovascular reflexes[21], but this topic needs further investigation. In cirrhosis the plasma concentration of CGRP, a powerful vasodilating neurotransmitter, is increased[18,32]. Circulating CGRP relates to cardiac output, systemic vascular resistance, arterial compliance, and liver dysfunction. In experimental studies, specific antagonists of CGRP partly reverse the vasodilatation and hyperdynamic circulation. Vallance and Moncada have proposed that NO could be implicated in the vasodilatation in cirrhosis[49]. Several human and animal studies have supported this concept, whereas others have been unable to do so. To support the hypothesis, blockade of NO formation significantly improves the systemic hyperdynamic circulation, and infusion of the NO donor, L-arginine, aggravates the systemic vasodilatation and hyperdynamic circulation. Other vasodilating substances, such as adrenomedullin, the natriuretic peptides, and cannabinoids, may also be involved[18]. Adrenomedullin, a polypeptide similar to CGRP, is increased in patients with decompensated cirrhosis. From the family of natriuretic peptides, ANP, BNP, CNP, urodilatin, the two cardiac peptides, ANP and BNP, may be raised in patients with cirrhosis[35,45]. ANP is often increased in patients with ascites, owing to an enlarged left atrium and atrial stretching caused by a change in anatomical location of the heart. Circulating BNP (and pro-BNP) is especially increased in patients with cirrhotic cardiomyopathy[36,44]. Urodilatin is often normal and CNP may be reduced in advanced cirrhosis[35]. The increased natriuretic peptides and other vasodilators in cirrhosis may contribute to vasodilatation, and thereby counteract any increased arterial blood pressure from the coexistence of essential or secondary arterial hypertension.

According to the peripheral arterial vasodilatation theory of Schrier and co-workers, peripheral and splanchnic vasodilatation leads to a reduction in systemic vascular resistance and arterial blood volume and pressure, which are primary events in the retention of sodium and water[6]. Over the past few years several vasodilators have been candidates, and those mentioned above are involved in the cirrhotic vasodilatation, but further research is needed in order to answer a number of unsolved questions, especially in relation to the vascular beds which are dilated and constricted.

KIDNEY FUNCTION AND FLUID RETENTION

Functional renal failure in cirrhosis (hepatorenal syndrome) is characterized by renal insufficiency consequent on hepatic failure[5,16,45,47]. No clinical or patho-anatomical signs of other known causes of renal failure are present. In general, the hepatorenal syndrome is characterized by a decrease in the renal blood flow and glomerular filtration rate, avid sodium-water retention, azotemia, and later oliguria. The condition may be considered as a progressive functional nephropathy, consequent on dyscirculatory and dysregulatory collapse[5,16]. The renal perfusion pressure is decreased, not only because of the low arterial blood pressure, but also because of the elevated renal venous pressure, especially in patients with tense ascites[16]. The enhanced sympathetic nervous activity may alter the autoregulation curve of the kidney with a shift to the right[50]. Patients with hepatorenal syndrome have the highest plasma levels of circulating catecholamines, and there are several indicators that enhanced renal SNS activity plays an important role in the renal vasoconstriction. The highly significant inverse relation between renal noradrenaline overflow on the one hand and renal blood flow on the other may illustrate this[16].

Activation of the RAAS may contribute to decreased renal perfusion, but the RAAS may also have more complex regulatory effects inside the kidney[16,18,26]. It has been shown that vasopressin does not substantially change renal perfusion[26]. Noradrenaline, adrenaline, and endothelin-1 are important elements in the renal hypoperfusion and sodium-water retention in advanced cirrhosis[16,46,47]. Local vasodilators, such as prostaglandins and NO, most likely compensate for, at least in part, the progressive renal vasoconstriction seen in advanced cirrhosis[5]. In decompensated patients, normalization of or an increase in arterial blood pressure may increase renal perfusion and improve renal function[50]. Accordingly, a combination of prolonged administration of ornipressin or terlipressin and albumin has recently been reported to improve functional renal failure in cirrhosis[45]. Treatment with alpha-adrenergic blockers and potentially with endothelin-1 blockers may reverse renal vasoconstriction, but their lowering effect on arterial blood pressure may overrule beneficial local effects on the kidney[16]. Improvement of the cardiac dysfunction in cirrhosis may also improve the renal dysfunction[51].

Loyke found that renal and neurogenic mechanisms able to elevate the blood pressure remained intact in patients with cirrhosis[25,28]. This conclusion was based on several patients with coexistent cirrhosis and glomerular nephritis. However, today this is a rather uncommon combination, at least in patients with alcoholic liver disease. Later, Loyke reported examples of renal hypertension, which returned to normotension when the patients developed cirrhosis[23].

The hemodynamic changes in cirrhosis point towards renal hypoperfusion as being, at least initially, a physiological response to the changes in the systemic circulation. Increased SNS activity is a pathogenic factor, but other systems, such as the RAAS and endothelins, may also play a role. Angiotensin II mainly acts on the efferent arteriole and a low dose of an ACE-inhibitor

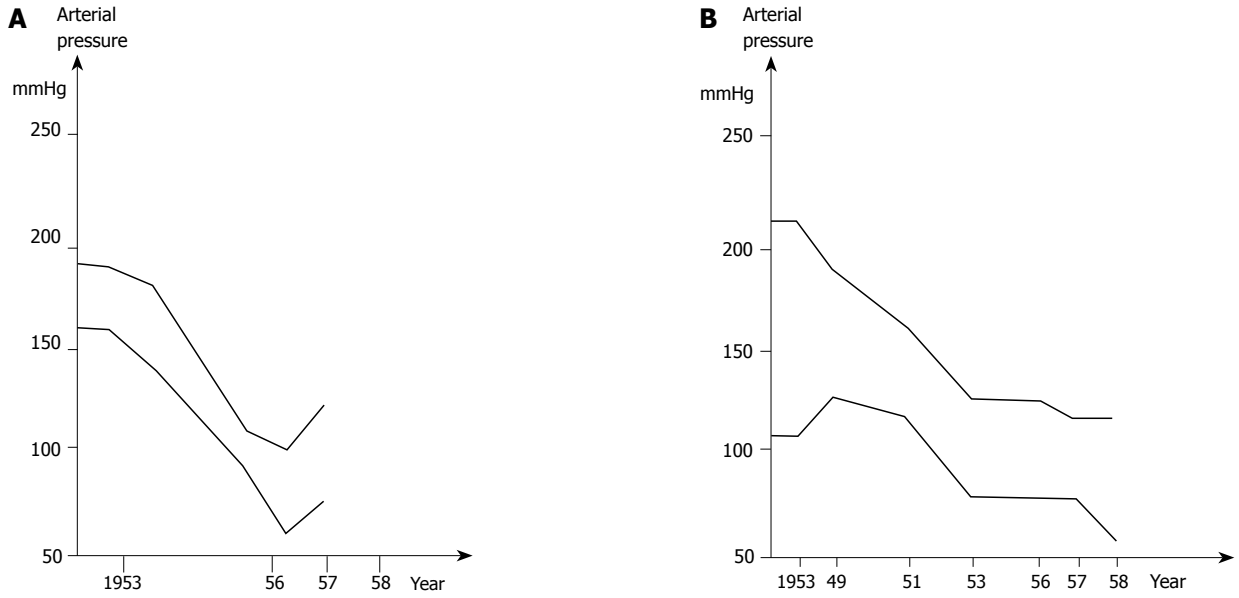

Figure 2 Change in arterial blood pressure in two patients with arterial hypertension. (**A**, 39-year-old woman with essential hypertension; **B**, 59-year-old woman with hypertension of renal origin.) A decrease in the arterial blood pressure is seen when the patients develop cirrhosis. From Loyke 1962 (reproduced with the permission of Archives of Internal Medicine, Ref. 23).

may induce a significant reduction in glomerular filtration and a further reduction in sodium excretion, even in the absence of a change in arterial blood pressure. This suggests that the integrity of the RAAS is important for the maintenance of renal function in cirrhotic patients, and that RAAS overactivity does not solely contribute to the adverse renal vasoconstriction. Infusion of terlipressin or ornipressin has, along with a rise in arterial blood pressure, demonstrate beneficial effects with increased GFR in patients with hepatorenal syndrome.

A few cases have been reported where patients with cirrhosis with renal involvement may develop arterial hypertension, especially in patients with early cirrhosis[52]. After the onset of severe cirrhosis most patients with renal hypertension become normotensive, like patients with essential hypertension (Figure 2). However, nephropathy in cirrhosis most often leads to low arterial blood pressure, in spite of the highly increased activity in the RAAS system. In most types of bilateral organic renal failure with vascular involvement and sodium retention, hypertension will be the outcome, but not in patients with cirrhosis, where the mechanisms are quite the reverse. Alterations in hemodynamic and homoeostatic mechanisms in renal hypertension and cirrhosis are summarized in Table 1.

ARTERIAL HYPERTENSION IN CIRRHOSIS

The prevalence of arterial hypertension in cirrhotic patients is substantially reduced, especially in advanced cirrhosis. Characteristic findings in patients with cirrhosis are vasodilatation with low overall systemic vascular resistance, high arterial compliance, increased cardiac output, secondary activation of counter-regulatory systems (RAAS, sympathetic nervous system, release of vasopressin), and resistance to vasopressors. The vasodilatory state is mediated through adrenomedullin, CGRP, NO, and other vasodilators, and is most pronounced in the splanchnic area. This constitutes an effective (although relative) counterbalance to raised arterial blood pressure. Subjects with arterial hypertension may become normotensive during the development of chronic liver disease, and arterial hypertension is rarely manifested in patients with cirrhosis, even in cases with renovascular disease and high circulating renin activity. There is much dispute as to the understanding of homoeostatic regulation in cirrhotic patients with manifest arterial hypertension. This condition most likely includes the combination of vasodilatation and vasoconstriction in parallel. As hypertensive patients are often effectively treated with diuretics, calcium channel antagonists, beta-blockers, ACE-inhibitors, etc., and some of these drugs are also applied in the treatment of cirrhosis and portal hypertension (Table 3), the natural history and prevalence of cirrhosis in patients with arterial hypertension, arterial hypertension in patients with cirrhosis, and the inter-relationship of these two diseases may be difficult to study today in prospective and untreated cases. Nevertheless, such studies are relevant, since there are many unsolved questions.

ACKNOWLEDGMENTS

The authors acknowledge the skillful assistance of Ms H.L. Hansen and Ms R. Sørensen. The study was supported by a grant from Savværksejer Jeppe Juhl and Hustru Ovita Juhl's Mindelegat.

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Table 3 Effects of propranolol, diuretics, terlipressin, and albumin on pressures, vascular resistance, vascular compliance, blood flow, blood volume distribution, portal pressure, and sodium excretion in patients with cirrhosis

	Non-selective beta-blocker	Diuretics	Terlipressin	Albumin
Arterial blood pressure	(↓)	(↓)	↑	→
Systemic vascular resistance	↑↑		↑↑	↓
Arterial compliance	→	→	↓	↑→
Total effective vascular compliance		↓→		
Cardiac output	↓↓	↓	↓	↑
Central and arterial blood volume	→	(↓)	↑	↑→
Plasma volume	→	↓	↓	↑
Hepatic venous pressure gradient	↓→	↓	↓	→
Urinary sodium excretion	→	↑	↑	↑

→ ↑ ↓, denote normal, increased, and decreased values, respectively. Parentheses denote minor changes.

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S- Editor Guo SY **L- Editor** Elsevier HK **E- Editor** Wu M

PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

World J Gastroenterol 2006 February 7; 12(5):686-690
World Journal of Gastroenterology ISSN 1007-9327

GASTRIC CANCER

Construction of retroviral vector of p^{125FAK} specific ribozyme genes and its effects on BGC-823 cells

Guo-Xian Guan, Hong-Xing Jian, Dong-Yin Lei, Hui-Shan Lu, Xiang-Fu Zhang

Guo-Xian Guan, Hong-Xing Jian , Dong-Yin Lei, Hui-Shan Lu, Xiang-Fu Zhang, Department of Oncology, Affiliated Union Hospital, Fujian Medical University, Fuzhou 350001, Fujian Province, China
Supported by the Natural Science Foundation of Fujian Province, No.C0010015
Correspondence to: Guo-Xian Guan, Department of Oncology, Affiliated Union Hospital, Fujian Medical University, Fuzhou 350001, Fujian Province, China. gxguan1108@163.com
Telephone: +86-591-83357896 Fax: +86-591-83321970
Received: 2005-06-22 Accepted: 2005-08-14

Abstract

AIM: To construct the retroviral vector of p^{125FAK} specific ribozyme genes and to explore the feasibility of ribozyme in BGC-823 gene therapy *in vitro*.

METHODS: A hammerhead ribozyme DNA targeting p^{125FAK} mRNA from nt 1010 to nt 1032 was synthesized and recombinated into the retroviral vector pLXSN forming pLRZXSN recon. Using the lipofectin-mediated DNA transfection technique, pLRZXSN was introduced into BGC-823 cells. The effects of ribozyme on the growth of BGC-823 cells and apoptosis were studied by cell colony assay, flow cytometry (FCM), reverse transcriptase-polymerase chain reaction (RT-PCR), detection of DNA fragmentation and electron microscopy.

RESULTS: The number of BGC-823 cell colonies was inhibited by 56% after the cells were treated for 48 h. The cell proliferation was inhibited effectively by p^{125FAK} ribozyme and the inhibitory effect depended on the concentration and the time of incubation. The expression of p^{125FAK} mRNA and protein P^{125} decreased sharply in BGC-823 cells treated with p^{125FAK} ribozyme. The characteristics of apoptosis, namely sub-G1 peak, DNA fragmentation and morphological changes, were revealed in BGC-823 cells treated with p^{125FAK} ribozyme.

CONCLUSION: p^{125FAK} ribozyme decreases p^{125FAK} gene expression and induces apoptosis of human gastric cancer cells *in vitro*.

Key words: Ribozyme; p^{125FAK} gene; Stomach neoplasm; Apoptosis

Guan GX, Jian HX, Lei DY, Lu HS, Zhang XF. Construction of retroviral vector of p^{125FAK} specific ribozyme genes and its effects on BGC-823 cells. *World J Gastroenterol* 2006; 12(5):686-690

http://www.wjgnet.com/1007-9327/12/686.asp

INTRODUCTION

p^{125FAK} is a non-receptor cytoplasm protein tyrosine kinase (PTK) with a molecular weight of 125 ku , which has unique structural and functional characteristics . Focal adhesion kinase (FAK) regulates multiple cellular processes including growth, differentiation, adhesion, motility and apoptosis[1-4]. The high expression of p^{125FAK} is possibly a part of cell incidents contributing to the invasion and metastasis of tumors[5-7]. Ribozyme (RZ) is defined as a kind of small RNA molecule that has catalytic activity and can inhibit the translation process of mRNA after RZ combines with the complementary sequences of mRNA and incises it[8-11]. In this study, a hammerhead ribozyme DNA targeting p^{125FAK} mRNA from nt 1010 to nt 1032 was synthesized according to p^{125FAK} cDNA sequences, and recombinated into the retroviral vector pLXSN forming pLRZXSN recon. Using the lipofectin-mediated DNA transfection technique, pLRZXSN was introduced into BGC-823 cells to study the expression of RZ and its effect on BGC-823 cells.

MATERIALS AND METHODS

Materials

BGC-823 cells were purchased from Shanghai Cytobiology Institute of Chinese Academy of Medical Sciences. *E. coli* DH-5 were donated by Hematopathy Laboratory of Fujian Medical University. Restriction enzymes (HindⅢ, pstⅠ, BamHⅠ, XhoⅠ)and T_4DNA ligase, X-gal, ITPG were all from Promega. pBluescript@SK plasmid was from Stratagene. RT-PCR kit was from Promega, Lipofectin™ was from Gibco/BRL, p^{125FAK} (H-1) was from American Santa Cruz. S-P kit and DAB kit were from Maxim Biotechnology and Zymed Lab Inc, respectively.

Design and synthesis of RZ template

GUU triplets of nt 1010 to nt 1032 in p^{125FAK} cDNA base sequences were used as cleavage sites. Two small nucleotide sequences complementary to the cleavage

Table 1 Primer sequence and fragment length

Primer	Sequence	Fragment length (bp)	Annealin Temp (℃)
p^{125FAK}	A: 5′TTCTTCTATCAACAGGTGAAG3′ B: 5′CTGCGAGGTTCCATTCACCAG3′	632	55
β-actin	A: 5′GGCATGGGTCAGAAGGATTCC3′ B: 5′ATGTCACGCACGATTTCCCGC3′	500	55

Note: A and B represent the sense and anti-sense primers, respectively.

sites of target RNA were put at the two ends of the conservative core sequence-(the hammerhead structure) to form a typical active incisive secondary structure of RZ as previously described[12]. The two limbs and complementary ribonucleotides of target RNA were all 10 nt. RZ gene consisted of 2 completely complementary trains called trains A and B, consisting of 60 bases respectively. Two cleavage sites and 3 protective bases were synthesized by Shanghai Sangon Technology Corporation. The sequences of RZ gene were 5'CGGACTCATCAGCAAGCTGGAT AAGCTTCGT-3' for train A and 3'GCCTGAGTAGTCG TTCGACCTATTCGAAGCA-5' for train B.

Constructtion of sequencing vector and DNA sequence analysis

Plasmid extraction, restrictive enzymolysis reaction, ligase coupled reaction, preparation of competent germ, *E coli* transfection, agarose gel electrophoresis and DNA fragment retrieval were routinely carried out. DNA sequencing was undertaken by Shanghai Sangon Technology Corporation.

Construction of pLRZSN recon

Sequencing of a fragment of 81 bp was carried out with Bam HI and Xho I, retrieved by polyacrylamide gel electrophoresis(PGE), recombinated into pLXSN cleavage sites of Bam H I and Xho I and then labeled as pLRZSN. The pLRZSN from QIAGEN was exactly quantified and sub-packaged for later use.

Transfection of BGC-823 cells

BGC-823 cells in log growth phase were divided into BGC 823 cell blank group, BGC-823 cells + Lipofectin™ group, BGC-823 cells + pLXSN group and BGC-823 cells + pLRZSN group. Liquor A was obtained by adding 4 μg DNA (pLXSN or pLRZSN) to 100 μL serum-free RPMI1640, while liguor B was obtained by adding 10μL Lipofectin™ to 90 μL serum-free RPMI1 640. Liguors A and B were mixed after 30 min and stood for 15 min. At the same time, 1×10^6 BGC-823 cells in log growth phase were washed. After resuspension in 0.8mL serum-free RPMI1640, the mixture of liquors A and B was added into the suspension and incubated at 37 ℃ in an atmosphere containing 5% CO_2 for 6 h . Four mL RPMI1640 containing 10% serum was added into the suspension and incubated for 48-72 h and then the BGC823 cells were collected.

Colony forming experiment

After incubated for 48 h, the 4 groups of cells were digested in monoplast suspensions and inoculated into 24-well plates (200/well). At the same time, 3 parallel wells were being designed. After being incubation for 7 d, colonies formed when the number of cells was over 50. The number of colonies was calculated under microscope and the colony forming efficiency was determined.

Detection of p^{125FAK} protein

After being incubated for 48 h, the 4 groups of cells were digested in monoplast suspensions and inoculated onto slides. When the slides were open-air dried and fixed in cold acetone for 10 min, immunochemistry dyeing was carried out by S-P method. Positive cell cytoplasm was buffy, nuclei and negative cells were not stained. p^{125FAK} positive cell labeling index (PI = p^{125FAK} positive cell number under one field of vision/1000×100%) was calculated. After being incubated for 48 h, the 4 groups of cells were digested in monoplast suspensions and prepared into specimens. p^{125FAK} protein was detected by flow cytometry (Bio-Rad, Bryte-HS).

Evaluation of p^{125FAK} mRNA

Total RNA was extracted from the cells after incubation for 48 h. cDNA synthesis and PCR amplification were performed as previously described[13]. p^{125FAK} gene nucleotide sequence to be amplified the primers used are listed in Table 1.

Detection of apoptosis of BGC-823 cells

After being incubated for 48 h, the 4 groups of cells were digested in monoplast suspensions, poached with PBS, centrifuged and incubated for 30 min in Kenesis50 kit (BioRad) and the DNA contents were analyzed by FCM. The ultra-structure of BGC-823 cells was observed under transmission electron microscope. The 4 groups of cells incubated for 48 h were prepared and photographed. Extraction and electrophoresis of DNA apoptotic fragments were carried out following the directions of apoptotic DNA ladder kit (Roche Corporation).

Statistical analysis

The data were expressed as mean ± SD. The variance analysis and significant difference test were crried out by SPSS 10.0.

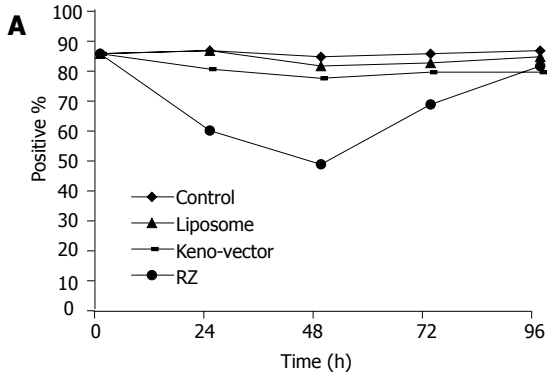
RESULTS

Effect of RZ on colony forming efficiency (CFE) of BGC-823 cells

The results of colony forming experiment are summarized in Table 2. RZ could inhibit colony formation of BGC-823 cells ($P < 0.05$).

Effect of RZ on expression of BGC-823 p^{125FAK} protein

After the cells were incubated for 48 h, the expression rate of p^{125FAK} protein was 88.4% in control group, 79.55% in liposome group, 77.08% in kenovector group, and 46.09% in ribozyme group. A significant difference was found among the 4 groups. The positive number of BGC823 cells expressed in the p^{125FAK} protein decreased sharply and the intensity of expression also weakened. PI was obviously smaller in RZ group than in other three groups



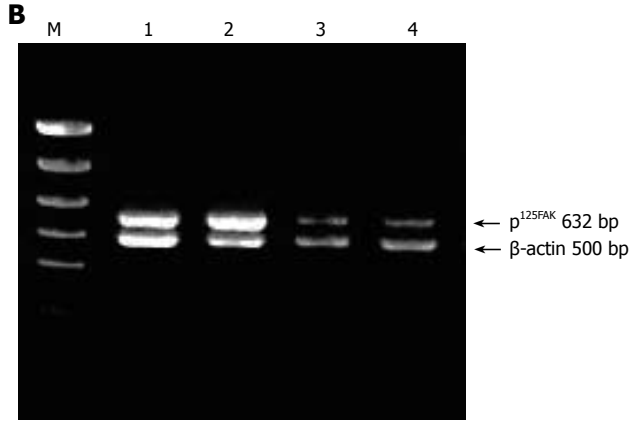


Figure 1 Expression of p^{125FAK} protein **(A)** and mRNA **(B)** in BGC 823 cells M: Marker; lane 1: control; lane 2: liposome; lane 3: keno-vector; lane 4: RZ

Table 2 Effect of RZ on colony formation of BGC-823 cells

Groups	CFE (%)	CIE (%)
Control group	51.24±6.8[a]	
Liposome group	46.43±8.6[a]	9.38
Kenovector group	45.32±5.2[a]	11.55
Ribozyme group	16.14±3.5[a]	68.50

[a]$P<0.05$ *vs* other groups.

(Figure 1A).

Effect of RZ on p^{125FAK} mRNA expression in BGC 823 cells

PCR product electrophoresis showed 632 bp and 500 bp bands. The fluorescence intensity value of p^{125FAK} and β-actin was 1.23 in control group, 0.98 in liposome group, 0.92 in keno-vector group, and 0.38 in RZ group, indicating that the level of p^{125FAK} mRNA in BGC-823 cells decreased sharply than that in other three groups (Figure 1B).

Apoptosis of BGC-823 cells induced by RZ

Apoptotic peak (sub-diploid peak) appeared in RZ group but not in other three groups (Figure 2).

Karyopynosis, chromatin margination, complete caryotheca, condensed kytoplasm with deep staining were found in RZ group. Larger karyoplasmic ratio, puffed chromotin, clear plasmosome, and abundant cytoplasm were seen in other three groups. These cells were poorly-differentiated BGC-823 cells which did not undergo apoptosis (Figure 3).

Typical DNA fragments were seen in RZ group but not in other three groups (Figure 4).

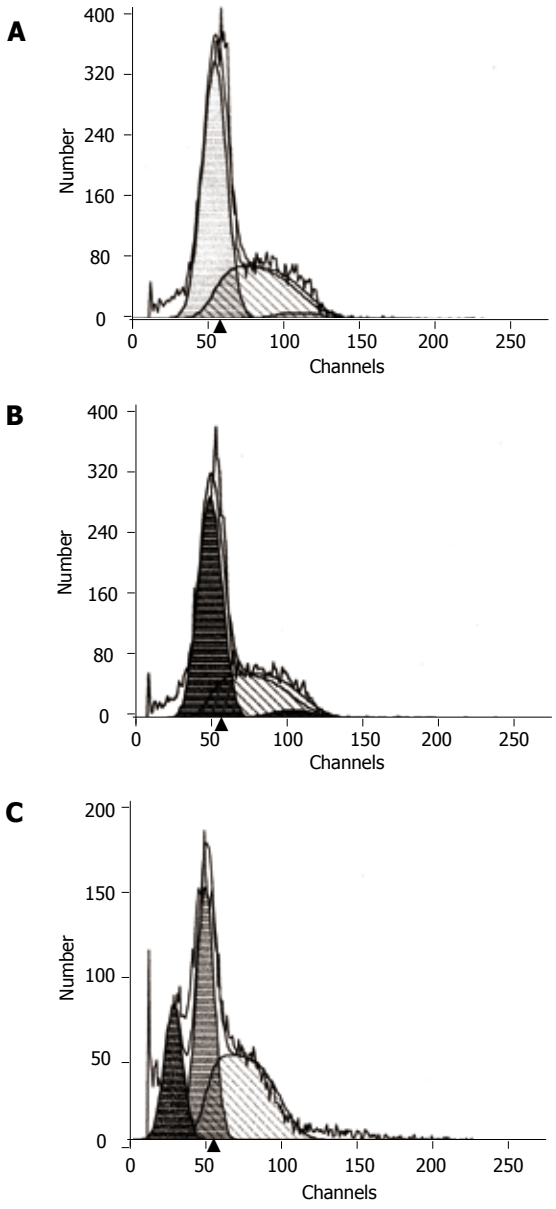


Figure 2 Cell cycle analysis of cells cultivated for 48 h in liposome group **(A)**, keno-vector group **(B)** and RZ group **(C)**.

DISCUSSION

Recent studies showed that when malignant cells adhere to extra-cellular matrix (ECM), p^{125FAK} tyrosine self phosphorylation takes place and its activity increases[14-16]. The precise mechanism of p^{125FAK} underlying apoptosis is not clear. But when malignant cells spread or migrate , p^{125FAK} regulates the formation of adhesion plaque or takes part in signal cascade conduction and inhibits apoptosis by informing karyoplasts to that cells are ECM-anchored[17,18]. Anti-sense oligonucleotides of p^{125FAK} have been used to

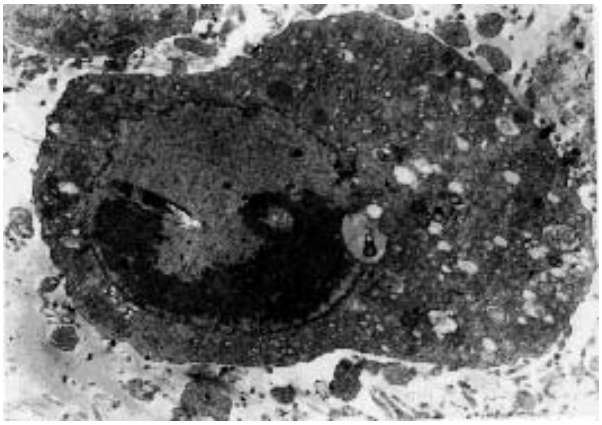


Figure 3 Apoptotic changes of BGC-823 cells in RZ group.

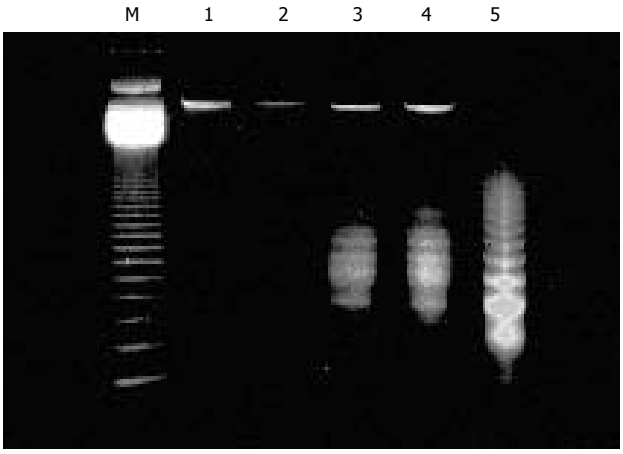


Figure 4 DNA fragments of BGC-823 cells in different groups M: 123 bp DNA ladder; lane1: liposome group; lane 2: keno-vector group; lane 3: RZ group for 24 h; lane 4: RZ group for 48 h; lane 5: RZ group for 72 h.

inhibit the expression of p^{125FAK} gene in order to induce apoptosis of malignant cells[19,20], but the result is not satisfactory because the number of target RNAs is so larger that complete blocking is impossible and anti-sense oligonucleotides are so easy to be degraded. RZ may solve the above problems. Ribozyme is a small RNA molecule that has catalytic activity. On the one hand, it can combine with complementary sequences of mRNA to block the translation of mRNA. moreover, it can incise mRNA and promote the degradation of mRNA[10,21]. Symons[8] reported that RZ can be used in treatment of viral disease and tumor. RZ genes such as c-erbB-2, P53, Ras, TGF-β, can be used in oncotherapy[22-25]. Qian *et al* [26] reported that FGFR3 RZ gene could inhibit the growth of myeloma cells and promote their apoptosis. But studies on p^{125FAK} RZ gene are relatively fewer. In the present study, a hammerhead ribozyme DNA targeting p^{125FAK} mRNA from nt 1010 to nt 1032 was synthesized according to p^{125FAK} cDNA sequences, and recombinated into the retroviral vector pLXSN forming pLRZXSN recon. Using the lipofectin-mediated DNA transfection technique, pLRZXSN was introduced into BGC-823 cells to inhibit the growth of BGC-823 cells to observe the typical changes of apoptosis under electron microscope. The results demonstrate that p^{125FAK} RZ gene is sequence specific and can be used in treatment of gastric carcinoma.

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S- Editor Wang XL and Guo SY **L- Editor** Elsevier HK **E- Editor** Cao L

PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

World J Gastroenterol 2006 February 7; 12(5): 691-696
World Journal of Gastroenterology ISSN 1007-9327

Down-expression of tumor protein p53-induced nuclear protein 1 in human gastric cancer

Pei-Hong Jiang, Yoshiharu Motoo, Stéphane Garcia, Juan Lucio Iovanna, Marie-Josèphe Pébusque, Norio Sawabu

Pei-Hong Jiang, Norio Sawabu, Department of Internal Medicine and Medical Oncology, Cancer Research Institute, Kanazawa University, Kanazawa, Japan
Yoshiharu Motoo, Department of Medical Oncology, Kanazawa Medical University, Ishikawa, Japan
Stéphane Garcia, Juan Lucio Iovanna, Marie-Josèphe Pébusque, INSERM U624, Marseille, France
Correspondence to: Yoshiharu Motoo, MD, Department of Medical Oncology, Kanazawa Medical University, Uchinada, Ishikawa 920-0293, Japan. motoo@kanazawa-med.ac.jp
Telephone: +81-76-218-8284 Fax: +81-76-218-8283
Received: 2005-05-30 Accepted : 2005-06-01

Abstract

AIM: Overexpression of tumor protein p53-induced nuclear protein 1 (TP53INP1) induces G_1 cell cycle arrest and increases p53-mediated apoptosis. To clarify the clinical importance of TP53INP1, we analyzed TP53INP1 and p53 expression in gastric cancer.

METHODS: TP53INP1 and p53 expression were examined using immunohistochemistry in 142 cases of gastric cancer. The apoptosis of gastric cancer cells was analyzed using the TUNEL method. The relationship between the expression of TP53INP1 and clinicopathological factors was statistically analyzed.

RESULTS: TP53INP1 was expressed in 98% (139/142 cases) of non-cancerous gastric tissues and was down-expressed in 64% (91/142 cases) of gastric cancer lesions from the same patients. TP53INP1 expression was significantly decreased (43.9%) in poorly differentiated adenocarcinoma compared with well or moderately differentiated adenocarcinoma (81.6%). Cancers invading the submucosa or deeper showed lower positively (59.1%) compared with mucosal cancers (85.2%). Decrease or loss of TP53INP1 expression was significantly correlated with lymphatic invasion (54.3% *vs* 82.0% without lymphatic invasion) and node-positive patients (31.3% *vs* 68.3% in node-negative patients). P53 was expressed in 68 (47.9%) patients of gastric cancer, whereas it was absent in normal gastric tissues. A significant association was also observed between TP53INP1 status and the level of apoptosis in tumor cells: the apoptotic index in TP53INP1-positive tissues was significantly higher than that in TP53INP1-negative portions. Finally, when survival data were analyzed, loss of TP53INP1 expression had a significant effect in predicting a poor prognosis ($P = 0.0006$).

CONCLUSION: TP53INP1-positive rate decreases with the progression of gastric cancer. TP53INP1 protein negativity is significantly associated with aggressive pathological phenotypes of gastric cancer. TP53INP1 is related to the apoptosis of gastric cancer cells. The decreased expression of the TP53INP1 protein may reflect the malignant grade of gastric cancer and is regarded as an adverse prognostic factor.

Key words: Tumor protein 53-induced nuclear protein 1; p53; Gastric cancer

Jiang PH, Motoo Y, Garcia S, Iovanna JL, Pébusque MJ, Sawabu N. Down-expression of tumor protein p53-induced nuclear protein 1 in human gastric cancer. *World J Gastroenterol* 2006; 12(5): 691-696

http://www.wjgnet.com/1007-9327/12/691.asp

INTRODUCTION

Tumor protein 53-induced nuclear protein 1 (TP53INP1) is a p53-inducible gene encoding two protein isoforms able to modulate p53 biological activities[1-4]. TP53INP1 expression is strongly induced *in vivo* in mice with acute pancreatitis[1], and *in vitro* in several cell lines submitted to various stress agents[2,4]. Over-expression of TP53INP1 induces cell cycle arrest in G1 phase and enhances the p53-mediated apoptosis[3]. TP53INP1 co-localizes with p53 and the serine-threonine p53-kinase HIPK2[5] within the promyelocytic leukemia protein nuclear bodies (PML-NBs) and physically interacts with these proteins modifying the p53 transcriptional activity on several p53 target genes[3]. *TP53INP1* thus appears as a key-element in p53-mediated cell death and cell cycle arrest, induced by cellular stresses. The multi-step model of carcinogenesis in gastric cancer, the second most common cancer leading to death in the world, suggests accumulation of genetic alterations, epigenetic changes and posttranslational modifications. It often metastasizes to other organs, including the liver, lung, and ovary[6]. Multiple factors are known to be related to gastric carcinogenesis, including *Epstein-Barr* virus[7] and *H pylori* infections[8], microsatellite instability[9]. From the molecular point of view, it has now been established that gastric carcinogenesis is involved the accumulation

of mutations in oncogenes and tumor suppressor genes controlling epithelial cell growth and differentiation[10-14]. In particular, TP53 mutations are frequently seen in gastric cancers and correlates with gastric cancer prognosis[15,16]. However, the molecular alterations and their role in gastric cancer still remain to be fully defined.

Previous works implied that *TP53INP1* is a pro-apoptotic gene induced by p53[2]. Overexpression of TP53INP1 promotes G1 arrest and apoptosis through the p53-mediated pathway[3]. The aim of the present study was to analyze the expression patterns of TP53INP1 in a large series of gastric carcinomas to (1) identify the possible modulation of TP53INP1 expression; (2) investigate the association with apoptotic activity; (3) analyze the relationship with clinicopathologic parameters, and evaluate its prognostic value.

MATERIALS AND METHODS

Patients and specimens

One hundred and forty-two patients with gastric cancer were enrolled in this study. The areas adjacent to cancer lesions were used as non-malignant gastric tissue. The patients underwent operation at the Cancer Research Institute Hospital, Kanazawa University. The histological classification was defined using the Japanese classification of gastric carcinoma[17]. Intestinal type was defined as either papillary or well to moderately differentiated tubular adenocarcinoma. Diffuse type was defined as poorly differentiated adenocarcinoma, signet-ring cell carcinoma, or mucinous adenocarcinoma. The series included 104 men and 38 women, and the mean age was 63.1±10.6 years. There were 76 and 66 cases of differentiated and undifferentiated type, respectively.

Immunohistochemistry

A standard avidin-biotin-peroxidase complex method (ABC) was used for immunostaining. Deparaffinized sections were treated by microwaving at a high power for 5 min twice in a 10 mmol/L citrate buffer to retrieve antigenicity. After washing with PBS, the sections were immersed in 3% hydrogen peroxide in methanol for 20 min to block any endogenous peroxides activity. Then the ABC staining system kit (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used for the detection. Sections were incubated with 10% normal serum for 1 h to inhibit nonspecific antibody binding. Then, sections were incubated overnight at 4 °C with 6 μg/mL of rat anti-human monoclonal antibody raised against *TP53INP1* (kindly provided by Carrier). After washing with PBS, detection was done by successively incubating the sections with biotinylated goat anti-rat IgG for 30 min, and avidin-biotin-HRP for 30 min. After extensive washings with PBS, sections were stained with 3,3-diaminobenzidine for 2-10 min. Then, sections were counterstained with hematoxylin, dehydrated, and mounted. Nuclei were lightly counterstained with Mayer's hematoxylin. TP53INP1-positive cells were counted in fields chosen at random (100 × magnification), and the percentage of the number of positive cells per 1 000 cells was expressed as TP53INP1-positive index (%). Using the same method we counted the TP53INP1-positive in nucleus and in cytoplasm under the microscopy with a 200× magnification. The normal IgG was used as a negative control.

Immunohistochemistry for p53 was performed using a DAKO LSAB kit (DakoCytomation, Kyoto, Japan). The primary antibody was mouse monoclonal antibody against human p53 (DO-7, Nichirei Inc., Tokyo, Japan). The procedure was according to the protocol from the company. Finally the sections were incubated with DAB substrate as a chromogen. The cell nuclei were also lightly counterstained with Mayer's hematoxylin.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)

TUNEL-positive epithelial cells were detected in the sections using ApopTag Plus peroxides *in situ* apoptosis detection kit (Chemicon International, Inc., Temecula, CA, USA). Briefly, after pretreatment with 20 μg/mL of proteinase K and 3% hydrogen peroxide, sections were incubated with a labeling mixture for 1 h at 37 °C. Then 55 μL of anti-digoxigenin-peroxidase was deposited on the sections and incubated for 30 min. The reaction products were visualized by 3,3-diaminobenzidine substrate. Nuclei were counterstained with methyl green for 10 min. After washing with *n*-butanol, the sections were dehydrated, and mounted. Apoptotic index (%) corresponding to the number of labeled nuclei per 1 000 nuclei was calculated.

Statistical analysis

Experimental results were expressed as mean±SE. Difference between the means was evaluated by the Mann-Whitney *U* test. $P<0.05$ was considered statistically significant. The statistical analysis in this paper such as Kaplan-Meier analysis and Cox regression model was performed by using the software of StatView-5.0 Macintosh (Tokyo, Japan).

RESULTS

TP53INP1 was expressed in non-malignant gastric tissues and its expression was reduced in gastric cancer tissues

In the non-neoplastic gastric mucosa, TP53INP1 was mainly located in the cytoplasm of epithelial cells (Figures 1A and 1B). Some nuclei were also stained for TP53INP1 (Figure 2). Similar patterns were observed for intestinal metaplasia samples (Figure 1A). To determine if TP53INP1 is differentially expressed in gastric carcinomas or if it is stage-related, we did immunohistochemical analysis on 142 samples (76 cases of intestinal type, and 66 cases of diffuse type). All the cancer samples had accompanying non-malignant tissues, 98% of them were positive for TP53INP1 expression (Table 1) and thus could be used as internal control. In contrast, the expression of TP53INP1 protein was seen in 91 cases (64%), the other 51 samples (36%) were TP53INP1-negative (Figure 1B, arrow). Overall, TP53INP1 expression in gastric cancers was significantly lower both in cell cytoplasm and nucleus than in non-malignant gastric tissue ($P<0.0001$, Table 1 and Figure 2). However, the expression of TP53INP1 was decreased in well-differentiated tubular adenocarcinoma (Figure 1C), and was markedly diminished in poorly

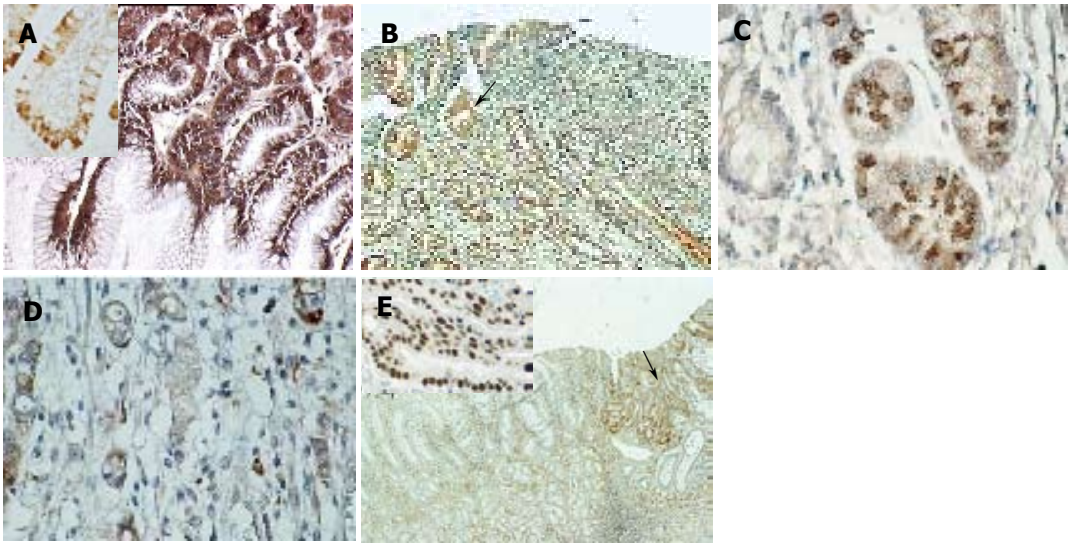

Figure 1 Immunohistochemical analysis of TP53INP1 expression in gastric carcinoma. **A:** TP53INP1 was strongly expressed in normal gastric mucosa or intestinal metaplasia foci (inset); **B:** TP53INP1 expression decreased in gastric carcinoma (arrow); **C:** well differentiated tubular carcinoma exhibited moderate alteration of TP53INP1 expression; **D:** poorly differentiated carcinoma showed weak staining with TP53INP1 antibody; **E:** P53 expression increased in gastric carcinoma (arrow).

Table 1 TP53INP1 expression in gastric cancer *n* (%)

		Non-malignant gastric tissue ($n=142$)	Gastric cancer ($n=142$)[1]	*p* value
TP53INP1	Positive	139 (98)	91 (64)	<0.0001
	Negative	3 (2)	51 (36)	
P53	Positive	0 (0)	68 (47.9)	<0.0001
	Negative	142 (100)	74 (52.1)	

[1]In gastric cancer the *P* value between TP53INP1 and P53 was 0.006.

differentiated-type cancer (Figure 1D).

We next examined whether TP53INP1 expression is associated or not with the development and progression of gastric carcinoma. The clinical details of the cohort of patients and the statistical analysis are listed in Table 2. Only two non significant associations were observed, i.e., age and gender. TP53INP1 negativity was associated with gastric body and antrum tumor location ($P=0.0193$), with poorly differentiated adenocarcinoma (diffuse type) ($P<0.0001$). With regard to the depth of invasion, the positive TP53INP1 expression rate was 100% in intramucosal tumors (5/5), 81.8% when mucosa was invaded (18/22), 76.5% in muscularis propria (26/34), 54.3% in subserosa (25/46), and 48.6% in serosa (17/35). These results showed that alteration of TP53INP1 expression was correlated to the staging of the tumors. The difference was statistically significant when T1 tumors were compared to the other stages ($P=0.0111$, Table 2). In addition, TP53INP1 was significantly expressed in node-negative patients ($P=0.0037$), and significantly associated with lymphatic invasion-negative patients ($P=0.0010$). Taken together, these results indicated that loss of TP53INP1 expression was significantly associated with poorly differentiated histology, deep invasion, lymph node invasion, and metastasis.

Table 2 Correlation between TP53INP1 expression levels and clinicopathologic features in gastric cancer

	TP53INP1-positive ($n=91$ of 142 patients)	TP53INP1-negative ($n=51$ of 142 patients)	*P* value
Age (years)			
<60	31	18	NS
⩾60	60	33	
Sex			
Male	68	36	NS
Female	23	15	
Location			
Cardia	20	22	
Body	58	26	0.0193
Antrum	13	3	
Histological type			
Differentiated[a]	62	14	
Undifferentiated[b]	29	37	<0.0001
Tumor invasion			
T1a+T1b	23	4	0.0111
T2+T3+T4	68	47	
Lymph node metastasis			
Positive	5	11	0.0037
Negative	86	40	
Liver metastasis			
Positive	0	5	0.0024
Negative	91	46	
Lymphatic invasion			
Positive	50	42	0.0010
Negative	41	9	

NS: not significant. [a]Differentiated type corresponds to well and moderately differentiated tubular and papillar tumors (intestinal type). [b]Undifferentiated type includes poorly differentiated and signet-ring cell carcinomas (diffuse type).

TP53INP1 and apoptosis

TP53INP1 modulates the cell cycle arrest and programmed cell death[3]. To investigate whether the modulation of TP53INP1 expression is associated with differences in apoptotic activity, TUNEL assays were done in all the 142 cases. TUNEL-positive nuclei were clearly seen in TP53INP1-positive (Figure 3A) and negative (Figure 3B) cancer lesions. As shown in Figure 3C, the apoptotic index in the TP53INP1-positive group ($7.48\pm2.66\%$) was significantly higher than that in the TP53INP1-negative group ($4.16\pm2.41\%$).

TP53INP1 expression and prognosis

On univariate analysis, patient survival according to

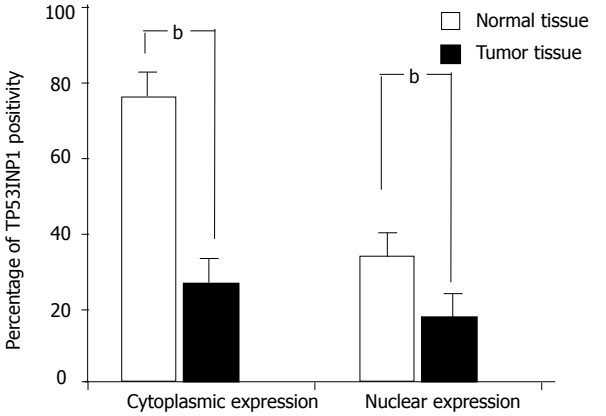

Figure 2 Comparison of TP53INP1 expression (in cell cytoplasm and nucleus) between normal gastric mucosa and gastric cancer tissues ($^bP < 0.0001$).

Table 3 Multivariate survival analysis using the Cox regression model

Factor	Reference	Odds Ratio	*p*
TP53INP1	+ *vs* -	1.250	0.3680
Age	<60 *vs* ≥60	1.388	0.1077
Gender	Male *vs* female	1.158	0.5217
Location	Body+cardia *vs* autrum	1.253	0.4847
Histological type	Poor *vs* well+moderately	2.043	0.0026
Tumor invasion	T2+T3+T4 *vs* T1	1.061	0.8485
Stage	III+IV *vs* II + I	1.269	0.2612
Apoptotic index	≤4% *vs* >4%	2.244	0.0008
Metastasis	+ *vs* -	18.688	0.0007
Lymphatic invasion	+ *vs* -	0.721	0.2124
Lymph node invasion	+ *vs* -	3.121	0.0032

pathological stage was significantly different between TP53INP1-positive and TP53INP1-negative groups. Those patients with TP53INP1-positive expression had significantly better survival than those without TP53INP1 expression ($P = 0.0006$, Figure 4A). Survival for TP53INP1-positive patients with poorly differentiated adenocarcinoma was significantly longer than that of TP53INP1-negative patients ($P = 0.0199$, Figure 4B), whereas the survival of TP53INP1-positive patients in well or moderately differentiated adenocarcinoma was not significantly different from that of TP53INP1-negative patients ($P = 0.1110$, Figure 4C). Taken together, the results indicated that alteration of TP53INP1 expression was associated with a poor prognosis. Nevertheless, no prognostic value for TP53INP1 expression was evidenced from the multivariate analysis (Table 3). Histological type, apoptotic index, metastasis, and lymph node invasion were the most important independent prognostic factors, TP53INP1 could not be considered as an independent prognostic marker.

P53 was not expressed in non-malignant gastric tissues but expressed in gastric cancer tissues

As shown in Table 1 and Figure 1E, the p53 protein was expressed in gastric cancer regions. whereas it was absent in non-malignant portions. The staining was nuclear. Cytoplasmic staining without nuclear staining was regarded as negative. The p53 positive rate in gastric cancer was significantly higher (47.9%) than that in non-malignant tissues ($P < 0.05$).

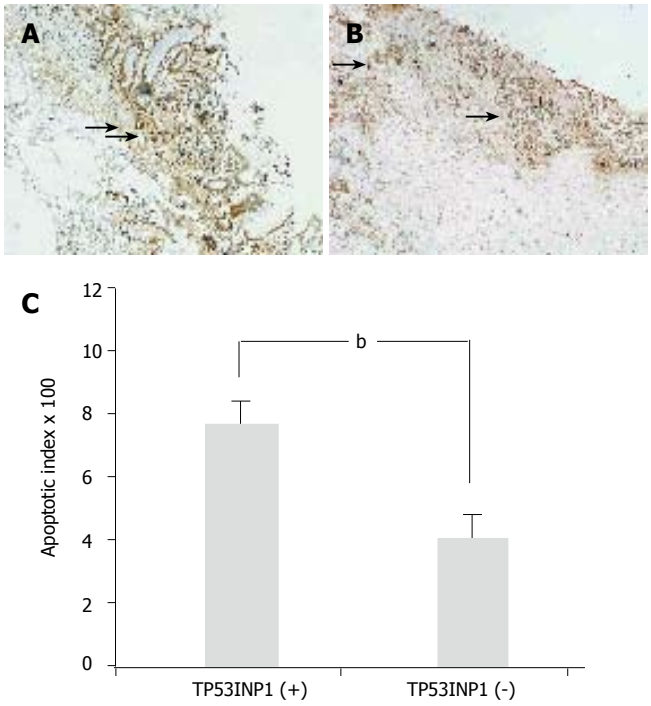

Figure 3 Apoptosis analysis in gastric cancer. **A and B:** Representative patterns of TP53INP1-positive (**A**) and negative (**B**) carcinoma in TUNEL staining. Arrows indicate TUNEL-positive nuclei (original magnification ×20); **C:** Statistical analysis of apoptotic index in TP53INP1-positive and -negative cancer tissues ($^bP < 0.0001$, positive *vs* negative).

DISCUSSION

The development and progression of gastric cancer involve many types of genes that need to be activated or inactivated in order to promote malignancy. Gastric cancer is a heterogeneous pathology, classified into two general subtypes: intestinal (differentiated) and diffuse (undifferentiated)[17]. The intestinal type gastric carcinoma presents tumor suppressor gene alterations similar to colorectal tumors and distinct from diffuse type gastric cancer[18]. An accumulation of multiple genetic and epigenetic alterations of oncogenes, tumor suppressor genes, DNA repair genes, cell cycle regulators, cell adhesion molecules, and growth factor/receptor systems are involved during the multi-step conversion from normal epithelial cells to clinical gastric cancer[10-14]. *TP53* gene alterations have been observed in both histological subtypes[19]. TP53INP1 is a tumor suppressor gene, located on the chromosome band 8q22[20]. Its expression is dependent on the activation of wide-type p53[3].

In this study, we have showed that TP53INP1 protein expression was significantly reduced in gastric cancer cells compared with non-cancerous adjacent tissues. We also reported that reduced TP53INP1 expression was associated with the diffuse cancer phenotype. Tomasini *et al*[2] have shown that TP53INP1 and HIPK2 are partners in regulating p53 activity. It is increasingly evident that methylation of CpG islands in the promoter of specific

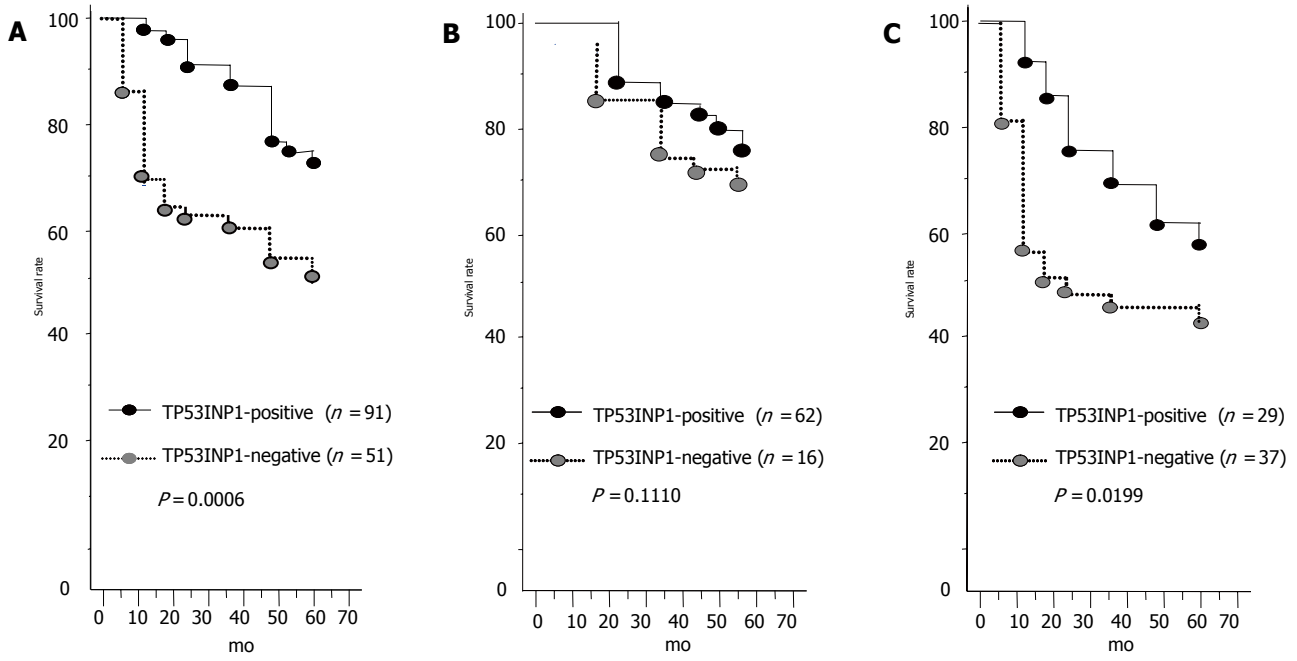

Figure 4 TP53INP1 expression and patient survival by Kaplan-Meier analysis. **A:** Survival curves for TP53INP1-positive and negative gastric cancers. The 60-mo survival rates were 74.7% and 54.9%, respectively. The difference between the values was highly significant ($P = 0.0006$). **B:** In well or moderately differentiated adenocarcinoma (intestinal-type), the survival of TP53INP1-positive patients was not significantly different from that of TP53INP1-negative group ($P = 0.1110$). **C:** In poorly differentiated adenocarcinoma (diffuse-type), the 60-mo patient survival rates were 62.1% and 45.9% for TP53INP1-positive and -negative gastric cancers, respectively. This difference was statistically significant ($P = 0.0199$).

tumor suppressor genes, such as *p16*, is associated with their silencing in human gastric cancer[21]. The 5' -upstream region of TP53INP1 contains a CpG island. The sequences from nucleotide -792, the region on exon 1 and part of the first intron, to nucleotide +839 have the highest content of CpG dinucleotides. However, there was no mutation of TP53INP1 gene in pancreatic carcinoma (unpublished data). In addition, the reduced TP53INP1 expression in gastric cancer especially in the diffuse type. may relate to the wide-type p53 inactivation in gastric cancer.

We showed that p53 was expressed in 68 cases of gastric cancer, whereas it was not present in normal gastric epithelial cells. We observed that in non-gastric cancer regions the expression of TP53INP1 was opposite to that of p53. The expression of TP53INP1 was dependent on the wild type p53 since the wild-type p53 protein is biologically unstable and has a shorter half-life than mutant p53 protein[24]. This characteristic of wide-type p53 protein does not allow it to be detected by immunohistochemical methods, but mutant p53 can be detected by immunostaining. Our data was similar to Carvalho *et al* [22] who showed that there was no difference of the p53 expression between the intestinal type and the diffuse type in gastric cancer. Whereas Lin *et al* [23] showed the expression of p53 in the intestinal type was more frequent than that in the diffuse type. The precise mechanisms of TP53INP1 suppression in gastric cancer need further research.

The mechanism of the suppression of these genes in poorly differentiated gastric carcinoma is not clear. Many studies suggested that most tumor suppressor genes play a role in mediating cell cycle arrest in the G_1 phase following DNA damage and also function in the removal of damaged cells by initiating apoptosis in certain physiological situations[10]. TP53INP1 and HIPK2 are partners in regulating p53 activity[2]. Overexpression of TP53INP1 induces G_1 cell cycle arrest. TP53INP1 expression was significantly decreased in advanced gastric cancers. These results suggest that decrease of TP53INP1 expression might play an important role in the progression of gastric cancer. Assessment of TP53INP1 expression level may serve as a novel biomarker for predicting the malignant grade of cancers, like another marker for poor prognosis genes[25,26].

Lymphatic involvement is thought to be important as an initial step of lymph node metastasis[27]. Our study showed that TP53INP1 expression was significantly reduced in lymphatic invasion-positive groups. TP53INP1 expression decreased in node-positive. Taken together, these results suggested that loss of TP53INP1 expression is associated with lymph node metastasis of gastric cancer.

Deregulation of genes involved in cell cycle and cell signaling pathways has been described and classified as early events for cyclin D1 and p16 genes or late events for p53, DPC4 and BRCA2 genes in the progression model studies[28]. *TP53INP1* is a pro-apoptotic gene strongly activated during cell stress. Overexpression of TP53INP1 is related to G_1 cell cycle arrest and induces p53-mediated cell death[3]. In the present study, we showed that the apoptotic index in TP53INP1-positive lesions was higher than that in TP53INP1-negative lesions in gastric cancer tissues as detected by TUNEL assay, indicating that TP53INP1 is related to apoptosis and tumor aggressiveness in gastric carcinoma.

TP53INP is an acute gene that is induced by various

stresses such as UV, heat shock, etc.[1]. Up to now, we have known little function of it. We for the first time revealed the impact of the TP53INP1 on the survival in gastric cancer. We showed that the survival rate of the TP53INP1-positive cases was longer than that of the TP53INP1-negative cases, especially in the diffuse type gastric cancer.

In conclusion, the present study has showed that the reduction of TP53INP1 expression might play roles in gastric carcinogenesis and tumor aggressiveness. Analysis of the TP53INP1 may be useful to evaluate the malignant grade of gastric cancer.

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S- Editor Guo SY **L- Editor** Elsevier HK **E- Editor** Cao L

PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

World J Gastroenterol 2006 February 7; 12(5): 697-702
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

Cyclosporine *versus* tacrolimus in patients with HCV infection after liver transplantation: Effects on virus replication and recurrent hepatitis

Philip Hilgard, Alisan Kahraman, Nils Lehmann, Cornelia Seltmann, Susanne Beckebaum, R Stefan Ross, Hideo A Baba, Massimo Malago, Christoph E Broelsch, Guido Gerken

Philip Hilgard, Alisan Kahraman, Susanne Beckebaum, Guido Gerken, Department of Gastroenterology and Hepatology, University Hospital Essen, Hufelandstrasse 55, 45122 Essen, Germany
Nils Lehmann, Institute for Medical Informatics, Biometry and Epidemiology, University Hospital Essen, Hufelandstrasse 55, 45122 Essen, Germany
Cornelia Seltmann, Susanne Beckebaum, Massimo Malago, Christoph E Broelsch, Department of General Surgery and Transplantation, University Hospital Essen, Hufelandstrasse 55, 45122 Essen, Germany
R Stefan Ross, Institute for Virology, University Hospital Essen, Hufelandstrasse 55, 45122 Essen, Germany
Hideo A Baba, Institute for Pathology, University Hospital Essen, Hufelandstrasse 55, 45122 Essen, Germany
Correspondence to: Dr Philip Hilgard, Universitätsklinikum Essen, Klinik für Gastroenterologie and Hepatologie, Hufelandstraße 55, 45122 Essen, Germany. philip.hilgard@uni-essen.de.
Telephone: +49-201-723-3618 Fax: +49-201-723-5970
Received: 2005-06-08 Accepted: 2005-07-08

Abstract

AIM: To determine the effects of the calcineurin inhibitors, cyclosporine and tacrolimus, on hepatitis C virus (HCV) replication and activity of recurrent hepatitis C in patients post liver transplantation.

METHODS: The data of a cohort of 107 patients who received liver transplantation for HCV-associated liver cirrhosis between 1999 and 2003 in our center were retrospectively analyzed. The level of serum HCV-RNA and the activity of recurrent hepatitis were compared between 47 patients who received either cyclosporine or tacrolimus as the primary immunosuppressive agent and an otherwise similar immunosuppressive regimen which did not lead to biliary complications within the first 12 mo after transplantation.

RESULTS: HCV-RNA increased within 3 mo after transplantation but the differences between the cyclosporine group and the tacrolimus group were insignificant ($P = 0.49$ at 12 mo). In addition, recurrent hepatitis as determined by serum transaminases and histological grading of portal inflammation and fibrosis showed no significant difference after 12 mo ($P = 0.34$).

CONCLUSION: Cyclosporine or tacrolimus as a primary immunosuppressive agent does not influence the induction or severity of recurrent hepatitis in HCV-infected patients after liver transplantation.

Key words: Cyclosporine; Tacrolimus; Liver transplantation; Recurrent hepatitis; HCV-RNA

Hilgard P, Kahraman A, Lehmann N, Seltmann C, Beckebaum S, Ross RS, Baba HA, Malago M, Broelsch CE, Gerken G. Cyclosporine *versus* tacrolimus in patients with HCV infection after liver transplantation: Effects on virus replication and recurrent hepatitis. *World J Gastroenterol* 2006; 12(5): 697-702

http://www.wjgnet.com/1007-9327/12/697.asp

INTRODUCTION

Hepatitis C virus (HCV) is a leading cause of chronic hepatitis with about 170-190 million people infected worldwide[1,2]. The treatment of choice for patients with end-stage liver disease associated with HCV infection is liver transplantation (LTx) and in fact, HCV is the main indication for liver transplantation among adults in Europe and USA, accounting for approximately 50% of cases[3]. Since HCV persists not only in hepatocytes but also in leukocytes, lymph nodes and most likely additional tissues[4-6], the virus is redistributed into the donor liver after LTx almost immediately in virtually all patients[7,8]. However, the severity and clinical course of the resulting reinfection hepatitis vary widely, ranging from deleterious fibrosing cholestatic hepatitis with failure of the allograft within 1 year to a rather mild hepatitis with progression to cirrhosis within 5 years in 20-30% of cases[9,10].

A main factor determining the severity of recurrent hepatitis C after transplantation may be immunosuppression[11]. Of particular concern is the fact that the prognosis of recurrent HCV infection seems to deteriorate in recent years, which has been attributed to the use of donor organs of less optimal quality as well as changes in the immunosuppressive therapy such as steroid-free regimens

or the combination of particular drugs[12,7]. Considering that retransplantation remains the only treatment for patients with graft failure due to HCV recurrence, optimization of the immunosuppressive regimens might be a key aspect to improve the prognosis of chronic hepatitis C after transplantation.

The two most frequently used basic immunosuppresive drugs are cyclosporine A (CsA) and tacrolimus (Tac). Although CsA and Tac are structurally different, they share a similar mode of immunosuppressive action by inhibition of the transcription factor NFAT and consecutive reduction of inflammatory gene expression in activated T cells[13-15]. The main mechanism by which immunosuppressive therapy with calcineurin inhibitors accelerates the clinical course of post-transplantation hepatitis C is their influence on the replication of the virus. Immunosuppressive therapy after LTx of patients with chronic HCV infection is associated with significantly increased serum viral load compared to the values of the same patients before transplantation[7,11], suggesting decreased systemic immune responses against the virus. Locally in the liver, the uncontrolled replication of the virus may cause an enhanced immune response, resulting in accelerated hepatocellular damage and fibrosis.

Whether a particular calcineurin inhibitor has an advantage in immunosuppressive therapy of HCV-infected patients post LTx is still highly controversial[16-19]. To further clarify this issue, the aim of this study was to determine the effects of CsA and Tac on serum HCV-RNA level as well as on the inflammatory activity of recurrent hepatitis in patients with chronic HCV infection after LTx.

MATERIALS AND METHODS

Patients

The data of 107 consecutive patients within the first year after liver transplantation due to hepatitis C-associated liver cirrhosis were retrospectively reviewed. Patients underwent LTx between June 1998 and December 2003 at our center either as cadaveric-(orthotopic) or as living donor-related liver transplantation. In order to compensate for missing randomization in this retrospective study, only 47 patients who received either cyclosporine or tacrolimus as the primary immunosuppressive agent and an otherwise similar immunosuppressive regimen which did not lead to acute or chronic rejection and biliary complications within the first 12 mo after transplantation, forming a relatively homogenous subgroup, were included in this study. A further exclusion criterion was early death after transplantation. During the first year all patients underwent routine liver biopsy 3-5 mo after LTx, independent from serum liver enzyme activities. Every rise of transaminases or cholestasis parameters was followed by additional liver biopsies to differentiate between acute or chronic rejection, cholestasis and hepatitis C reinfection. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Local Ethics Committee.

The basic immunosuppressive agent used in all the patients was either CsA or Tac. The dose of the drugs was controlled in light of the serum level, which was adjusted between 140 and 180 ng/mL for CsA, and 10 and 15 ng/mL for Tac within the first 3 mo. After 3 mo, the serum level of CsA and Tac was reduced to 100-120 ng/mL and 7-10 ng/mL, respectively. During the first 3 mo. after LTx, CsA, and Tac were routinely combined with corticosteroids (prednisolone) at an initial dose of 1 mg/kg body weight and reduced to 5 mg daily within 4 weeks. After 3 mo steroids were discontinued. In addition, all the patients included in this study received 1000-2000 mg mycophenolate mofetil (MMF), per day for the first 12 mo post LTx.

Determination of HCV infection

HCV infection status was evaluated by the examination of the patients' sera for the presence of anti-HCV antibodies and HCV-RNA. Anti-HCV antibodies were detected by a commercially available enzyme immunoassay (Monolisa anti-HCV plus, Biorad, Munich) according to the manufacturer's instructions. Qualitative detection of serum HCV-RNA was performed by a RT-PCR-based (Cobas Amplicor HCV 2.0 assay, Roche Diagnostics, Mannheim, Germany) assay or a transcription-mediated amplification (TMA)-based assay (VERSANT HCV-RNA qualitative, Bayer Diagnostics, Fernwald, Germany). HCV-RNA concentrations were determined by commercially available second and third generation branched DNA (bDNA) assays (VERSANT HCV-RNA 2.0 and 3.0, Bayer Diagnostics, Fernwald, Germany). To obtain HCV-RNA titers in IU/mL, the bDNA 2.0 assay was calibrated against the WHO HCV Standard 96/790[20], as described in full details elsewhere[21,22]. Values below the lower limit of sensitivity of 615 IU/mL were set to 615 IU/mL divided by 2.

Determination of inflammatory activity

Inflammatory activity in the graft due to hepatitis induced by HCV reinfection was in all patients under surveillance determined by routine measurement of transaminases (ALT or GPT and AST or GOT) as well as alkaline phosphatase and gamma-glutamyl-transferase and bilirubin (every 7-30 d, interval depending on the time after LTx and level of elevation). Every unexplained rise was immediately followed by B-mode and duplex-/Doppler ultrasonographic analysis of liver parenchyma and the supplying vessels. If thrombosis of the hepatic artery and congestion of the biliary system were excluded, liver biopsy was performed. In analogy to patients pre LTx, recurrent hepatitis C severity was routinely determined by the modified Knodell hepatic activity index (HAI) with subscores for piecemeal necrosis (0-4), confluent necrosis (0-6), lytic necrosis/apoptosis/focal inflammation (0-4) and portal inflammation (0-4), as well as fibrosis staging (0-6)[23].

Statistical analysis

Virological, biochemical, and histological data were collected in a computer database (Microsoft Excel). Statistical analyses were performed utilizing SAS System software, release 8.2 (SAS Institute Inc., Cary, NC, USA). Qualitative characteristics were analyzed by χ^2 test or

Table 1 Baseline characteristics of 47 HCV-infected patients after LTx, orthotopic liver transplantation (OLT), and living donor liver transplantation (LDLT)

	CsA	Tac	*P*-value
Number of patients	35	12	
Age	28-64	27-61	0,20
Gender	f=9, m=26	f=3, m=9	0.96
OLT	31	10	
LDLT	4	2	0.64
HCV genotype	gt1=32/35, gt2=1/35, gt3=2/35	gt1=10/12, gt2=0/12, gt3=2/12	0.46

Fisher's exact test as appropriate. Two-sided *P*-values and changes in HCV-RNA concentrations and ALT activities were evaluated by Mann-Whitney *U*-test. Correlation coefficients were reported as rank (Spearman) correlations. $P < 0.05$ was considered statistically significant. In this explorative analysis, no adjustment for multiple testing was performed.

RESULTS

Effect of CsA and Tac on HCV serum RNA levels in HCV-infected patients post LTx

Of the 47 patients included in this study, 35 received CsA and 12 received Tac as the primary immunosuppressive agent. The baseline characteristics of the two patient groups showed no statistically significant differences (Table 1). Patients underwent liver transplantation at least 12 mo before the study. In both groups of patients, serum HCV-RNA level was quantitatively assessed at 1, 3, 6, and 12 mo after liver transplantation and compared to the HCV-RNA level prior to the operation. Figure 1 depicts these data on logarithmic scale (log10) as a box-whisker's plot of HCV-RNA in relation to the primary immunosuppressive drug (CsA or Tac) and the time after LTx. *P*-values (Mann-Whitney *U*-test) at the top of the Figure express the group differences of changes in HCV serum RNA level (virus load), after 1, 3, 6, and 12 mo and the last measurement pre transplantation as indicated. *P*-values at the bottom of the Figure are the group differences between HCV-RNA concentrations at the given time of measurement. Boxes indicate the lower and upper quartiles and median, '+' indicates mean.

Serum HCV-RNA concentrations showed a substantial rise (between 10- and 1 000-fold) after LTx. The elevation of HCV-RNA levels developed between 1 and 3 mo post LTx, at which point the elevation was the most pronounced and continued then throughout the entire observation period of 12 mo. The patients who received tacrolimus for immunosuppression had a lower mean HCV-RNA level pre LTx than those who received CsA, but the difference was not statistically significant ($P = 0.09$). One month post LTx this group difference in the HCV-RNA levels was still present and even slightly accentuated, reaching statistical significance at this point ($P = 0.006$). However, 3 mo post LTx the difference was significantly diminished neither at this time ($P = 0.84$) nor at 6 ($P = 0.37$) or 12 mo ($P = 0.40$) post LTx. The group difference reached statistical significance.

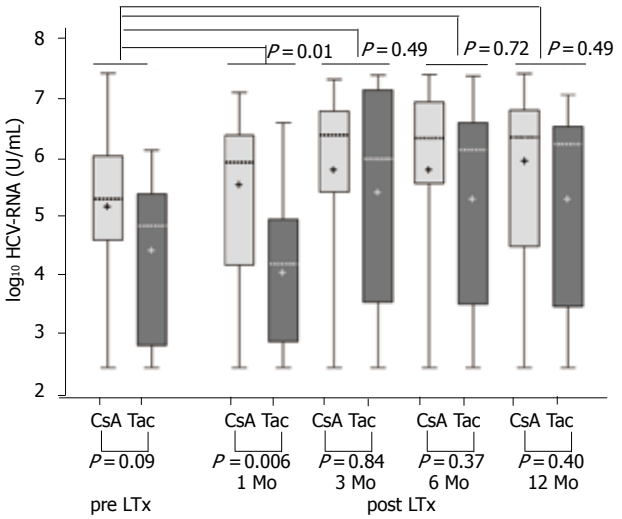

Figure 1 Box–whisker's plot of $\log_{10}$-transformed HCV-RNA concentrations in dependence on group (immunosuppression) and time.

A statistical significance between the levels of HCV-RNA in CsA and Tac groups was evaluated by comparison of the group difference at each given time point after LTx with the group difference before LTx. This analysis revealed no relevant dissimilarity between serum HCV-RNA concentrations in patients who received CsA or Tac for immunosuppression. A slightly higher rise in the tacrolimus group particularly 3 mo post LTx was statistically insignificant ($P = 0.49$). The differences at 6 and 12 mo between both the groups were also not significant ($P = 0.72$, $P = 0.49$). Table 2 lists the net group differences of the changes on a $\log_{10}$-scale in serum HCV-RNA between the pre-LTx and post LTx values.

A power analysis revealed that with the given patient number, the group differences of the changes in serum HCV-RNA concentrations should be on the order 10-fold higher in order to be detectable with a power of 80%. In fact, the strongest deviation from the null hypothesis of no group difference of changes was not a significantly ($P = 0.11$) stronger increase by a factor of 2.5 from pre LTx to the 1 mo measurement in the CsA as compared to the tacrolimus group, opposing a possible superiority of CsA.

Activity of recurrent hepatitis C in dependence of immunosuppression

The value of HCV serum RNA level in predicting the inflammatory activity and progression of fibrosis was analyzed by a scatter plot of serum ALT, mirroring the activity of recurrent hepatitis, in dependence of serum HCV-RNA levels (Figure 2). The correlation coefficient was almost equal to zero ($r = 0.01$, $P = 0.92$), suggesting no significant association between levels of HCV-RNA and intrahepatic inflammatory activity. Therefore HCV-RNA levels post transplantation may not be a valid predictor of allograft inflammation. Differences in the progression of recurrent hepatitis were examined by serum transaminase level and histology in the two patient groups. Figure 3 shows the ALT levels (logarithmically scaled) as a box-whisker's plot of enzyme activity in correlation to

Table 2 Net group difference (CsA *vs* FK 506) of changes in virus load (log_{10}-scale) between pre-LTx measurement and indicated time points post LTx

Mo	Mean	95%CI	*P*-value[1]
1	0.40	(–0.28; 1.08)	0.11
3	–0.50	(–1.46; 0.47)	0.49
6	–0.43	(–1.50; 0.64)	0.72
12	–0.56	(–1.64; 0.52)	0.49

[1]Mann–Whitney *U* test.

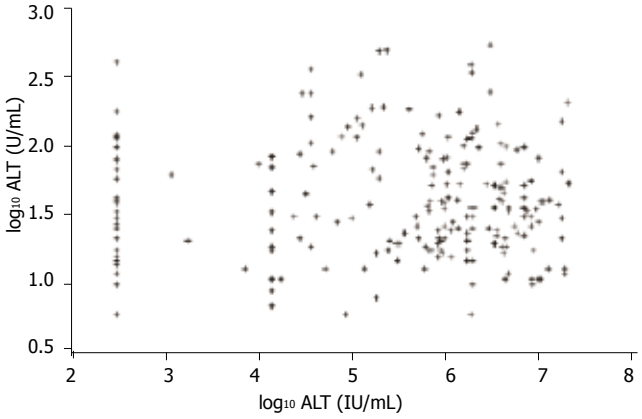

Figure 2 Correlation of HCV-RNA level in serum with ALT activity. Scatter plot with HCV-RNA concentrations on the *y*-axis and ALT activity on the *x*-axis, both on log_{10}-scale.

immunosuppression (CsA group or FK 506 group) and the time after LTx. *P*-values (Mann-Whitney *U*-test) at the bottom of the Figure are for group differences between ALT activity at the time of measurement. Boxes indicate the lower and upper quartiles and median, '+' indicates mean.

In comparison to the level before LTx, the ALT level fell significantly immediately after the operation, with the lowest values 1 mo post LTx. After 3 mo especially after 6 mo ALT levels rose again and stayed elevated over the entire observation period of 12 mo, indicating the development of recurrent chronic hepatitis C. The ALT activity after 3 and 6 mo was increased in the CsA group. but the difference was not statistically significant (3 mo: $P=0.36$, 6 mo: $P=0.65$). Analysis of AST activity revealed similar kinetics post LTx with lower absolute values and also no statistically significant group differences (data not shown).

In conjunction with these analyses, examination of the proportion of patients with normal activities of transaminases post LTx revealed that 70% of patients in the CsA group and 83% in the Tac group had normal ALT activities 1 mo post LTx (Figure 4A). Recurrent hepatitis led to a significant decrease of the proportion of patients with normal ALT values after 3 and 6 mo (48% in the CsA group and 42% in the Tac group). While the proportion of patients with normal ALT at these time points was slightly higher in the CsA group than in the Tac group which was reversed after 12 mo, only 49% of patients in

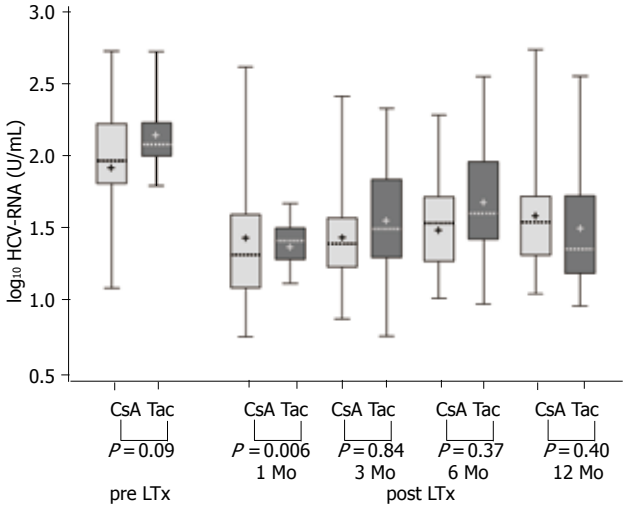

Figure 3 Box–whisker's plot of log_{10}-transformed ALT activities in dependence on group (immunosuppression) and time.

the CsA group but 58% of patients in the Tac group had normal ALT values. However, the group differences were statistically significant neither at 6 mo ($P=0.69$) nor at 12 mo ($P=0.56$). The proportion of patients with normal AST changed similarly (Figure 4B), indicating no advantage for CsA in suppressing inflammatory activity.

To further evaluate the potential differences in the activation and maintenance of recurrent hepatitis C between the two groups, we compared necroinflammatory activity and grade of fibrosis according to HAI scores in the liver histology taken 3-5 mo post LTx. The majority of patients in both groups had a grade II inflammation (74% of patients in the CsA group and 83% of patients in the Tac group), indicating that recurrent hepatitis occurred in a significant proportion of patients in the absence of elevated serum transaminases (Figure 5A). The total group differences including all inflammation grades between the CsA and Tac groups were statistically negligible ($P=0.85$). Examination of fibrosis revealed a larger proportion of patients in the CsA group than in the Tac group scored with grade 0 (26% *vs* 8.3%, Figure 5B). However, at grade I this relation was reversed (2.9% *vs* 25 %). In addition, more patients in the CsA group were scored with grade II fibrosis, while patients in the Tac group were more frequently scored with grade III fibrosis. The number of patients presenting with grade IV fibrosis was very small in both groups. Due to the changes in grades 0 and I, these overall group differences were statistically significant ($P = 0.03$). However, if grades 0 and I were analyzed as one group, no statistical differences were found ($P=0.34$).

DISCUSSION

The marked increase in serum HCV-RNA levels after LTx is closely associated with the immunosuppressive therapy[7,11,24]. Our study also confirmed that serum HCV-RNA levels increased in the early (3 mo) post-transplant period, when immunosuppression was the highest. Although there is evidence that immunosuppression accelerates the progression of chronic HCV infection to cirrhosis, no clear

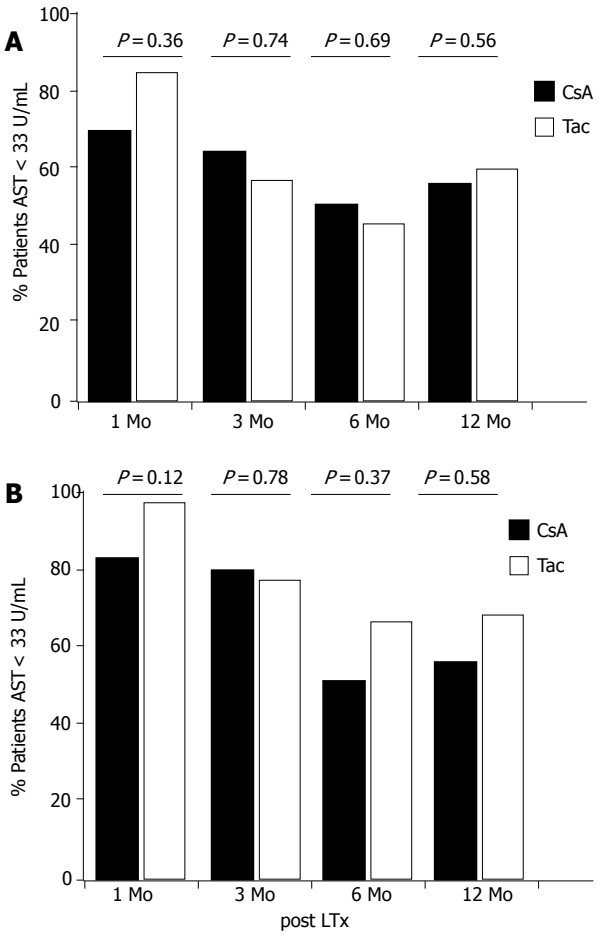

Figure 4 Proportion of HCV-infected patients under CsA or Tac medication with normal transaminase activities at different time points post LTx. **A:** Percentage of patients with normal ALT; **B**: percentage of patients with normal and AST. *P*-values were calculated by x^2 test.

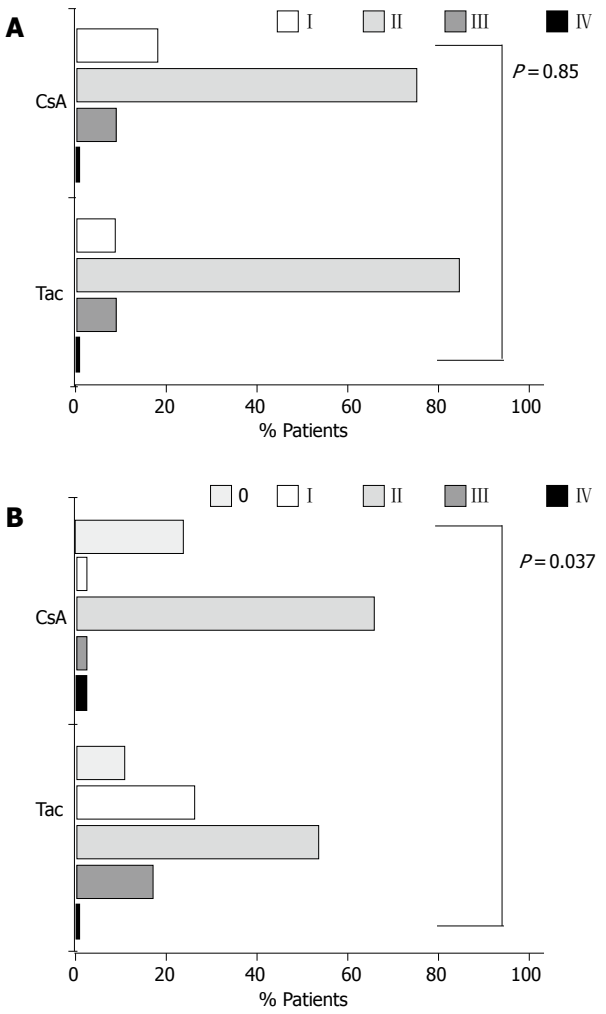

Figure 5 Intrahepatic inflammatory activity and fibrosis post LTx depending on immunosuppression. **A:** Necroinflammatory activity histologically scored by the subscores for portal inflammation of the Knodell's hepatic activity index (HAI); **B**: grade of fibrosis in the graft. Fibrosis was scored according to the appropriate subscores of the HAI.

association has been found between the type or dose of immunosuppressive regimen and the outcome of post-transplant HCV recurrence. CsA and Tac are the most common immunosuppressive drugs used after LTx, but their exact impact on the incidence, severity, time of onset and outcome of HCV recurrence is unclear[10]. In this context, Watashi *et al* [17] showed that CsA but not Tac can inhibit HCV replication *in vitro*, which raises the question whether HCV-infected patients treated with CsA have an advantage with regard to the progression of recurrent hepatitis and therefore graft survival as compared to those treated with Tac.

Our results did not confirm the function of CsA in inhibiting HCV replication as compared to Tac. Neither the differences at any time of examination nor the differences in serum HCV-RNA levels after LTx were in favor of CsA. In addition, there were no significant differences in inflammation of the allograft between the CsA and Tac groups. as determined by serum transaminase activities and histological grading of intrahepatic necroinflammatory activity. The slight differences in the grade of fibrosis, suggesting a lower grade of fibrosis in the CsA group were not statistically significant. While a study showed that HCV-infected patients treated with Tac have an improved survival in comparison to those treated with CsA[16], our results are in accordance with a recently published study, which also found no advantage for either immunosuppressive agent[19]. However, Watashi *et al* [17] showed that CsA can inhibit HCV replication in hepatocytes. In the last several years. a number of cell culture propagation systems based on the infection of primary human[25,26] or chimpanzee[27] hepatocytes or hepatic cell lines[28,29] have been reported. There is a general agreement that all these systems have a poor validity and reproducibility. Viral proteins and particles have never been convincingly detected and viral RNA can be quantified only by the sensitive PCR methodology[30,31]. In HCV research, these limitations of the infection-based cell culture systems have led to the development of a selectable HCV replicon system[32]. In this system, a subgenomic part of HCV-RNA can replicate in a human hepatoma cell line (HuH-7) to unphysiologically high levels, suggesting that this system is highly artificial and may not adequately mirror the situation *in vivo*. An additional important aspect regarding both cell culture systems is the absence of the immune system. Even if CsA has a HCV suppressing effect *in vivo*, this effect is by far overweighed by the immunosuppressive effects of the drug, resulting in a decreased control of HCV replication by the immune system.

In conclusion, our study does not justify a recommendation for CsA as a primary immunosuppressive agent for the treatment of HCV-infected patients post LTx. Further prospective studies should be conducted to clarify this issue. To vigorously improve the graft survival and prognosis of HCV-infected patients post LTx, development of new therapeutic agents directly modifying HCV-replication and/or inflammatory activity of recurrent hepatitis C in combination with alternative immunosuppressive regimens remains a high priority goal.

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S- Editor Wang XL and Guo SY **L- Editor** Elsevier HK **E- Editor** Cao L

PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

World J Gastroenterol 2006 February 7; 12(5): 703-708
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

STI571 (Glivec) suppresses the expression of vascular endothelial growth factor in the gastrointestinal stromal tumor cell line, GIST-T1

Toufeng Jin, Hajime Nakatani, Takahiro Taguchi, Takumi Nakano, Takehiro Okabayashi, Takeki Sugimoto, Michiya Kobayashi, Keijiro Araki

Toufeng Jin, Department of Tumor Surgery, Kochi Medical School, Kochi University, Nankoku, Kochi 783-8505, Japan and Department of General Surgery, College of Medical, Yanbian University, 119 Juzijie, Yanji, Jilin Province, China
Hajime Nakatani, Takumi Nakano, Takehiro Okabayashi, Takeki Sugimoto, Michiya Kobayashi, Keijiro Araki, Department of Tumor Surgery, Kochi Medical School, Kochi University, Nankoku, Kochi 783-8505, Japan
Takahiro Taguchi, Department of Human Health and Medical Science, Graduate school of Kuroshio Science, Kochi University, Nankoku, Kochi 783-8503, Japan
Correspondence to: Hajime Nakatani, MD, PhD, Department of Tumor Surgery, Kochi Medical School, Kochi University, Nankoku, Kochi 783-8505, Japan. nakatanh@med.kochi-u.ac.jp
Telephone: +81-88-880-2370 Fax: +81-88-880-2371
Received: 2005-08-09 Accepted: 2005-08-26

Abstract

AIM: To estimate whether STI571 inhibits the expression of vascular endothelial growth factor (VEGF) in the gastrointestinal stromal tumor (GIST) cells.

METHODS: We used GIST cell line, GIST-T1. It has a heterogenic 57-bp deletion in exon 11 to produce a mutated c-KIT, which results in constitutive activation of c-KIT. Cells were treated with/without STI571 or stem cell factor (SCF). Transcription and expression of VEGF were determined by RT-PCR and flow cytometry or Western blotting, respectively. Activated c-KIT was estimated by immunoprecipitation analysis. Cell viability was determined by MTT assay.

RESULTS: Activation of c-KIT was inhibited by STI571 treatment. VEGF was suppressed at both the transcriptional and translational levels in a temporal and dose-dependent manner by STI571. SCF upregulated the expression of VEGF and it was inhibited by STI571. STI571 also reduced the cell viability of the GIST-T1 cells, as determined by MTT assay.

CONCLUSION: Activation of c-KIT in the GIST-T1 regulated the expression of VEGF and it was inhibited by STI571. STI571 has antitumor effects on the GIST cells with respect to not only the inhibition of cell growth. but also the suppression of VEGF expression.

Key words: c-KIT; Vascular endothelial growth factor (VEGF); STI571; Gastrointestinal stromal tumor; GIST-T1

Jin T, Nakatani H, Taguchi T, Nakano T, Okabayashi T, Sugimoto T, Kobayashi M, Araki K. STI571 (Glivec) suppresses the expression of vascular endothelial growth factor in the gastrointestinal stromal tumor cell line, GIST-T1. *World J Gastroenterol* 2006; 12(5): 703-708

http://www.wjgnet.com/1007-9327/12/703.asp

INTRODUCTION

Gastrointestinal stromal tumors (GIST) are characterized by the expression of both c-KIT and CD34 on the cell surface. Mutations in c-*kit* have recently been implicated with oncogenic activation associated with GISTs[1,2]. c-KIT, a type III receptor tyrosine kinase, is activated upon binding of the ligand, stem cell factor (SCF) to initiate a signaling pathway that is critical for the growth and development of mast cells, melanocytes, hematopoietic stem cells, and the interstitial cells of Cajal[3,4]. Gain-of-function mutations in c-*kit* are associated with a number of cancers in human beings[1,5-9]. The majority of GISTs show constitutive c-KIT phosphorylation due to a gain-of-function mutation in exon 11 (cytoplasmic juxtamembrane domain), with other mutations known to occur in exon 9 (extracellular membrane domain), exon 13 (first part of the split tyrosine kinase domain), and exon 17 (phosphotransferase domain)[8].

STI571 is a specific tyrosine kinase inhibitor that acts on Bcr-Abl, platelet-derived growth factor receptor (PDGFR), and c-KIT. STI571 has been used successfully in patients with unresectable or metastatic GISTs that show constitutive activation of c-KIT[9].

Vascular endothelial growth factor (VEGF) is a highly specific mitogen for vascular endothelial cells that is induced by hypoxia, oncogene activation, and a variety of cytokines. VEGF is important in angiogenesis and neovascularization of solid tumor growth[10]. Expression of VEGF in GISTs was reported by Takahashi *et al* [11], who suggested a correlation between this expression

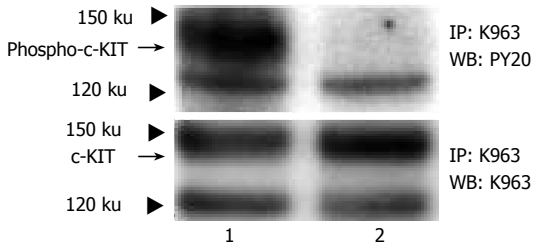

Figure 1 4×10^6 of GIST-T1 cells were treated with STI571 (1 μg/mL) for 1 h. Treated cells were lysed in RIPA buffer and immunoprecipitation with anti-c-KIT antibody as described in "Materials and methods". Western blotting was performed using anti-phosphotyrosine antibody (upper panel) or anti-c-KIT antibody (lower panel). Lane 1, non-treated; lane 2, STI571 1 μg/mL. WB, Western blot; IP, immunoprecipitation.

and poor prognosis. It is thought that the assessment of VEGF expression in GISTs is important clinically.

In the present study, we have analyzed the expression of VEGF in the GIST cell line, GIST-T1, with or without STI571 treatment. To our knowledge, there are only two GIST cell lines, including GIST-T1. The GIST-T1 line was established from a patient with metastatic GIST[12], which showed the c-*kit* mutation in exon 11. In addition, we have analyzed the effect of STI571 on cell growth of the GIST-T1 cells.

MATERIALS AND METHODS

Materials

STI571, also known as Glivec capsule (Novartis, Basel, Switzerland), was dissolved in water (5 μg/μL) and stored at –20 °C. Antibodies were used to detect the c-KIT (K963, rabbit polyclonal IgG, Immuno-Biological Laboratories, Japan), phosphotyrosine (PY20, mouse monoclonal IgG, Zymed, USA), HIF-1 alpha (H-206, rabbit polyclonal IgG, Santa Cruz Biotechnology, USA), and VEGF (A-20, rabbit polyclonal IgG, Santa Cruz Biotechnology, USA).

Cells and cell culture

The human GIST cell line, GIST-T1 has been characterized in detail by Taguchi *et al* [12]. GIST-T1 cells and DLD-1 cells (colon adenocarcinoma cell line) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin, streptomycin, and 80 mL/L fetal bovine serum maintained in a 50 mL/L CO_2 atmosphere at 37 °C in a humidified incubator.

Western blot analysis

Cells were washed thrice with ice-cold phosphate buffered saline (PBS) and then lysed in RIPA buffer containing 20 mmol/L sodium pyrophosphate, 20 mmol/L NaF, 1 mmol/L orthovanadate, 2 mmol/L pyrophosphate, 1 mmol/L PMSF, 10 μg/mL aprotinin, and 10 μg/mL leupeptin. Cell lysates containing comparable amounts of proteins, estimated by a Bradford assay (BioRad, Munchen, Germany), were analyzed by Western blotting using antibodies as listed above.

Immunoprecipitation analysis

A measure of 4×10^6 cells was seeded on 10-cm dishes and incubated for 24 h, prior to the treatment with STI571. Cells were treated with or without STI571 (1 μg/mL) for 1 h. Treated cells were collected and washed with ice-cold PBS thrice and lysed in RIPA buffer as described above. Cells lysates in RIPA buffer were subjected to immunoprecipitation with c-KIT antibody. The immunoprecipitates were reacted with protein A-agarose and washed with Tris buffered saline. They were then finally resuspended in 3×SDS sample buffer containing 30×DTT and boiled at 95 °C for 5 min. Samples were separated by 75 g/L SDS-PAGE and transferred to a membrane for immunoblot analysis.

Isolation of RNA and detection of VEGF or HIF-1 alpha mRNA

A measure of 1×10^6 cells was seeded on 6-cm dishes and incubated for 24 h prior to the treatment with or without the reagents. Prior to the treatment with SCF, medium was changed to the serum-free medium. Treated cells were washed twice in ice-cold PBS and the total cellular RNA was isolated by using TRIzol reagent (Gibco-BRL) according to the manufacturer's protocol. Specific mRNA was assayed using the reverse transcription polymerase chain reaction (RT-PCR). VEGF PCR condition[13]: denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and elongation at 72 °C for 1 min, for 35 cycles; HIF-1 alpha[14]: denaturation at 94 °C for 10 min-1 cycle, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C; beta-actin: denaturing, 94 °C for 30 s, annealing at 50 °C for 40 s and elongation, 72 °C for 1 min, for 30 cycles; and final elongation at 72 °C for 10 min-1 cycle. PCR products were subjected to electrophoresis on 20 g/L agarose gel. The primer sequences were VEGF sense: 5' -CGAAGTGGTGAAGTTCATGG ATG-3'; VEGF antisense: 5' -TTCTGTATCAGTCT TTCCTGGTGA-3'; HIF-1 alpha sense: 5' -CTCAAA GTCGGACAGCCTCA-3'; HIF-1 alpha antisense: 5' -CCCTGCAGTAGGTTTCTGCT-3'; beta-actin sense: 5'-A TTGGCAATGAGCGGTTCCGC-3'; beta-actin antisense: 5'-CTCCTGCTTGCTGATCCACATC-3'.

Flow cytometry

A measure of 2×10^6 cells was seeded on 6-cm dishes and incubated for 24 h, prior to the treatment with STI571 for 4 h. Following this, a Goldi plug (BD Bioscience, San Diego, CA, USA) was added to each dish for 2-h incubation. Cells were then washed with ice-cold PBS twice before the treatment with cytoplex (BD Bioscience) and cytoperm (BD Bioscience) according to the manufacturer' s protocols. Anti-VEGF, anti-HIF-1 alpha, and FITC-labeled anti-rabbit antibodies were used. Normal rabbit IgG antibody and FITC-labeled anti-rabbit antibody were used for control staining.

MTT assay

A measure of 1×10^4 cells/100 μL was seeded in each well of a 96-well plate. After 24 h with or without STI571 treatment, 100 μL of a 2.5 mg/mL solution in PBS of the MTT (3,-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) tetrazolium substrate was added and incubated for 4 h at 37 °C. The resulting violet

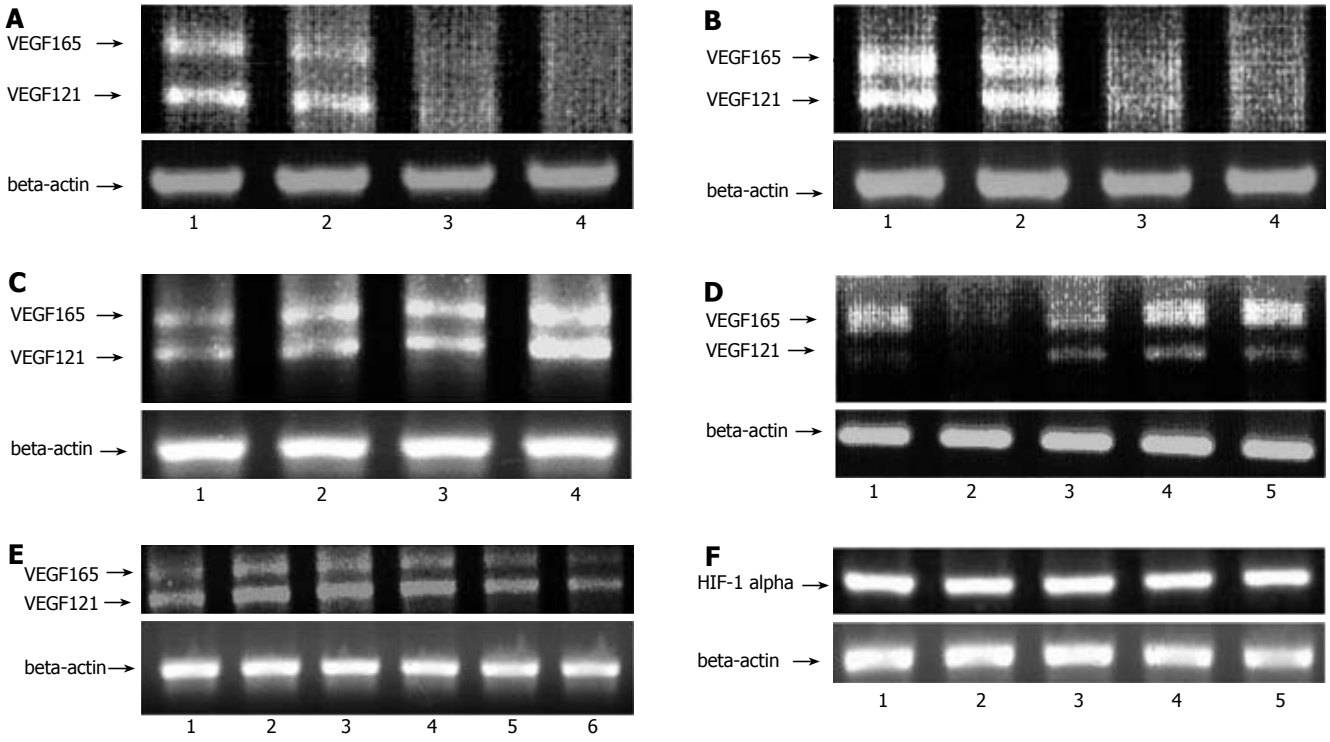

Figure 2 RT-PCR, 1×10^6 GIST-T1 cells were treated with STI571 (0.01, 0.1, and 1 μg/mL) for 6 h (A) or 1 μg/mL of STI571 for 60, 120, and 360 min (B). **A**: Lane 1, non-treated; lane 2, 0.01 μg/mL; lane 3, 0.1 μg/mL; lane 4, 1 μg/mL; **B**: lane 1, 0 min; lane 2, 60 min; lane 3, 120 min; lane 4, 360 min. GIST-T1 cells were treated with SCF (10 ng/mL) for 1, 2, 6 h (C). **C**: lane 1, 0 h; lane 2, 1 h; lane 3, 2 h; lane 4, 6 h. GIST-T1 cells were treated with STI571 (1 μg/mL), LY294002 (50 μmol/L), PD98059 (50 μmol/L), or Wortmannin (100 nmol/L) for 6 h (D). **D:** Lane 1, non-treated; lane 2, STI571; lane 3, LY294002; lane 4, PD98059; lane 5, Wortmannin. 1×10^6 DLD-1 cells were treated with SCF in different concentrations for 6 h (E). **E**: Lane 1, non-treated; lane 2, SCF 5 ng/mL; lane 3, SCF 10 ng/mL; lane 4, SCF 15 ng/mL; lane 5, SCF 20 ng/mL; lane 6, SCF 100 ng/mL. GIST-T1 cells were treated with STI571, SCF, or combination of both for 6 h to determine the transcriptional level of HIF-1 alpha (F). **F:** Lane 1, non-treated; lane 2, STI571 1 μg/mL; lane 3, SCF 10 ng/mL; lane 4, SCF 10 ng/mL+STI571 1 μg/mL; lane 5, SCF 10 ng/mL+STI571 10 μg/mL.

formazan precipitate was solubilized by the addition of 100 μL of a 50% *N*,*N*,-dimethyl formamide/10% SDS solution, and incubated for 4 h at room temperature. Sample absorbances were then measured on a plate reader at 570 nm.

Statistical analysis

Data were reported as mean ± SD. Statistics were analyzed with the Student's *t*-test. A P value of less than 0.05 was considered statistically significant.

RESULTS

Tyrosine phosphorylation of c-KIT

c-KIT in the non-treated cells was tyrosine phosphorylated (Figure 1, upper panel, lane 1) in the GIST-T1 cells. This phosphorylation was inhibited by STI571 treatment (Figure 1, upper panel lane 2). c-KIT protein is also shown by Western blotting (Figure 1, lower panel).

Transcription of VEGF and HIF-1 alpha

RT-PCR detected the transcription of VEGF or HIF-1 alpha in the GIST-T1 cells. In the GIST-T1 cells, VEGF121 and VEGF165 isoforms were detected (Figures 2A-2D). STI571 inhibited VEGF in a dose- and time-dependent manner (Figures 2A and 2B). On the contrary, STI571 could not inhibit HIF-1 alpha (Figure 2F, lane 2). SCF up-regulated VEGF (Figure 2C) but not HIF-1 alpha (Figure 2F, lane 3). PD98059, MEK inhibitor, did not inhibit the transcription of VEGF (Figure 2D, lane 4). LY294002 and Wortmannin. both the inhibitors of phosphatidylinositol 3-kinase (PI3K). also did not inhibit the transcription of VEGF in the GIST-T1 cells compared to STI571 (Figure 2D, lanes 3 and 5). SCF (10 ng/mL) upregulated the transcription of VEGF in the colon adenocarcinoma cell line, DLD-1 (Figure 2E, lane 3). However, in the case of high concentration of SCF stimulation (100 ng/mL for 6 h), transcriptions of VEGF were downregulated in the DLD-1 cells (Figure 2E, lane 6).

Intracellular expression of VEGF and HIF-1 alpha in the GIST-T1

Flow cytometry was used to show that VEGF or HIF-1 alpha was expressed in the non-treated GIST-T1 cells (Figures 3B and 4B). STI571 reduced the expression of VEGF (Figure 4C). SCF upregulated the expression of VEGF in the GIST-T1 cells (Figure 4D) and STI571 inhibited the upregulation of VEGF by SCF stimulation in the GIST-T1 cells (Figure 4E). HIF-1 alpha could not be influenced by STI571 treatment (Figure 3C). SCF also did not influence the expression of HIF-1 alpha (data not shown). Western blotting revealed that VEGF was expressed as a 21-ku band, which was downregulated by ST1571 in a dose-dependent manner (Figure 5).

Effect of ST1571 on GIST-T1 cell viability

Cell viability of the GIST-T1 cells was measured by MTT

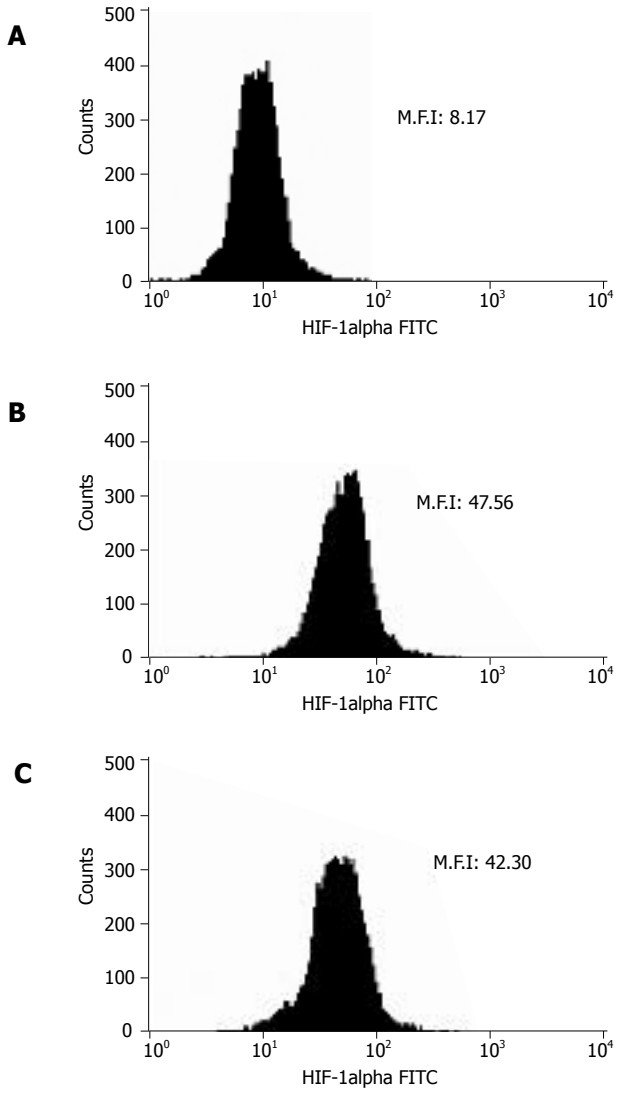

Figure 3 Expression of HIF-1 alpha was determined by flow cytometry. 2×10^6 GIST-T1 cells were treated with/without STI571 as described in "Materials and the methods. **A:** Control (normal rabbit IgG); **B**: non-treated (HIF-1 alpha antibody); **C:** STI571 (1 μg/mL). MFI, mean fluorescent intensity.

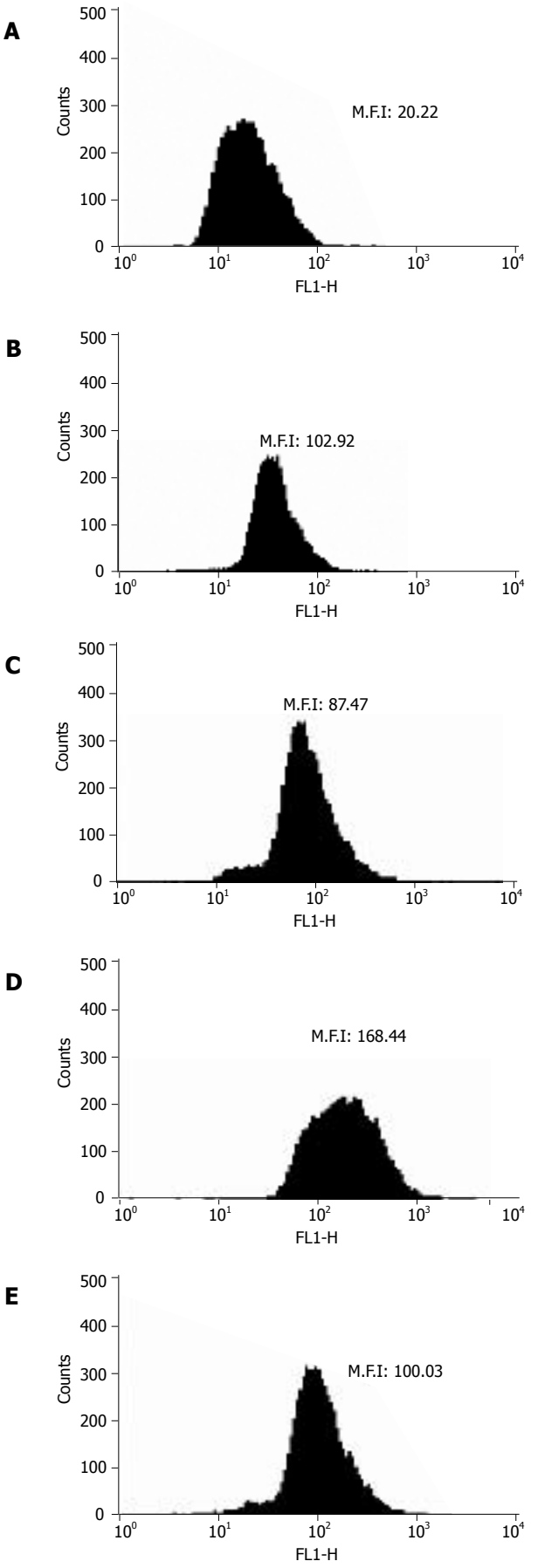

Figure 4 Expression of VEGF determined by flow cytometry. 2×10^6 GIST-T1 cells were treated with/without STI571 or SCF as described in "Materials and methods". **A**: Control (normal rabbit IgG); **B**: non-treated (VEGF antibody); **C:** STI571 (1 μg/mL); **D:** SCF (10 ng/mL); **E:** SCF (10 ng/mL)+STI571 (1 μg/mL). MFI, mean fluorescent intensity.

assay, which showed a reduction in viability following the treatment with STI571 to 37.9 ± 1.3%, 36.5 ± 1.7%, and 36.3 ± 0.3% at the concentrations of 0.1, 1, and 2 μg/mL, respectively ($P < 0.01$ *vs* non-treated). Cell viability in the case of non-treated cells was indicated as 100 ± 0.5%.

DISCUSSION

The present study demonstrated that the recently developed compound STI571 inhibited the tyrosine phosphorylation of c-KIT in GIST-T1 tumor cells, as well as inhibiting both the transcription and translation of VEGF. Tyrosine phosphorylation of c-KIT was inhibited after 30 min treatment with STI571 at different concentrations (0.1 and 1 μg/mL), as assessed by Western blot analysis (data not shown). In contrast, Tuveson *et al* [15] reported that tyrosine phosphorylation of another GIST cell line, GIST882, was not inhibited by a 1-h treatment with STI571 when used at 1 μmol/L (0.59 μg/mL), as assessed by Western blot analysis. GIST882 has a

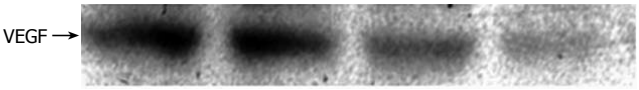

Figure 5 Assessment of expression of VEGF by Western blotting. 3×10^6 cells were treated with STI571 (0.01, 0.1, and 1 μg/mL) for 12 h, and then cells were rinsed with ice-cold PBS and lysed in RIPA buffer as described in "Materials and methods". Fifty micrograms of protein was applied in each lane. Lane 1: non-treated; lane 2: STI571 0.01 μg/mL; lane 3: STI571 0.1 μg/mL; lane 4: STI571 1 μg/mL.

homogenic mutation in the c-*kit* gene resulting in a K642E amino acid change and the constitutive activation of c-KIT[15]. On the other hand, GIST-T1 has a heterogenic 57-bp deletion in exon 11 of the c-*kit* mutation, and the c-KIT protein in the GIST-T1 cells constitutively activated. Furthermore, Frolvo *et al*[16] reported that STI571 inhibits the activation of p44/42 mitogen activated protein kinase (p44/42MAPK) in GIST882 cells, but we could not detect the same effect in our cells following STI571 treatment (data not shown). Oncogenic Ras family proteins activate the p44/42MAPK pathway, and this activation contributes to the increased proliferative rate of tumor cells[17]. Phosphorylated c-KIT also activates the p44/42MAPK pathway as well as plays a role in the process of oncogenic activation[18]. MEK is upstream of p44/42MAPK, and MEK inhibitors could inhibit the activation of p44/42MAPK. In our hands, the MEK inhibitor, PD98059, partially inhibited the activation of p44/42MAPK (data not shown). These results suggested that activation of p44/42MAPK was independent of the activation of c-KIT in GIST-T1 cells.

In a previous study, Ebos *et al* [19] reported that STI571 reduced VEGF expression in the Bcr-Abl positive chronic myelogenous leukemia cells. They reported that activation of Bcr-Abl played an important role in VEGF expression. In our study, RT-PCR and flow cytometry analysis revealed that both the transcription and translation of VEGF in GIST-T1 cells were suppressed by STI571 treatment. Western blot analysis revealed that STI571 inhibited the expression of VEGF for 12-h incubation. SCF activated c-KIT and upregulated the expression of VEGF. These results suggested that activation of c-KIT was an important role in both the transcription and the translation of VEGF in the GIST-T1 cells. Furthermore we investigated the signal transduction cascade which was involved in the expression of VEGF in the GIST-T1 cells using MEK inhibitor or PI3K inhibitors. In our study, PD98059, LY294002 and Wortmannin had no such effect on these cells. LY294002 and Wortmannin are both inhibitors of PI3K and thereby inhibit activation of the AKT pathway[20]. AKT promotes angiogenesis through eNOS activation[20]. STI571 inhibited the activation of AKT in GIST882 cells[16], but the same result was not obtained in our GIST-T1 cells by Western blotting (data not shown). These results suggested that neither the p44/42MAPK nor PI3K signaling pathways are involved in the regulation of VEGF expression in GIST-T1 cells.

Hypoxia inducible factor-1 (HIF-1) plays an important role in the expression of VEGF in the malignant tumors. HIF-1 activity is primarily regulated by the levels of HIF-1 alpha in the cells[21]. In our study, transcriptional or translational levels of HIF-1 alpha were not regulated by STI571 treatment or SCF stimulation.

c-KIT is expressed in the colon adenocarcinoma cell line, DLD-1[22]. It is clear that c-KIT regulates the expression of VEGF in other cells. So, we observed whether c-KIT was involved in the upregulation of the transcriptional levels of VEGF using SCF in DLD-1 cells. SCF induced VEGF transcription at a concentration of 5–10 ng/mL. These results suggested that the expression of VEGF might be concerned with the activation of c-KIT.

MTT assay revealed that STI571 inhibited the cell viability of GIST-T1 cells. Activation of c-KIT in the GIST-T1 cells played an important role in cell survival signaling.

In conclusion, our results on the treatment of GIST-T1 cells with STI571 suggest that the expression of VEGF in these cells might be regulated via the c-KIT signal transduction cascade. STI571 inhibited not only cell viability but also the expression of VEGF in GIST-T1 cells, and it could be a useful compound for GIST therapy. Further studies are required to investigate the mechanism underlying VEGF regulation in more detail.

ACKNOWLEDGMENTS

We thank Ms Motoko Miyata and Ms Yuka Takezaki for their technical assistance.

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S- Editor Guo SY **L- Editor** Elsevier HK **E- Editor** Cao L

PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

World J Gastroenterol 2006 February 7; 12(5): 709-715
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

Effect of increased hepatic platelet activating factor and its receptor portal hypertension in CCl_4-induced liver cirrhosis

Yong-Ping Yang, Xue-Mei Ma, Chun-Ping Wang, Jun Han, Yin-Ying Lu, Yi Xiang, Shu-Hui Su, Yong-Yi Feng

Yong-Ping Yang, Xue-Mei Ma, Chun-Ping Wang, Jun Han, Yin-Ying Lu, Yi Xiang, Shu-Hui Su, Yong-Yi Feng, Department of Gastroenterology, 302 Hospital of PLA, Beijing 100039, China
Supported by the Key Scientific and Technological Research Foundation of the National 863 Program, No. 2003AA208106; Medical Outstandard Foundation of Army, No. 04J020
Correspondence to: Dr Yong-Ping Yang, Department of Gastroenterology, 302 Hospital of the Chinese PLA, Beijing 100039, China. yongpingyang@hotmail.com
Telephone: +86-10-66933429 Fax: +86-10-63806508
Received: 2005-07-11 Accepted: 2005-07-29

Abstract

AIM: To evaluate the changes in hepatic platelet activating factor (PAF) and its receptors and their effect on portal pressure of cirrhotic rats induced by CCl_4.

METHODS: A model of liver cirrhosis was replicated in rats by intra-peritoneal injection of CCl_4 for 8 wk. We determined the effect of hepatic PAF and its receptor level on portal and arterial pressure by EIA, saturation binding and RT-PCR technique.

RESULTS: Compared to control rats, cirrhotic rats had higher hepatic PAF levels and output as well as higher plasma PAF levels ($P < 0.01$, $P < 0.01$, $P < 0.05$, respectively). Both hepatic PAF receptor mRNA levels and PAF binding were nearly 3-fold greater in cirrhotic rats ($P < 0.01$). Portal injection of PAF (1 g/kg WT) increased the portal pressure by 22% and 33% in control and cirrhotic rats, respectively. In contrast, the arterial pressure was decreased in the both groups (54% in control rats and 42% in cirrhotic rats). Injection of the PAF antagonist BN52021 (5 mg/kg WT) decreased the portal pressure by 16% in cirrhotic rats but had no effect in the control rats.

CONCLUSION: The upregulation of the PAF system contributes to hepatic hemodynamic and metabolic abnormalities in cirrhosis, and the increased release of PAF into the circulation has impacts on the systemic hemodynamics.

Key words: Platelet activating factor; PAF receptors; Endothelin; Portal hypertension; Cirrhosis

Yang YP, Ma XM, Wang CP, Han J, Lu YY, Xiang Y, Su SH, Feng YY. Effect of increased hepatic platelet activating factor and its receptors on portal hypertension in CCl_4-induced liver cirrhosis. *World J Gastroenterol* 2006; 12(5): 709-715

http://www.wjgnet.com/1007-9327/12/709.asp

INTRODUCTION

Platelet activating factor (PAF: 1-*O*-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a phospholipid exhibiting diverse biological actions. PAF acts both as a multifunctional, soluble, proinflammatory agent and as a specific membrane-bound adhesion molecule. PAF receptors have been identified in smooth muscle cells, cardiomyocytes, endothelial cells, neutrophils, monocyte-macrophages, Kupffer cells and eosinophils[1]. The physiological actions of PAF play a role in platelet secretion and aggregation, bronchoconstriction, vascular permeability and systemic arterial hypotension[1-4]. Exogenous PAF administration via the portal vein increases portal venous pressure and activates glycogenolysis both in perfused organs[5] and *in vivo*[6]. In contrast, systemic infusion of PAF reduces arterial blood pressure[7,8]. Portal hypertension and hyperdynamic systemic circulation are two prominent clinical features of human and animal experimental liver cirrhosis[9-11]. Circulatory responses to intravenous infusion of PAF resemble the hemodynamic change in advanced liver cirrhosis. Excessive deposition of extracellular matrix is a cause of the increased resistance to hepatic blood flow and consequent portal hypertension in cirrhosis, but studies in animals indicate that increased vascular tone is also a contributing factor[12].

The PAF content in intact liver is elevated by various types of injury, including ischemia-reperfusion[13], obstructive jaundice[14], and endotoxin exposure *in vivo*. Based on these observations, we hypothesized that increased hepatic synthesis of PAF in cirrhosis could lead to increased plasma levels, and that PAF might play a role in portal hypertension. Our results have confirmed that hepatic PAF levels are elevated and PAF receptors are upregulated in cirrhosis. The reactivity of the hepatic vasculature to PAF is consequently increased, while that of the systemic vasculature is attenuated.

MATERIALS AND METHODS

Induction of cirrhosis

Cirrhosis was induced in male Sprague-Dawley rats

weighing 230-250 g as described previously[10,15,16]. Briefly, intraperitoneal injection of CCl_4 (0.15 mL/kg WT, twice a week for 8 wk) was combined with drinking water containing phenobarbital (0.4 g/L). Control rats received injection of the vehicle (peanut oil) and phenobarbital water.

Determination of portal venous and systemic arterial pressure

Rats were anesthetized with 50 mg/kg (i.p.) pentobarbital, and placed on a heated water blanket maintained at 38°C. A PE-50 catheter (Thomas Scientific, Swedesboro, NJ, USA) was inserted into the femoral artery to monitor arterial blood pressure via a strain gauge pressure transducer connected to a 4-channel Grass polygraph (Model no. 79E, Quincy, MA, USA). The hepatic trigone was exposed via laparotomy, and the portal vein was skeletonized. The intestines and the abdominal cavity were covered with warm saline-soaked sponges, and a catheter was inserted into the portal vein and connected to a pressure transducer coupled to a 4-channel Grass polygraph to monitor portal pressure. After stable recordings of both portal and arterial blood pressure were obtained, 1 mL of blood was withdrawn from the femoral artery, portal vein and suprahepatic vena cava, respectively. Then, the liver was rapidly excised and washed in ice-cold phosphate buffered saline containing 0.1 mmol/L EDTA and 0.1 mmol/L EGTA. A portion of the liver tissue was stored in 10% buffered formalin, a portion in OCT, and the rest was snap-frozen in liquid nitrogen and stored at -80 °C.

Morphometric analysis

An established histological grading system was employed for the determination of pathological scores of liver injury[17-19]. Paraffin-embedded liver sections of 4-μm thickness were stained with hematoxylin-eosin and Masson' s trichrome. Steatosis, inflammation, necrosis and fibrosis were scored in at least three random fields of view in each tissue section and a score for each specific parameter was estimated. Steatosis was assessed by estimating the percentage of cells with micro- and macrovesicular fat as follows: 0 (absent), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%). Necrosis was scored as follows: 0 (absent), 1 (1-10 necrotic cells per view), 2 (11-20 necrotic cells per view), 3 (21-30 necrotic cells per view), 4 (31-40 necrotic cells per view), and 5 (41-50 necrotic cells per view). Inflammation was assessed as follows: 0 (absent), 1 (rare), 2 (scattered), 3 (scattered with localized foci), 4 (abundant with foci), and 5 (extensive). Architectural change, fibrosis and cirrhosis were estimated as follows: 0 (absent), 1 (rare), 2 (scattered deposition), 3 (scattered with localized deposition), 4 (abundant with minor bridging fibrosis), 5 (bridging fibrosis) and 6 (cirrhosis). A total pathology score was calculated by combining and summing the scores for the above pathological parameters, and the average score per animal/treatment group was calculated.

Determination of PAF in liver and blood

Lipids were extracted as previously described[20]. Briefly, 100 mg of liver or 1 mL of blood was homogenized in 9.5 mL of a mixture of methanol, chloroform and water (2:1:0.8, v:v:v). The homogenates were kept at room temperature for 1 h, followed by the addition of chloroform (2.5 mL) and water (2.5 mL). After being thoroughly mixed, the mixture was kept at room temperature for 1 h and centrifuged at 1200 r/min for 15 min. The chloroform layer was aspirated and dried under nitrogen at 35°C. The residue was dissolved in 200 μL chloroform, and applied to the Bond Elut SI column (Amprep silica mini-columns; Amersham Pharmacia Biotech, Piscataway, NJ, USA). The column was washed with 3 mL of chloroform, 2 mL of chloroform-methanol (6:4; v/v) and 3 mL of chloroform-methanol-28% aqueous ammonia (70:85:7, v:v:v). PAF was eluted with 2 mL of chloroform-methanol-28% aqueous ammonia (50:50:7, v/v). The elute was evaporated to dryness under nitrogen, and the residue was dissolved in 200 μL of saline containing 0.01% Triton X-100. PAF concentration was determined by [^{3}H]-PAF scintillation proximity assay (Amersham-Pharmacia Biotech).

Determination of hepatic PAF binding

Hepatic membranes were prepared as described previously[10,16] and suspended in 50 mmol/L Tris-HCl (pH 8.0) containing 5 mmol/L $MgCl_2$, 125 mmol/L choline chloride, 0.1 mol/L PMSF, 0.1 μg/mL leupeptin and 1 μg/mL pepstatin. The membrane suspension was stored at -80°C in aliquots after the protein concentration was adjusted to 10 mg protein/mL. Membranes (100 μg protein) were incubated in 50 mmol/L Tris-HCl (pH 7.2) containing 5 mmol/L $MgCl_2$, 125 mmol/L choline chloride, 0.25% BSA and 0.125-32 nmol/L 1-*O*-[^{3}H]octadecyl-2-acetyl-sn-glycero-3-phosphocholine (151 Ci/mmol, 9.96 GBq/mg; Amersham) ± 10 μmol/L unlabeled PAF (Bachem Americas, King of Prussia, PA, USA) at 30°C for 1 h. The reaction was terminated with the addition of 5 mL ice-cold assay buffer and filtration through Whatman GF/C filters (Whatman, Hillsboro, OR, USA) presoaked in assay buffer for 1 h. Filters were washed thrice with 4 mL of assay buffer, and the radioactivity was determined in a β-scintillation counter.

Determination of hepatic PAF receptor mRNA levels

The mRNA expression of PAF receptors was determined by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR). RNA was isolated from the livers using a RNA isolation kit (ToTALLY RNA™, Ambion, Austin, TX, USA). Two micrograms of total RNA was used for the preparation of cDNA by reverse transcription as described. cDNA equivalent of 5 ng original RNA was used in PCR. The reaction mixture (50 μL) contained 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, 20 pmol of PCR primers and 2U platinum Taq DNA polymerase (GIBCO-Invitrogen, Carlsbad, CA, USA). Thirty-five cycles of reaction were carried out as follows: denaturation at 94°C for 1 min, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The PCR primers used for PAF cDNA were: 5'-GCCACAACAGAGGCTTGA-3'(forward) and 5'-TCCATTGCTCTGGGCAGGAA-3'(reverse) [product size, 121 bp[21]]. For normalization, β-actin mRNA levels were measured as described previously[15], using cDNA primers:

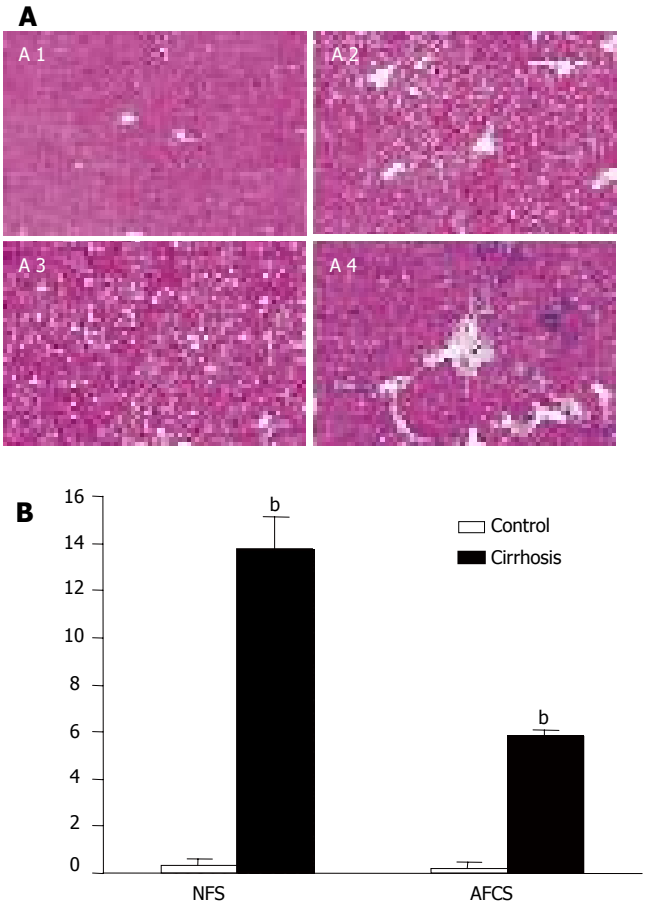

Figure 1 Morphometric analysis of cirrhotic liver 8 wk after CCl_4 or vehicle treatment **(A)** and scores for necroinflammatory NFS, architectural change, fibrosis and cirrhosis (AFCS) **(B)**. $^bP<0.01$ *vs* control.

Table 1 Characteristics of cirrhosis (mean ± SD)

	Control	Cirrhotic rats	*P*
Liver/body weight ratio (%)	4.80±0.25	4.90±0.44	NS
Weight of spleen (g)	0.73±0.06	1.36±0.15	0.001
Portal pressure (kPa)	0.71±0.08	1.63±0.09	0.001
MAP (kPa)	15.2±1.2	10.9±1.3	0.01
PAF (ng/g)	2.74±0.49	3.95±0.43	0.01

NS: not significant.

5'-TTCTACAATGAGCTGCGTGTG-3'(forward) and 5' -TTCATGGATGCCACAGGATTC-3'(reverse) [product size, 561 bp[22]]. The PCR products were resolved in a 25 g/L agarose gel and stained with SYBR Green I (FMC Bioproduct, Rockland, ME, USA). The gels were scanned under blue fluorescent light using a phosphorimager and the band intensity was quantified using ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA, USA).

Determination of PAF effect on portal venous and systemic arterial pressure

The experiment was essentially conducted for the measurement of baseline pressure. After a stable recording of portal and systemic mean arterial pressure (MAP) was obtained, 1 mL of a solution containing PAF (1 μg/kg) was infused over 1 min from a 975 Harvard apparatus compact infusion pump into the portal vein via a 23 gauge needle/PE50 catheter. Portal venous pressure and MAP were monitored continuously for 15 min.

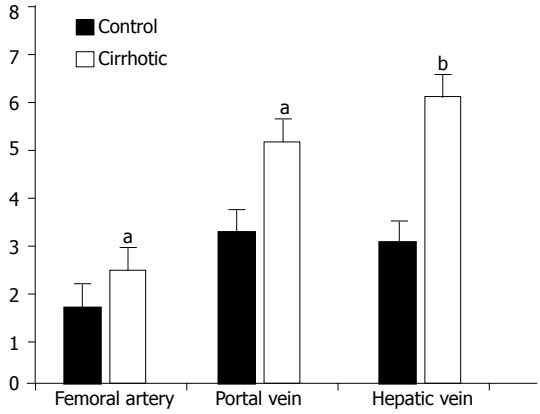

Figure 2 Effect of cirrhosis on circulating PAF levels. Concentrations of PAF in blood from femoral artery, portal and hepatic vein (suprahepatic vena cava) were determined. Values are mean±SD. $^aP<0.05$ *vs* control; $^bP<0.01$ *vs* control.

Statistical analysis

The values were presented as mean ± SD. Student's *t*-test was employed for statistical comparison between the groups. $P<0.05$ was considered statistically significant.

RESULTS

Characteristics of cirrhosis

There were no notable changes in liver histology throughout the 8 wk in control rats (Figure 1A1). In contrast, CCl_4-treated rats demonstrated extensive changes in liver morphology, including steatosis, inflammation, hepatocyte ballooning, and necrosis. Extensively distorted architecture due to excessive deposition of extracellular matrix was observed, which caused bridging of fibrosis, and infiltration of inflammatory cells in sinusoids and their accumulation around the islands of hepatocytes (Figure 1A3). Hepatic fibrosis was further validated histologically with Masson's trichrome staining (Figures 1A2 and A4).

The liver/body weight ratio was not significantly different between control and cirrhotic rats, whereas spleen weight of cirrhotic rats was nearly doubled (Table 1). MAP was significantly lower in cirrhotic rats (10.9 ± 1.3 kPa) than in control rats (15.2 ± 1.2 kPa). Portal venous pressure was higher in cirrhotic rats (1.63 ± 0.09 kPa) than in control rats (0.71 ± 0.08 kPa), indicating portal hypertension (Table 1).

Effect of cirrhosis on hepatic and circulating PAF and hepatic PAF receptor levels

Hepatic PAF concentrations were increased by 44% in cirrhotic rats (Table 1). Concentrations of PAF in the femoral arterial, portal venous, and hepatic arterial blood were significantly greater in cirrhotic rats than in control rats (Figure 2). Moreover, the concentration of PAF was significantly greater in hepatic venous blood than in

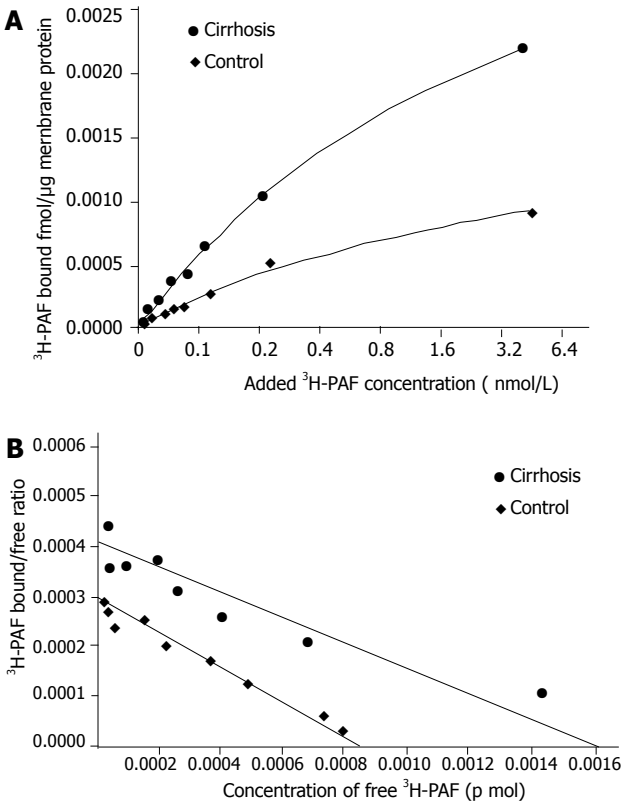

Figure 3 Effect of cirrhosis on hepatic PAF binding. **A:** Results of the saturation binding assay. ^{3}H-PAF was incubated with 100 μg membrane protein, in the presence or absence of 10 μmol/L at 30 °C for 1 h; **B**: Scatchard plot analysis of ^{3}H-PAF binding to hepatic tissue of cirrhotic and control rats. Values are mean of separate experiments. Cirrhosis: $R = 0.98$, Kd = 8.013 nmol/L, Bax = 2.8 ± 0.213 fmol/μg membrane protein; control: $R = 0.99$, Kd = 5.794 nmol/L, Bax = 0.9 ± 0.061 fmol/μg membrane protein.

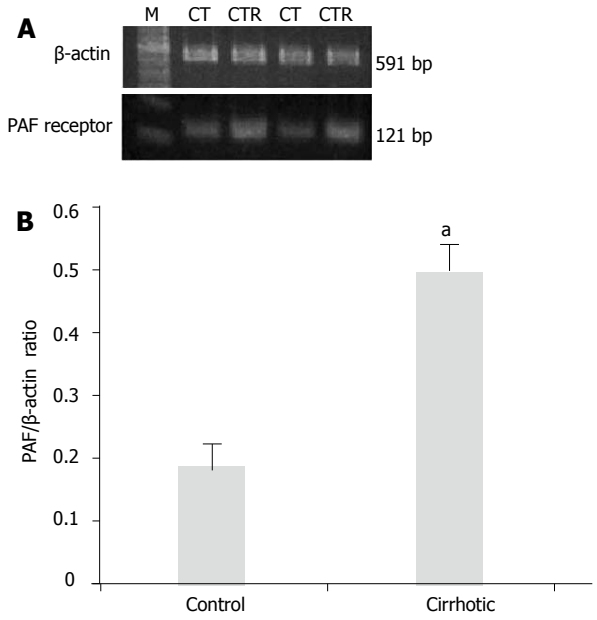

Figure 4 Effect of cirrhosis on mRNA expression of hepatic PAF receptor. **A:** PCR products of PAF and β-actin from control (CT) and cirrhotic (CR) rat livers; **B**: ratio of the PAF receptor and β-actin mRNA from six samples. $^aP < 0.01$ *vs* control.

portal venous blood in cirrhotic rats (Figure 2). No such difference in hepatic venous and portal venous blood of control rats was observed.

Scatchard analysis of the saturation binding data revealed a 3-fold increase in PAF binding capacity in cirrhotic liver as compared to the control (Bmax of 2.8 ± 0.21 *vs* 0.9 ± 0.06 fmol/μg protein, $P < 0.01$), whereas receptor affinity was unaltered (Kd: 8.01 ± 1.33 nmol/L for cirrhotic rats and 5.79 ± 0.96 nmol/L for control rats, $P < 0.05$, Figure 3). Consistent with the increased receptor density, a similar increase in the PAF receptor mRNA transcript was observed in the cirrhotic liver as determined by RT-PCR (PAFR/β-actin mRNA ratio of 0.51 ± 0.03 *vs* 0.16 ± 0.015, $P < 0.01$, Figure 4).

Effect of PAF on portal and arterial pressure in cirrhotic rats

A separate set of control and cirrhotic rats were used to determine the PAF-induced changes in portal and systemic blood pressure. Infusion of PAF (1 g/kg WT) via the portal vein after a short delay caused a greater increase ($P < 0.01$) in portal pressure in cirrhotic rats (from 1.61 ± 0.08 to 2.13 ± 0.09 kPa; 33% increase) than in control rats (from 1.07 ± 0.10 to 1.30 ± 0.09 kPa; 22% increase) (Figures 5 and 6). Following the initial rise, the portal pressure decreased progressively with time and was lower than the basal value 9 min after the administration of PAF.

The effect of PAF administered into the portal vein on arterial pressure was opposite to that observed on portal pressure (Figures 5 and 6). In contrast to the delay onset of the rise in portal venous pressure, MAP fell immediately after the administration of PAF. The decrease in MAP ($P < 0.01$) was greater in control rats (54%) than in cirrhotic rats (42%). With the fall in MAP, pulse pressure fell markedly and did not recover, suggesting a reduction in cardiac contractility.

Effect of PAF antagonist BN52021 on portal and arterial pressure in cirrhotic rats

Administration of BN52021 (5 mg/kg WT) via the portal vein caused a 16% decrease in the portal pressure of cirrhotic rats (from 1.95 ± 0.21 to 1.64 ± 0.15 kPa, $P < 0.01$). This effect occurred 2 to 3 min after the administration of BN52021. On the other hand, arterial pressure did not change upon treatment with BN52021 (from 11.0 ± 0.8 to 11.3 ± 0.67 kPa). In control rats, BN52021 had no effect on either portal or arterial pressure(Figure 7).

DISCUSSION

This investigation showed that increased production of PAF in the cirrhotic liver could lead to an increased circulating level of PAF, which is likely responsible for the arterial hypotension associated with the disease. In addition, hepatic PAF receptor mRNA expression was upregulated with a concomitant increase in PAF binding. Since PAF potently increases portal pressure and hepatic vascular resistance[6,7,23], it can be concluded that the up-regulated PAF system in the liver plays an important role in portal hypertension.

In the present study, the source of the increased hepatic PAF and the site of increased PAF receptors were not

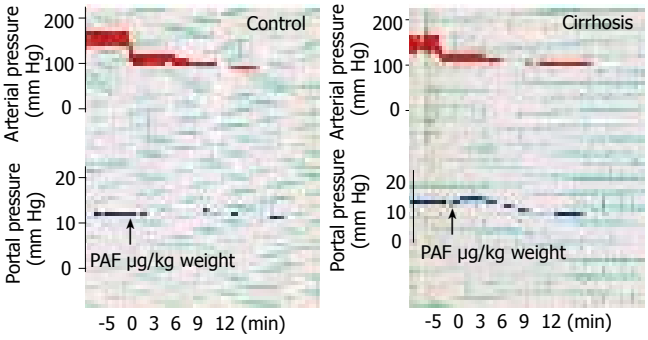

Figure 5 Tracings of the femoral arterial and portal venous pressure. After stabilization of arterial and portal venous pressure, PAF (1 μg/kg WT) was infused and the pressure was recorded continuously.

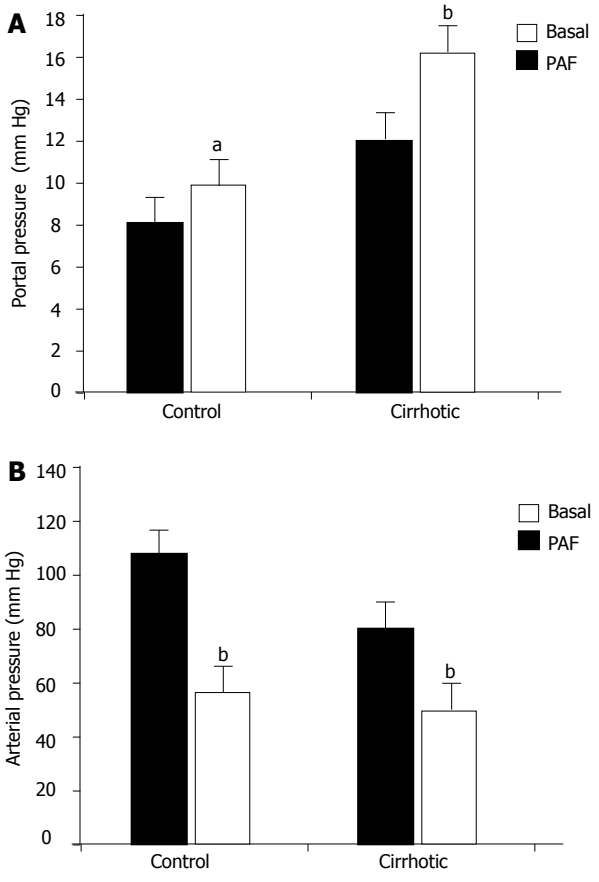

Figure 6 Effect of cirrhosis on PAF-induced changes in arterial and portal pressure. Arterial and portal venous pressure before and after portal administration of PAF were measured. Values are mean ± SD of six determinations. $^{a}P < 0.05$ *vs* basal value; $^{b}P < 0.01$ *vs* basal value.

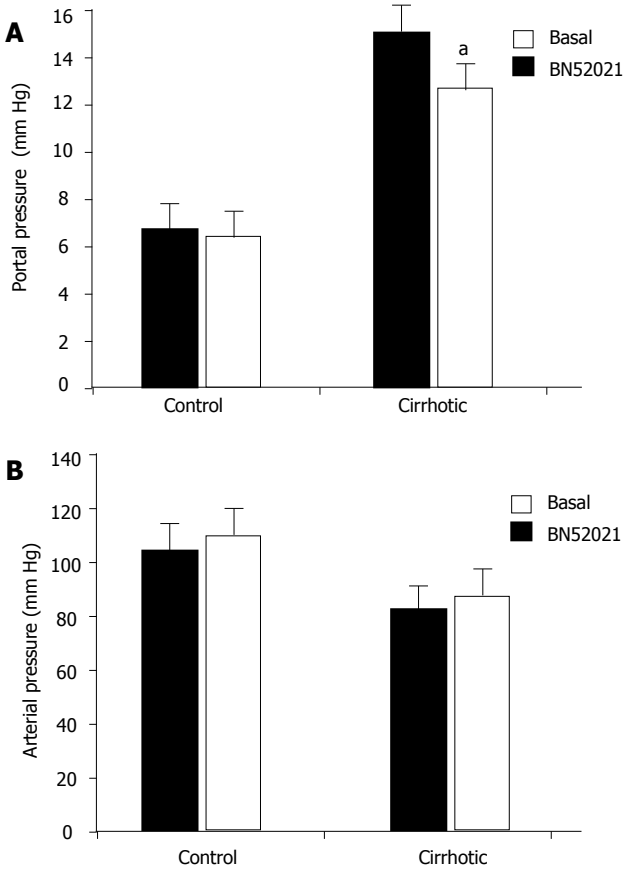

Figure 7 Effect of BN52021 on femoral arterial and portal venous pressure of cirrhotic rats 8 wk after vehicle or CCl_4 treatment. Values are mean±SD 5-6 min after introduction of BN52021. $^{a}P < 0.05$ *vs* basal value.

determined. Yang *et al* [10] reported that Kupffer cells are a major source of increased PAF in CCl_4-induced cirrhotic rat liver. Synthesis of PAF by Kupffer cells is stimulated by a number of inflammatory mediators and bacterial endotoxin (ET)[2,24,25]. Increased circulating ET is a strong candidate contributor to the elevation of hepatic portal pressure in cirrhosis[15,26]. We have previously reported that hepatic ET-1 and its receptors increase in rats 24 h after the administration of CCl_4[15] and progressively thereafter during the development of cirrhosis. In the present study, hepatic PAF did not increase during the first week of CCl_4 treatment (2.70 ± 0.75 ng/g in control rats and 2.74 ± 0.46 ng/g in CCl_4-treated rats, $P > 0.05$) but increased after 2 wk of treatment. Since PAF synthesis by Kupffer cells is increased by ET-1[24], the early elevation of ET-1 may contribute to the long-term increase in hepatic PAF. To further investigate the interaction between these two potent mediators during cirrhosis, we infused the powerful vasoconstrictor ET-1 into some experimental animals. After stabilization of the portal and arterial pressures following the administration of PAF, ET-1 (0.75 nmol) was infused as a bolus into the portal vein. The magnitude of ET-1 effect was much greater in control rats (200% increase) than in cirrhotic rats (80% increase, $P < 0.01$). We have previously reported that portal infusion of ET-1 alone causes a 150% increase in portal pressure in control rats and a 23% increase in cirrhotic rats[15], suggesting that with or without prior infusion of PAF, the qualitative responses to ET-1 are similar between control and cirrhotic rats. Quantitatively, prior infusion of PAF appears to sensitize the hepatic vasculature to ET-1 moderately in control rats (200% *vs* 150%) and greatly in cirrhotic rats (80% *vs* 23%). The interaction between these two potent mediators during cirrhosis warrants further investigation.

Potentially, PAF could be increased by a decrease in the activity of plasma PAF-acetylhydrolase, which is predominantly responsible for the hydrolysis of PAF to lyso-PAF[27,28]. Plasma PAF-acetylhydrolase activity is similar

in normal individuals and patients with alcohol-induced liver cirrhosis[29]. Patients with primary and secondary biliary cirrhosis have elevated levels of circulating PAF and serum PAF-acetylhydrolase activity[30], suggesting that synthesis of PAF increased by plasma PAF-acetylhydrolase is the mechanism underlying the elevated circulating levels of PAF.

The augmented effect of PAF on the vasculature of chronically injured liver appears to be independent of the hemodynamic state of the liver (i.e. normotensive or portal hypertensive). The vasoconstrictor effect of PAF in the perfused livers is greater in rats with thioacetamide-induced hepatic fibrosis than in control rats, even though the fibrosis rats have no portal hypertension[31]. In the present study, PAF-induced portal hypertension was of greater magnitude in the cirrhotic rats than in the control rats. These results suggest that the increased reactivity to PAF is associated with the pathology of hepatic cirrhosis or injury rather than the pre-existing presence or absence of portal hypertension. That is to say, the response to PAF is not dependent upon the already increased hepatic vascular tone.

The reduction of portal pressure in cirrhotic rats induced by the PAF antagonist BN52021 provides convincing evidence that endogenous PAF is involved in the development of portal hypertension. The mechanisms of PAF-induced hepatic vasoconstriction are very complex and remain to be defined. PAF receptors have been found in smooth muscle cells[32]. PAF elevates cytosolic free calcium in smooth cells[33,34], which is necessary for muscle contraction. Exogenous PAF causes contraction of smooth muscle from ileal and peripheral lung strips[35,36] and contributes to endothelin-induced vascular constriction in rat mesentery[37]. Therefore, the contractile effect of PAF on the hepatic vasculature may be elicited by its direct action on smooth muscle cells. PAF may also act by stimulating the synthesis of eicosanoids such as thromboxane, PGE_2, and PGD_2, which are known to cause portal vasoconstriction[38-41].

The combination of portal hypertension and peripheral vasodilatation with systemic arterial hypotension is characteristic of liver cirrhosis and implicates PAF. The observation that PAF induces smooth muscle contraction of ileal[35] and pulmonary strips[36], and micro-vessels[37] is apparently in contradiction with the observation that it induces hypotension when administered intravenously to rats[42]. In fact, PAF produces hypotension in all animal species studied[8]. Increased plasma levels of PAF in cirrhosis are associated with low peripheral vascular resistance that is reversed by PAF antagonist BN52021[9,29,43]. Sakaguchi *et al*[44] and Hines *et al*[6] reported that intravenous administration of PAF (1.5 μg/kg WT) causes arterial hypotension. The mechanisms of PAF-induced systemic arterial hypotension are not clearly understood. PAF-induced hypotension is not mediated by the central nervous system, renin-angiotensin system, β-adrenergic and dopaminergic eicosanoids, as well as Ca^{2+} influx and thyrotropin releasing steroids[45,46]. However, PAF-induced delay and persistent hypotension is inhibited by the nitric oxide synthase inhibitor, N^{ω}-nitro-L-arginine[47] while nitric oxide plays a role in PAF-induced relaxation of rat thoracic aorta[48]. This can be partially explained by the observation that PAF released by the liver may regulate systemic hemodynamics or stimulate the release of very powerful hypotensive agents such as nitric oxide[47,48]. The involvement of nitric oxide requires clarification.

In conclusion, PAF is an important mediator of hepatic pathology during chronic liver injury. Cirrhotic liver is a source of circulating PAF and a major contributor to the systemic hypotension associated with liver cirrhosis.

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S- Editor Wang XL and Guo SY **L- Editor** Elsevier HK **E- Editor** Cao L

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World J Gastroenterol 2006 February 7; 12(5): 716-722
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

IL-1β activates p44/42 and p38 mitogen-activated protein kinases via different pathways in cat esophageal smooth muscle cells

Tai Sang Lee, Hyun Ju Song, Ji Hoon Jeong, Young Sil Min, Chang Yell Shin , Uy Dong Sohn

Tai Sang Lee, Hyun Ju Song, Ji Hoon Jeong, Young Sil Min, Uy Dong Sohn, Department of Pharmacology, College of Pharmacy, Chung-Ang University, Seoul 156-756, Republic of Korea
Chang Yell Shin, Research Laboratory, Dong-A Pharmaceuticals Co. Ltd, Kyunggido, Republic of Korea
Correspondence to: Dr Uy Dong Sohn, Department of Pharmacology, College of Pharmacy, Chung-Ang University, Seoul 156-756, Republic of Korea. udsohn@cau.ac.kr
Telephone: +82-2-820-5614 Fax: +82-2-826-8752
Received: 2005-05-20 Accepted: 2005-07-15

Key words: IL-1β; MAP kinase; Esophageal smooth muscle cells

Lee TS, Song HJ, Jeong JH, Min YS, Shin CY, Sohn UD. IL-1β activates p44/42 and p38 mitogen-activated protein kinases via different pathways in cat esophageal smooth muscle cells. *World J Gastroenterol* 2006; 12(5): 716-722

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Abstract

AIM: To examine the pathway related to the IL-1β-induced activation of mitogen-activated protein (MAP) kinases in cat esophageal smooth muscle cells.

METHODS: Culture of the esophageal smooth muscle cells from cat was prepared. Specific inhibitors were treated before applying the IL-1β. Western blot analysis was performed to detect the expressions of COX, iNOS and MAP kinases.

RESULTS: In the primary cultured cells, although IL-1β failed to upregulate the COX and iNOS levels, the levels of the phosphorylated forms of p44/42 MAP kinase and p38 MAP kinase increased in both concentration- and time-dependent manner, of which the level of activation reached a maximum within 3 and 18 h, respectively. The pertussis toxin reduced the level of p44/42 MAP kinase phosphorylation. Tyrphostin 51 and genistein also inhibited this activation. Neomycin decreased the density of the p44/42 MAP kinase band to the basal level. Phosphokinase C (PKC) was found to play a mediating role in the IL-1β-induced p44/42 MAP kinase activity. In contrast, the activation of p38 MAP kinase was inhibited only by a pretreatment with forskolin, and was unaffected by the other compounds.

CONCLUSION: Based on these results, IL-1β-induced p44/42 MAP kinase activation is mediated by the Gi protein, tyrosine kinase, phospholipase C (PLC) and PKC. The pathway for p38 MAP kinase phosphorylation is different from that of p44/42 MAP kinase, suggesting that it plays a different role in the cellular response to IL-1β.

INTRODUCTION

Esophageal reflux is a common condition that affects both children and adults. If left untreated, it can result in chronic esophagitis, aspiration pneumonia, esophageal strictures and Barrett's esophagus, which is a premalignant condition[1]. Reflux esophagitis (RE) is a multifactorial disease that may depend on the relaxation of the transient lower esophageal sphincter (LES), the speed of esophageal clearance, the mucosal resistance and other factors, and is often associated with the LES pressure[2]. Cytokines exhibit potent chemotactic activity toward different populations of leukocytes, and are essentially involved in the induction of acute and chronic inflammatory reactions[3,4]. The selective infiltration of various leukocyte subsets into the esophagus indicates the participation of chemokines in the immune and inflammatory processes of RE.

Interleukin-1 (IL-1) has several biological effects on many cell types, and plays a key role in regulating the inflammatory reaction[5]. The IL-1 family is an important part of the innate immune system, which regulates functions of the adaptive immune system. In the presence of excess IL-1, inflammatory and autoimmune diseases can develop in many organs, including the joints, lungs, gastrointestinal track, central nervous system, or blood vessels. Stimulation of the cells by IL-1 initiates the transcription of many pro-inflammatory genes, including IL-6 and IL-8. The levels of the inflammatory genes are higher in the esophageal mucosa of those patients with RE[6]. IL-1 has its own identical biological functions and it is induced by gastric ulceration in rats[7,8]. Increased mucosal levels of IL-1 are constantly observed during acute and chronic intestinal inflammation in human beings[9,10] and animals[11,12]. The immunoneutralization of the IL-1 activity has been reported to greatly reduce the severity of colitis in a murine model of the disease,

suggesting that this cytokine plays an important role in initiating inflammation[12]. The IL-1 mRNA and protein were strongly expressed in the gastric mucosa of gastritis and ulcer patients[13,14]. Therefore, it is suggested that the pro-inflammatory effects of IL-1 are due to the induction of COX-2 gene expression in many tissues, including mucous[14]. However, there are few reports about the effect on the smooth muscle.

In mammalian cells, there have been at least three different subfamilies of mitogen-activated protein (MAP) kinases reported. They include the extracellular signal-regulated kinases (ERKs, p44/42 MAP kinase), *c*-Jun N-terminal kinase (JNK), and p38. These kinases are activated by distinct upstream MAP kinases (MEKs), which phosphorylate the residues within a tripeptide motif (Thr-X-Tyr) on the MAP. Once activated, MAP kinases, in turn, phosphorylate a variety of intracellular substrates, including certain transcription factors[15]. IL-1β is known to activate all three MAP kinase subfamilies in human airway smooth muscle cells. IL-1β stimulation has been associated with several MAP kinases cascades involved in the transmission of a signal from the cell surface to the nucleus. Although these pathways have not been demonstrated to be involved in the IL-1β-mediated effects on the esophageal smooth muscle, p38 and ERK have been shown to contribute to such effects in human[16] or canine airway[17] or myometrial[18] smooth muscle. Therefore, in this study, we examined the mechanism associated with IL-1β-induced MAP kinases activation in cat esophageal smooth muscle cells.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS) was purchased from Biofluids (Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM), antibiotic-antimycotic (penicillin, streptomycin, amphotericin B), and trypsin-EDTA were obtained from Invitrogen (Grand Island, NY, USA). Phospho-p44/p42 monoclonal MAP kinase antibody, phospho-SAPK/JNK monoclonal antibody, phospho-p38 monoclonal MAP kinase antibody, p44/42 MAP kinase antibody, SAPK/JNK antibody and p38 MAP kinase antibody were acquired from Cell Signaling (Beverly, MA, USA). Goat anti-mouse IgG-HRP was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The rainbow prestained molecular weight marker was obtained from Amersham (Arlington Heights, IL, USA). The enhanced chemiluminescence agents were purchased from PerkinElmer Life Sciences (Boston, MA, USA). Sodium dodecyl sulfate (SDS) sample buffer was acquired from Owl Scientific, Inc. (Woburn, MA, USA). Nitrocellulose membrane, Tris/glycine/SDS buffer and Tris/glycine buffer were purchased from BioRad (Richmond, CA, USA). Phosphate-buffered saline (PBS) was acquired from Boehringer Mannheim (Indianapolis, IN, USA). The PD 98059 and SB202190 were obtained from Calbiochem (La Jolla, CA, USA). The GF 109203X was purchased from Tocris (Ellisville, MO, USA). Restore™ Western Blot Stripping Buffer was obtained from Pierce (Rockford, IL, USA). Horseradish peroxidase-conjugated goat anti-rabbit antibody, phorbol-12-myristate-13-acetate (PMA), genistein, tyrphostin 51 and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of feline esophageal smooth muscle tissue and cell culture

Adult cats of either gender, weighing between 2.5 and 3.5 kg, were used in this study. The cats were anesthetized with ketamine (50 mg/mL per kg) and the abdomen was then opened with a midline incision. The esophagus and stomach were excised together, cleaned and maintained in Krebs buffer with the following composition (mmol/L): NaCl 116.6, $NaHCO_3$ 21.9, NaH_2PO_4 1.2, KCl 3.4, $CaCl_2$ 2.5, glucose 5.4 and $MgCl_2$ 1.2. The esophagus and stomach were opened along the lesser curvature. The location of the squamocolumnar junction was identified and the mucosa was peeled off. The high-pressure zone was identified by a visible thickening of the circular muscle layer in conjunction with the squamocolumnar junction and immediately proximal to the sling fibers of the stomach. It has been shown that a 5- to 8-mm band of tissue coinciding with the thickened area constitutes the LES, and has distinct *in vivo* characteristics in an organ bath or as single cells after enzymatic digestion[19,20].

After opening the esophagus and stomach and identifying the LES, the mucosa and submucosal connective tissues were removed via a sharp dissection. The LES was excised and a 3- to 5-mm wide strip at the junction of the LES and esophagus was discarded in order to avoid potential overlap. The circular muscle layer from the esophagus was cut into 0.5-mm-thick slices using a Stadie Riggs tissue slicer (Tomas Scientific Apparatus, Philadelphia, PA, USA). The last slices containing the myenteric plexus, the longitudinal muscle and the serosa were discarded, and the remaining slices were then cut into 2 mm × 2 mm tissue squares by hand.

The sliced tissue was then placed into DMEM supplemented with 500 mL/L FBS containing 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 μg/mL amphotericin B and incubated at 37 ℃ in a humidified atmosphere containing 50 mL/L CO_2 and 950 mL/L air. On the following day, fresh DMEM containing 100 mL/L FBS was added. Ten days later, the tissue explants were removed and the medium was exchanged with fresh DMEM containing 100 mL/L FBS. After the cells reached confluence, the cells were detached with 10 g/L trypsin-EDTA in HBSS with sodium bicarbonate. The cells were then counted, seeded at 1×10^6 cells/mL on 100-mm culture dishes, and maintained in DMEM containing 100 mL/L FBS. The medium was changed every 48 h until the cells reached confluence. The experiments were performed on the cells of passage 2.

In order to characterize the isolated and cultured esophageal smooth muscle cells as well as to exclude contamination by epithelial cells and fibroblasts, the cells were identified using an indirect immunofluorescent staining method with a monoclonal antibody of a light chain myosin[21]. More than 95% of the cell preparation was found to be composed of smooth muscle cells.

Preparation of cell extracts and Western blot analysis

For the animal esophagitis model, esophageal smooth

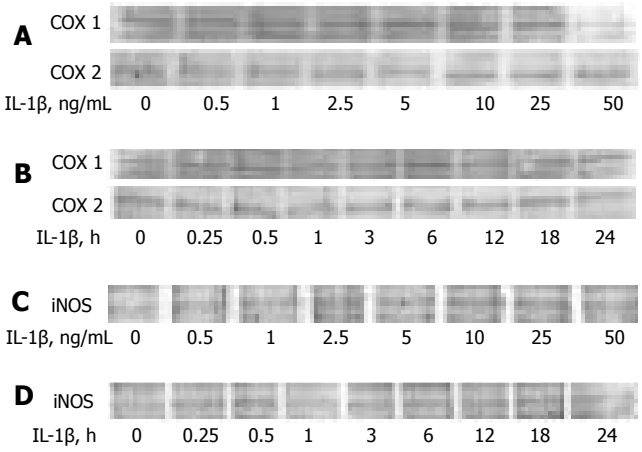

Figure 1 No increase of COX and iNOS expression levels by IL-1β. (A) The cultured cat ESMCs were treated with IL-1β for 18 h at each dose scale. The COX-1 and COX-2 expression levels were not altered by IL-1β at concentrations up to 50 ng/mL. (B) The IL-1β (25 ng/mL) treatment also failed to elevate the level after 24 h. (C) iNOS expression was unaffected by IL-1β.

muscle tissue squares were homogenized in an homogenizing buffer containing 20 mmol/L Tris (hydroxymethyl) aminomethane (Tris), 160 mmol/L HCl (pH 7.5), 0.5 mmol/L EGTA, 0.5 mmol/L EDTA, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 mmol/L β-mercaptoethanol and centrifuged at 14000 *g* for 15 min at 4 °C. The supernatants were used as the whole cell extract.

For the IL-1β treatment experiments, the cells were plated in six-well culture plates and made quiescent at confluence by incubating them in serum-free DMEM for 24 h. The growth-arrested esophageal smooth muscle cells were incubated with or without IL-1β at 37 °C at various times. When the inhibitors were used, they were added 1 h before applying the IL-1β according to the inhibitors, with the exception that the cells were pretreated with the pertussis toxin 24 h prior to IL-1β. After incubation, the cells were rapidly washed with ice-cold PBS and lysed for 5 min in an ice-cold lysis buffer containing 25 mmol/L Tris-HCl (pH 7.4), 25 mmol/L NaCl, 25 mmol/L NaF, 25 mmol/L sodium pyrophosphate, 1 mmol/L sodium vanadate, 2.5 mmol/L EDTA, 2.5 mmol/L EGTA, 0.5 g/L Triton X-100, 5 g/L SDS, 0.5 g/L deoxycholate, 0.5 g/L NP-40, 5 μg/mL leupeptin, 5 μg/mL aprotinin, and 1 mmol/L PMSF. After incubation, the lysates were scrapped and collected by centrifugation at 45000 *g* for 1 h at 4 °C to yield the whole cell extract.

Equal amounts of the protein from each sample were resolved on a SDS-polyacrylamide gel by electrophoresis. Prestained molecular mass markers were also run in an adjacent lane to determine the molecular mass. The separated proteins were transferred to a 0.45-μm nitrocellulose membrane in 25 mmol/L Tris (pH 8.3), 192 mmol/L glycine, and 100 mL/L methanol using a power supply (Power Pac 1000, BioRad, Melville, NY, USA). The membranes were incubated in a PBS buffer containing 50 g/L non-fat dry milk for 1 h at room temperature in order to block the nonspecific binding. After washing thrice with PBS, the blots were incubated with the primary antibodies in a PBS solution containing 1 g/L BSA at 4 °C overnight. The membranes were washed using PBS containing 0.5 g/L Tween 20 and then incubated with the horseradish peroxidase-conjugated secondary antibody (1:1000 dilution) for 1 h. The immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham). The total MAP kinase expression level was determined by subsequently stripping the same blot with a stripping buffer and reproving the blot with p44/42 MAP kinase antibodies. Developed films from ECL were scanned and analyzed densitometrically using Scion Image.

Protein determination

The protein concentration of the supernatant in each reaction vial was measured spectrophotometrically at a wavelength of 595 nm using a BioRad assay (BioRad Chemical Division, Richmond, CA, USA).

Statistical analysis

The data were expressed as mean ± SE of three separate experiments, and the statistical differences between means were determined by using Student's *t* test. A $P<0.05$ was considered statistically significant.

RESULTS

The treatment of the esophageal smooth muscle cells (ESMC) with IL-1β did not increase the COX-2 expression level (Figure 1). In other cell types such as canine tracheal smooth muscle cells[22], human gastric cancer cells[23], and human myometrial smooth muscle cells[18], it has been reported that IL-1β exerts its biological effects partly by inducing COX-2 expression. However, the cells treated with IL-1β at a concentration of 0.5-50 ng/mL for 18 h did not show an increased COX-1 and COX-2 expression levels (Figure 1A). In addition, when treated with 25 ng/mL IL-1β for 15 min to 24 h, the COX expression level in the cat ESMC was not altered (Figure 1B). These results corresponded to previous results showing that an experimentally induced rat esophagitis did not cause an increase in the COX-2 level, suggesting that COX-2 might not mediate esophagitis (unpublished data).

It is also known that IL-1β mediates inflammation, at least in part, by inducing the expression of inducible nitric oxide synthase (iNOS)[7], with the concomitant enhanced synthesis of NO, which is a critical effector molecule, in rat pulmonary artery smooth muscle cells. The cat ESMCs were treated with IL-1β (25 ng/mL) for 18 h and the iNOS level was determined by using Western blotting analysis (Figures 1C and 1D). This treatment did not increase the iNOS expression level, suggesting that iNOS, like COX-2, might not be a mediator of the cellular response to IL-1β in ESMC.

Although there was no change in the COX and iNOS levels, which is similar to other reports, IL-1β activated p44/42 MAP kinase with increasing concentration (Figure 2). After a treatment with 0.5-50 ng/mL IL-1β for 3 h, p44/42 MAP kinase was phosphorylated at concentrations above 10 ng/mL. Densitometric assessments demonstrated that the exposure of ESMCs to 25 ng/mL IL-1β for 3 h significantly increased the level of

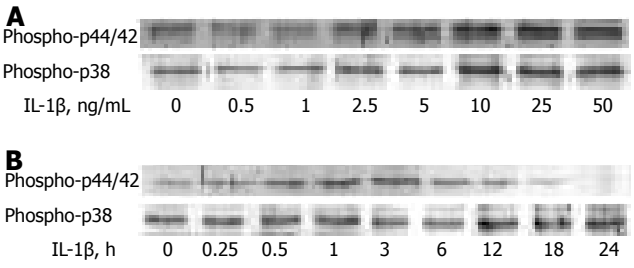

Figure 2 Concentration- and time-dependent activation of MAP kinases after IL-1β treatment. **(A)** To obtain dose-dependent course of p44/42 MAP kinase and p38 MAP kinase, the cat ESMCs were treated with IL-1β for 3 and 18 h, respectively. The levels of p44/42 MAP kinase and p38 MAP kinase phosphorylation were increased above a dose of 10 ng/mL, which was maintained up to 50 ng/mL. **(B)** After treatment the cells with IL-1β (25 ng/mL), the level of p44/42 MAP kinase phosphorylation reached a maximum within 3 h with a subsequent decrease to the basal level (upper panel). The phosphorylated forms of p38 MAP kinase were increased within 12 h, which were sustained to 24 h.

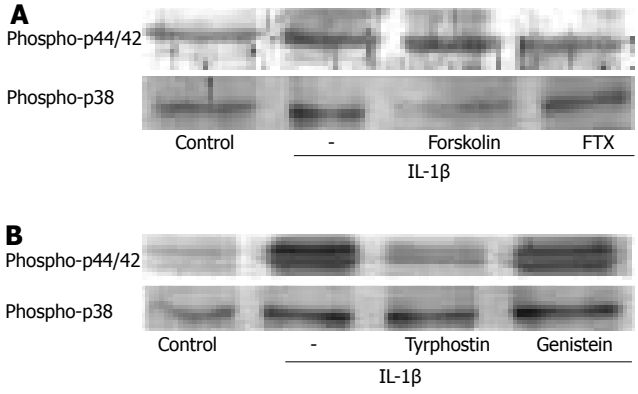

Figure 3 Relation of Gi protein and tyrosine kinase with activation of p44/42 MAP kinase, and negative regulation of p38 MAP kinase by adenylate cyclase. (**A**) Pretreatment with the pertussis toxin (100 ng/mL, 24 h) decreased the density of phosphorylated p44/42 MAP kinase only. On the other hand, forskolin (10 μmol/L) attenuated p38 MAP kinase phosphorylation by IL-1β. (**B**) The receptor tyrosine kinase inhibitor, tyrphostin 51 (30 μmol/L), markedly reduced the IL-1β-induced phosphorylation of p44/42 MAP kinase. The activation of p38 MAP kinase was unrelated to the tyrosine kinases.

activation of p44/42 MAP kinase by 2.9 ± 0.1 times over the level of control samples (Figure 2A). Furthermore, IL-1β (25 ng/mL) produced a time-dependent increase in p44/42 MAP kinase phosphorylation (Figure 2B). The effect of IL-1β on p44/42 MAP kinase was observed within 30 min. The maximum increase (2.6 ± 0.1-fold increase over the control) in this response was observed within 3 h, and a gradual decrease to the basal level within 18 h was observed. Based on the concentration-response and time-course data, the ESMCs were exposed to IL-1β at a final concentration of 25 ng/mL for 3 h in later experiments, aiming at identifying the signaling pathway of p44/42 MAP kinase activation. Similarly, p38 MAP kinases were activated by IL-1β in the cat ESMCs. As shown in Figure 2A, an 18-h IL-1β treatment caused phosphorylation of p38 MAP kinase in a concentration-dependent manner. A clear increase (1.9 ± 0.1-fold) was observed at concentrations above 10 ng/mL IL-1β. The time course of p38 MAP kinase phosphorylation indicated that the IL-1β increased the activity within 12 h, which was maintained up to 24 h (1.5 ± 0.04-fold increase over the control). Therefore, even though IL-1β activated p38 MAP kinase as well as p44/42 MAP kinase, a difference in the activated time of the two MAP kinases was also observed. This suggests that IL-1β can evoke these cellular responses via at least two pathways, involving p38 MAP kinase and p44/42 MAP kinase, in the cat ESMCs.

In order to determine if the effects of IL-1β on p44/42 and p38 MAP kinases were mediated through the activation of a receptor coupled to the pertussis toxin (PTX)-sensitive G protein, the ESMCs were pretreated with 100 ng/mL PTX for 24 h, followed by stimulation with IL-1β for 3 and 18 h, respectively (Figure 3A). Previous studies showed that various types of G proteins, such as Gs, Gq, Go and $G_{i1\text{-}3}$, could be detected in the cat ESMCs[24]. As shown in Figure 3A, pretreatment of these cells with PTX caused a slight attenuation of p44/42 phosphorylation, indicating that IL-1β exerts its effects through the receptor-coupled Gi protein. However, p38 MAP kinase phosphorylation was maintained without being mediated by the Gi protein. In order to determine the involvement of adenylate cyclase (AC) in the activation of MAP kinases, the cells were pretreated with forskolin 24 h before IL-1β in order to activate the AC (Figure 2A). AC had little effect on p44/42 MAP kinase activation, but was found to decrease the phosphorylated forms of p38 MAP kinase. Therefore, it is believed that IL-1β-induced p38 MAP kinase activation is negatively regulated by cAMP.

Recently, it has been proposed that IL-1β causes the activation of tyrosine kinase and regulates protein tyrosine phosphorylation[22]. There are two classic tyrosine kinases, namely, receptor and nonreceptor tyrosine kinase. The receptor tyrosine kinases are actually the growth factor receptors located in the inner side of the cytoplasmic membrane and undergo dimerization and autophosphorylation upon activation. In this study, we examined the possibility that the observed increase in the level of MAP kinases phosphorylation after an IL-1β treatment might be mediated by tyrosine kinase (Figure 3B). Therefore, the cells were pretreated with genistein for 1 h prior to adding IL-1β. This resulted in a decrease in IL-1β-induced p44/42 MAP kinase activation. In addition, tyrphostin 51, which is a member of the genistein family of tyrosine kinase inhibitors and is specific to the receptor tyrosine kinase, significantly decreased the level of p44/42 MAP kinase phosphorylation, suggesting that tyrosine kinase might participate in these responses to IL-1β.

The involvement of phospholipase in the IL-1β-induced p44/42 MAP kinase activation was examined using a phospholipase C (PLC) inhibitor (neomycin), a PLA_2 inhibitor (DEDA), and a PLD inhibitor (ρCMB). As shown in Figure 4A, pretreatment of the ESMCs with neomycin attenuated the p44/42 MAP kinase activation. DEDA also slightly decreased the band density, but could not reach statistical significance. In addition, pretreatment of the ESMCs with ρCMB did not decrease the level of p44/42 MAP kinase activation induced by IL-1β, suggesting that IL-1β-stimulated p44/42 activation in ESMC might be mediated via the activation of PLC. In this study, we therefore investigated whether or not

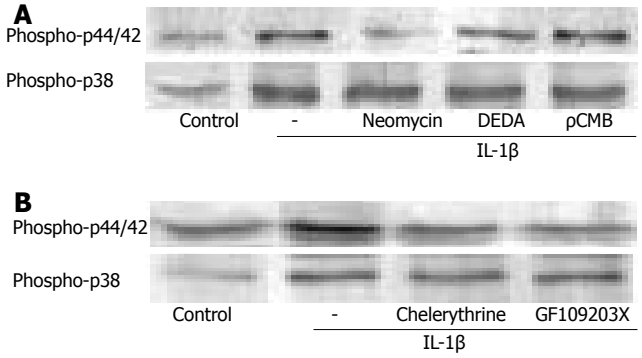

Figure 4 IL-1β-induced activation of p44/42 MAP kinase is mediated by PLC and PKC. (**A**) Neomycin (10 μmol/L, 1 h) decreased the phosphorylated forms of p44/42 MAP kinase. DEDA (10 μmol/L, 1 h) and ρCMB (10 μmol/L, 1 h) had no effect on the IL-1β-induced activation of p44/42 MAP kinase. (**B**) PKC inhibitors chelerythrine (10 μmol/L, 1 h) and GF109203X (10 μmol/L, 1 h) decreased the active form of p44/42 MAP kinase. All the phospholipase inhibitors and PKC inhibitors failed to regulate the activation of p38 MAP kinase by IL-1β.

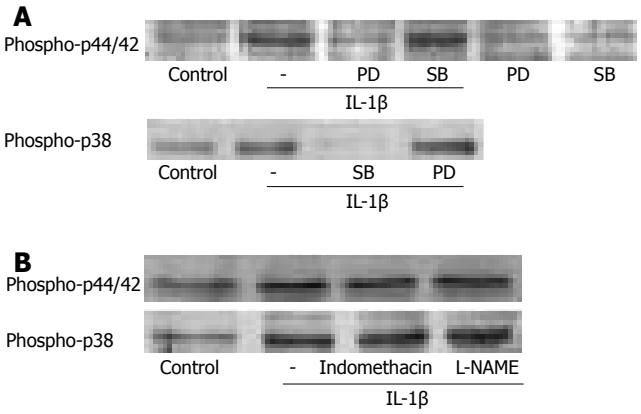

Figure 5 MAP kinase activation by IL-1β was not affected by other kind of MAP kinase, COX and iNOS. (**A**) MEK inhibitor PD98059 (10 μmol/L, 1 h) reduced the level of p44/42 MAP kinase phosphorylation by IL-1β. SB202190 (30 μmol/L, 1 h), p38 MAP kinase inhibitor, had no effect on the p44/42 MAPK phosphorylation, which blocked p38 MAP kinase activation. (**B**) Indomethacin (10 μmol/L) and L-NAME (10 μmol/L) had no effect on the activations of p44/42 MAP kinase and p38 MAP kinase by IL-1β.

phosphokinase C (PKC) activation enhanced the p44/42 MAP kinase levels. The ESMCs were pretreated with the PKC inhibitor, GF109203X and chelerythrine (Figure 4B), which reduced the level of p44/42 MAP kinase phosphorylation induced by IL-1β. It was assumed that there was no relationship between p38 MAP kinase phosphorylation and the phospholipases (Figure 4A). As the pretreatment with the phospholipase inhibitors had no effect on phospho-p38 MAP kinase band density, the PKC inhibitors did not decrease the level of activation by IL-1β (Figure 4B). Overall, IL-1β might activate p38 MAP kinase in cat ESMCs via a pathway other than the phospholipase- and PKC-associated pathway.

The relationship between the activation of the two MAP kinases was identified after treating the ESMCs with either 10 μmol/L PD98059 (a synthetic inhibitor of MEK1/2 activation) or 30 μmol/L SB202190 (a p38 MAP kinase inhibitor) for 1 h (Figure 5A). PD98059 resulted in inhibition of the IL-1β-induced p44/42 MAP kinase activation. However, SB202190 had little effect, indicating that p44/42 MAP kinase activation in ESMCs was mediated by the upstream kinase MEK, which was not affected by p38 MAP kinase. SB202190 markedly decreased the level of p38 MAP kinase phosphorylation to almost the basal control level, whereas PD98059 had little effects on this activation. This means that the IL-1β-induced p38 MAP kinase and p44/42 MAP kinase activation might be accomplished by independent pathways.

Although IL-1β did not enhance the COX-2 and iNOS expression levels, the participation of COX and NOS in the activation of p44/42 MAP kinase by IL-1β is still unclear. Therefore, the cells were treated with a COX inhibitor, indomethacin, and a NOS inhibitor, L-NAME, for 1 h before IL-1β stimulation in order to determine whether COX and NOS play a role in the activation (Figure 5B). The increase in the phosphorylated forms of MAP kinases was maintained even with the pretreatment of inhibitors. This suggested that COX and NOS played no role in IL-1β-induced MAP kinases activation.

DISCUSSION

It is evident that the proinflammatory cytokines and chemokines play an important role in the pathogenesis of various inflammatory conditions[3,4,25]. Recently, it was reported that there was a significantly higher expression level of IL-8 mRNA in biopsy specimens obtained from RE patients than that from non-inflamed samples, which was determined by competitive reverse transcriptase polymerase chain reaction[26]. Furthermore, in addition to IL-8, the MCP-1 and RANTES levels were also significantly higher in esophagitis patients. In that report, although there were no significant differences in the mucosal IL-1β levels between the RE samples and the control group, the tissue IL-1β levels correlated significantly with the level of IL-8 production[6]. Cytokines, such as IL-1β and TNF-α, act locally and systemically to recruit and activate the target cells that can produce additional cytokines. IL-1 induces the gene expression of neutrophils and monocyte chemotactic cytokines, such as IL-8, IL-9, and macrophage inflammatory proteins that in turn stimulate migration and degranulation of neutrophils *in vivo*[27]. The surgically induced esophagitis rats resulted in an approximately two-fold increase in the level of TNF-α and IL-1β production[28].

Previous studies demonstrated that COX-2 and iNOS were the effectors of the IL-1β-induced cellular responses, products of which were PGE2 and NO, respectively. In our previous study, although iNOS expression and nitrite levels in the tissue were higher after inducing esophagitis in rats (unpublished data), the COX-2 level was unaffected by each IL-1β concentration ranged from 0.5 to 50 ng/mL. In addition, the IL-1β treatment by 24 h failed to increase the iNOS concentration in the cultured cat ESMCs. Therefore, IL-1β did not play a direct role in the inflammatory process in the esophagus, suggesting that IL-1β might be related to the induction of other cytokines, which would be an immediate cause of the inflammatory cellular response, such as COX-2 and iNOS, in cat ESMC.

IL-1β plays its own role in the cellular signaling components in the cat ESMC. The MAP kinase pathway has been suggested to be a mechanism by which various signals are transduced from the cell surface to the nucleus in response to a variety of different stimuli. These proteins participate in several intracellular processes by further inducing the level of phosphorylation of various intracellular substrates, such as other protein kinases and transcription factors. This signaling mechanism is believed to control a wide spectrum of cellular physiological and pathophysiological processes including cell growth, differentiation, and the stress response[29]. The treatment of the ESMC with >10 ng/mL IL-1β resulted in the activation of p44/42 MAP kinase, which was generated by 30 min and reached a maximum within 3 h, with a subsequent decrease. A similar pattern was observed with p38 phosphorylation, which was also concentration-dependent above 10 ng/mL. This increase was observed after 12 h, suggesting that p44/42 MAP kinase might participate in the early phase response to IL-1β, and p38 MAP kinase might be a mediator of the late phase IL-1β effects.

In order to determine whether IL-1β interacts with the G proteins, the requirement of the G protein was evaluated using ESMC pretreated with the pertussis toxin (PTX). PTX has been shown to inhibit the intrinsic GTPase activity of the Gα subunit by the ADP-ribosylation of specific residues. A previous report demonstrated that Gq, G11, Gi1, Gi2, and Gi3 were present in the esophagus and LES[24]. In this study, the IL-1β-induced activation of p44/42 MAP kinase was affected by the PTX pretreatment, suggesting the involvement of a PTX-sensitive G protein in these processes. However, PTX had little effect on the IL-1β-induced activation of p38 MAP kinase. This inhibitory effect of the toxin might result from the increases in the cyclic AMP (cAMP) level via the abrogation of the Gi protein in ESMC. A previous report demonstrated that IL-1β activated AC in vascular smooth muscle via COX-2[30]. Therefore, a cAMP elevating agent, forskolin, was used to activate adenylate cyclase in order to evaluate its role in IL-1β-induced responses. Forskolin failed to attenuate the IL-1β-induced p44/42 MAP kinase activation, excluding the involvement of elevated cAMP. In contrast, the cAMP concentration affected the level of p38 MAP kinase phosphorylation, suggesting that the delayed activation of p38 MAP kinase is due to the elevation of cAMP by IL-1β.

It is well known that IL-1β triggers the activation of PI-PLC and PC-PLC in U937 cells and tracheal smooth muscle cells to generate DAG and IP_3[31,32]. DAG and IP_3 activate PKC and release Ca^{2+} from the intracellular stores, respectively, and may play an important role in regulating the cellular functions in several cell types[33]. PKC is a major component in the kinase cascade that is initiated by ligand attachment to both the G protein-coupled receptors and the receptors possessing intrinsic tyrosine kinase activity. Different PKC isozymes appeared to mediate the LES tone as well as the phasic contraction of the ESMC[34]. In order to confirm the participation of phospholipase in the IL-1β-induced activation of MAP kinases, the cells were pretreated with inhibitors of each phospholipase. Neomycin markedly decreased the activity of p44/42 MAP kinase, but failed to reduce the activity of p38 MAP kinase. Therefore, p44/42 MAP kinase and p38 MAP kinase are on a different IL-1β signaling pathway at least downstream from the receptors. The regulatory mechanisms involved in IL-1β-induced MAP kinase activation by PKC were further investigated. These results showed that pretreatment with the PKC inhibitors attenuated the IL-1β-induced p44/42 MAP kinase activation in the ESMC. These results were consistent with those showing that cytokine activated the PLC-coupling signaling pathways in the human histiocytic lymphoma cell line, U937[31]. Despite the relationship between p44/42 MAP kinase activation by IL-1β and PKC, the PKC inhibitors did not exert any inhibitory effects on p38 MAP kinase activation.

The implication of a tyrosine kinase in the activation MAP kinases by IL-1β was investigated using tyrosine kinase inhibitors, genistein and tyrphostin 51. Genistein and tyrphostin 51 inhibited p44/42 MAP kinase phosphorylation, indicating the involvement of tyrosine kinase in this response. In contrast, these inhibitors did not block the phosphorylation of p38 MAP kinase by IL-1β.

The activation of p44/42 MAP kinase requires both tyrosine and threonine phosphorylation as a result of the dual specificity MEK1/2. PD98059, a synthetic and highly specific MEK1/2 inhibitor, has been shown to inhibit p44/42 MAP kinase activation by several stimuli[35]. In this study, a PD98059 pretreatment attenuated the p44/42 MAP kinase activation, leaving p38 MAP kinase phosphorylated. In addition, IL-1β has been shown to phosphorylate p38 MAP kinase, which appears to be distinct from p44/42 MAP kinase in several cell types[36,37]. SB202190 inhibited the IL-1β-induced p38 MAP kinase activation, but did not show any inhibitory effect on p44/42 MAP kinase activation. Overall, the activation of p44/42 MAP kinase and p38 MAP kinase appears to involve independent pathways.

The absence of a role of COX and iNOS in these pathways was confirmed by their respective inhibitors, indomethacin and L-NAME. Indomethacin plays a role in preventing COX-2 from producing prostaglandin, and L-NAME blocks NO synthesis by NOS. The IL-1β-induced activity of p44/42 MAP kinase and p38 MAP kinase remained unchanged even after the indomethacin and L-NAME treatment, thereby excluding the role of prostaglandin and NO in this responses.

In conclusion, IL-1β, which is believed to act as a pro-inflammatory mediator by evoking other cytokines in the esophagitis, can induce p44/42 MAP kinase and p38 MAP kinase activation. The pathway related to p44/42 MAP kinase is composed of a PTX-sensitive G protein, tyrosine kinase, PLC and PKC. These components are believed to play a role in the early cellular responses via IL-1β-induced p44/42 activation, but have little effect on p38 MAP kinase phosphorylation, which is generated later and is regulated negatively by cAMP.

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S- Editor Kumar M and Guo SY **L- Editor** Elsevier HK **E- Editor** Cao L

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World J Gastroenterol 2006 February 7; 12(5): 723-730
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

GFAP promoter directs *lacZ* expression specifically in a rat hepatic stellate cell line

Gunter Maubach, Michelle Chin Chia Lim, Chun-Yan Zhang, Lang Zhuo

Gunter Maubach, Michelle Chin Chia Lim, Chun-Yan Zhang, Lang Zhuo, Institute of Bioengineering and Nanotechnology, 31 Biopolis Way, The Nanos, #04-01, 138669, Singapore.
Supported by the Biomedical Research Council and the Institute of Bioengineering and Nanotechnology, the Republic of Singapore
Correspondence to: Dr Lang Zhuo, Institute of Bioengineering and Nanotechnology, 31 Biopolis Way, The Nanos, #04-01, 138669, Singapore. lzhuo@ibn.a-star.edu.sg
Telephone: +65-68247114 Fax: +65-64789080
Received: 2005-07-25 Accepted: 2005-08-26

Abstract

AIM: The GFAP was traditionally considered to be a biomarker for neural glia (mainly astrocytes and non-myelinating Schwann cells). Genetically, a 2.2-kb human GFAP promoter has been successfully used to target astrocytes *in vitro* and *in vivo*. More recently, GFAP was also established as one of the several makers for identifying hepatic stellate cells (HSC). In this project, possible application of the same 2.2-kb human GFAP promoter for targeting HSC was investigated.

METHODS: The GFAP-*lacZ* transgene was transfected into various cell lines (HSC, hepatocyte, and other non-HSC cell types). The transgene expression specificity was determined by X-gal staining of the β-galactosidase activity. And the responsiveness of the transgene was tested with a typical pro-fibrotic cytokine TGF-β1. The expression of endogenous GFAP gene was assessed by real-time RT-PCR, providing a reference for the transgene expression.

RESULTS: The results demonstrated for the first time that the 2.2 kb hGFAP promoter was not only capable of directing HSC-specific expression, but also responding to a known pro-fibrogenic cytokine TGF-β1 by upregulation in a dose- and time-dependent manner, similar to the endogenous GFAP.

CONCLUSION: In conclusion, these findings suggested novel utilities for using the GFAP promoter to specifically manipulate HSC for therapeutic purpose.

Key words: GFAP; Astrocytes; Hepatic stellate cells; Fibrosis; TGF-β1; *LacZ*.

Maubach G, Lim MCC, Zhang CY, Zhuo L. GFAP promoter directs *lacZ* expression specifically in a rat hepatic stellate cell line. *World J Gastroenterol* 2006; 12(5): 723-730

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INTRODUCTION

Hepatic stellate cell (HSC, also known as Ito cell) is a minor cell type (roughly 5-8% of the total liver cells) most commonly found in the sinusoidal area of adult livers. The major physiological functions of HSC include fat storage, vitamin A uptake and metabolism, and the production of extracellular matrix (ECM) proteins. It has been demonstrated in the past decade that HSC is a major player in mounting defense during hepatic injury, and mediating hepatic fibrogenesis by over-producing pro-fibrotic cytokines and consequently the ECM molecules. Hence the HSC itself also became a target for the development of anti-fibrotic therapy[1-3].

Traditionally antibodies against desmin[4], vimentin and smooth muscle-alpha-actin (SMAA)[5,6], despite their poor tissue and cell specificity, remained the common battery for identifying rat HSC both *in vitro* and *in vivo*, until the re-introduction of GFAP in the mid-1990s[7-9], even though GFAP was first documented as a marker for rat HSC in 1985[10]. It was suggested that the GFAP could be a more reliable marker for marking quiescent HSCs[7]. More recently, the GFAP was also adopted as a marker for human HSCs[11-13].

Several gene promoters have been investigated for their ability to genetically manipulate HSC both *in vitro* and *in vivo*, which include the human collagen alpha 1 (ColI)[14-16], SMAA[17], LIM domain protein CRP2 (CSRP2), tissue inhibitor of metalloproteinases-1 (TIMP-1) and smooth muscle-specific 22-ku protein (SM22 alpha)[18,19]. However, none of the promoters tested thus far showed sufficient tissue and cell specificity.

In our own efforts, we constructed a transgene consisting of a 2.2-kb hGFAP promoter[20,21] and the *E. coli lacZ* coding sequence and tested its expression in a rat HSC line HSC-T6[22]. Our results showed that the 2.2-kb hGFAP promoter was not only capable of directing HSC-specific expression *in vitro*, but also responding to a known pro-fibrogenic cytokine transforming growth factor (TGF-β1) by upregulation in a dose- and time-dependent

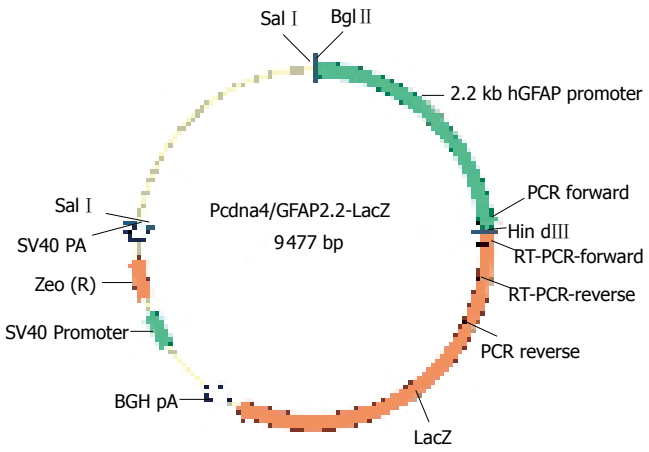

Figure 1 GFAP-*lacZ* transgene construct. The bacterial *lacZ* reporter coding sequence was under the control of a 2.2-kb human GFAP promoter. The SV40-Zeocin gene provides a resistance to antibiotic selection for stable clones. Primer locations for PCR and RT-PCR were also indicated.

manner. Similarly, an induction of the endogenous GFAP by TGF-β1 was also observed. These findings suggested novel utilities for the 2.2 kb hGFAP promoter for specifically manipulating HSC, and developing anti-fibrotic therapy. Furthermore, these findings raised fundamental questions on possible GFAP function in the HSC development and pathobiology.

MATERIALS AND METHODS

Construction of the 2.2 kb hGFAP-lacZ transgene

The plasmid vector pcDNA4/TO/LacZ (Invitrogen, CA, USA) was used as a cloning backbone, providing the *E. coli β-galactosidase* coding sequence and the Zeocin resistance gene. The 2.2-kb hGFAP promoter, which was originally mapped for its astrocytic specificity[20,21], was excised from another transgene GFAP-GFP-S65T[23] by *Bgl*II/*Hin*dIII digest and used to replace the CMVTetO2 promoter in the pcDNA4/TO/lacZ vector. The cloning junction sequences were verified by DNA sequencing. The resultant 9.5 kb plasmid (as depicted in Figure 1) was designated as pcDNA4/GFAP2.2-LacZ in our vector depository.

Cell line and culture conditions

The rat HSC-T6 cell line[22] was a generous gift from Dr Scott Friedman of the Mount Sinai School of Medicine in New York through Dr Alex Hui (Chinese University of Hong Kong). The HepG2, C3A, HeLa and NIH/3T3 were from American Type Culture Collection (ATCC, VA, USA), and the C6 was from Japanese Collection of Research Bioresources (JCRB, Osaka, Japan). All the cell culture media and reagents were from Invitrogen, unless specified otherwise. All cell lines were routinely cultured in full Dulbecco's modified Eagle medium (DMEM), supplemented with 10% FBS, 100 units penicillin/100 μg streptomycin per mL at 37°C in a humidified atmosphere of 50 mL/L CO_2. The cells were routinely split twice weekly in a ratio of 1:3.

Cell transfection and selection

The T6, C6, and HeLa cells were stably transfected with 0.5-1.0 μg of the SalI-linearized transgene plasmid using the Lipofectamine 2000 kit, according to the manufacturer's instructions (Invitrogen). Twenty-four hours after the transfection, the medium was changed and supplemented with 250-500 μg/mL Zeocin for selection for at least 6 wk before further analyses (as described below) were performed. The final concentration for maintaining the stable transfected cells was 500 μg/mL Zeocin. Alternatively, HepG2, C3A, and NIH/3T3 were transiently transfected with the circular transgene plasmid using the FuGene 6 kit (Roche Diagnostics).

Genomic detection of the transgene

In order to confirm the transgene integrity and its integration into the genome, genomic DNA was isolated from cell clones stable for at least 2 mo under 500 μg/mL Zeocin selection, using the NucleoSpin blood kit (Macherey-Nagel, Düren, Germany). A PCR method was used to verify the structural integrity of the inserted construct(s), with a forward primer 5'-ACTCCTTCATAAAGCCCTCG-3' (complementary to the GFAP promoter), and a reverse primer 5'-AACTCGCCGCACATCTGAACTTCAGC-3' (complementary to the lacZ coding sequence), using the Platinum PCR SuperMix High Fidelity (Invitrogen) on a thermal cycler (MJ Research, FL, USA). The expected PCR product was 944 bp in size.

RT-PCR GFAP-lacZ transcript

Total cellular RNAs were extracted from cells grown in 6-well plates by using the NucleoSpin RNAII kit (Macherey-Nagel) following the manufacturer's instructions. The RNA concentration was determined on a ND-100 spectrophotometer (Nanodrop Technologies, DE, USA). Fifty nanograms of total RNA was used to perform a one-step RT-PCR (Qiagen, Hilden, Germany) according to the user's manual, using forward primer (5'-TCAGCTTG GAGTTGATCCCGTCG-3') and reverse primer (5'-AAC AAACGGCGGATTGACCGTAATGG-3'). The thermal cycling profiles were 50°C 30 min (cDNA synthesis), 95°C 15 min (denaturation), and followed by 94°C 10 s 55°C 10 s, and 72°C 19 s for 35 cycles (PCR). The target product size was 337 bp, and the β-actin reference was 332 bp in size.

X-gal staining of β-galactosidase activity in fixed cells

A *lacZ* reporter cell staining kit (InvivoGen, CA, USA) was used to detect the reporter gene activity in both stable and transient transfectants. Development of the blue end product was monitored at different time intervals (15, 30, 60, 90, and 120 min). The staining results were documented with a digital camera (DP12) attached to an Olympus inverted bright field microscope (IX51).

Quantitative solution assay of β-galactosidase activity in cell extracts

To quantify the β-galactosidase activity in cell extracts, an enzyme assay kit (E2000, Promega, WI, USA) was used to measure the specific activity of β-galactosidase, according to the manufacturer's instructions in a 96-well format. Briefly, the cells were washed twice with 1×PBS buffer (Ph7.4), lysed for 15 min at room temperature with the reporter lysis buffer, and harvested using a cell scraper. The total cell extracts were appropriately diluted

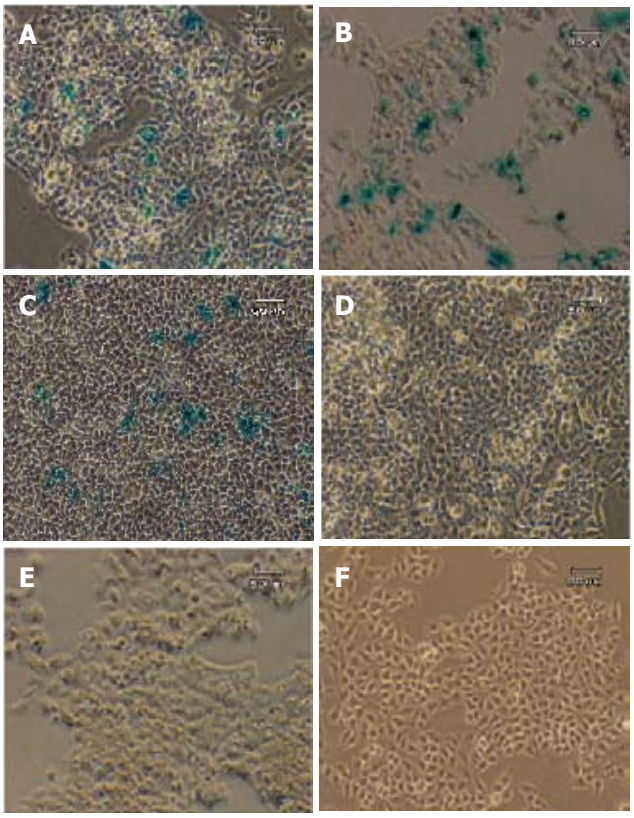

Figure 2 Transient transfection with CMV-*lacZ* and GFAP-*lacZ* transgenes. Hepatocyte cell lines C3A (**A**), HepG2 (**B**), and fibroblast cell line NIH/3T3 (**C**) transfected with the ubiquitous CMV-*lacZ* transgene displayed blue color after 2 h of X-gal staining. In contrast, C3A (**D**), HepG2 (**E**) and NIH/3T3 (**F**) transfected with the GFAP-*lacZ* transgene did not show any blue color. Scale bar = 60 μm.

(5-10 folds), and assayed for the enzyme activity. The specific β-galactosidase activity was presented in milliunit per milliliter (mU/mL) using a standard curve established from the β-galactosidase standard (provided with the kit). The protein concentration was measured with a BCA protein assay kit (Pierce, IL, USA), and used to further convert the specific β-galactosidase activity from mU/mL to mU/μg total cellular protein. Final data were summed from six independent experiments.

Treatment of cells with TGF-β1

Cells were seeded into 12-well plates in full DMEM with 500 μg/mL Zeocin. Twelve hours prior to the cytokine treatment, medium was changed so that the cells were allowed to grow in low serum (0.5% FBS) DMEM. The cells at a confluence of 70-80% were incubated with various concentrations (0, 0.1, 1, and 10 ng/mL) of rhTGF-β1 (BioVision, CA, USA) for 0, 2, 8, 16, 24, 48, and 72 h before they were harvested for β-galactosidase activity assay and real-time RT-PCR GFAP assay.

Real-time RT-PCR quantification of rat GFAP mRNA

Total RNA was reversely transcribed to cDNA using Taqman's reverse transcription reagent (Cat. # N808-0234). A total of 400 ng of RNA in 7.7 μL nuclease-free water was added to 2 μL 10× reverse transcriptase buffer, 4.4 μL 25 mmol/L magnesium chloride, 4 μL deoxyNTP mixtures, 1 μL random hexamers, 0.4 μL RNase inhibitor and 0.5 μL reverse transcriptase (50 U/μL) in a final reaction volume of 20 μL. The reaction was performed for 10 min at 25 °C (annealing), 30 min at 48 °C (cDNA synthesis) and 5 min at 95 °C (enzyme denaturation).

Real-time quantitative PCR was carried out with an ABI 7500 Real Time PCR System (Applied Biosystems, CA, USA). One microliter of sample cDNA was used in each PCR reaction, with the actin gene as a reference. The primers and probes for rat β-actin and GFAP were purchased from Taqman's assay-on-demand database. The PCR reaction was performed under a default profile consisting of 50 °C for 2 min (UNG activation), 95 °C for 10 min (enzyme denaturation) and 40 cycles of amplification (denaturation 15 s, annealing and extension 60 s).

Relative quantitation of the target mRNA was calculated using the comparative threshold cycle (C_T) methods as described in the User Bulletin #2 (ABI Prism 7700 Sequence Detection System). C_T indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold within the linear phase of gene amplification. ΔC_T, which reflects the difference between $C_{T\ \text{target}}$ and $C_{T\ \beta\text{-actin}}$, is inversely correlated to the abundance of mRNA transcripts in the samples. ΔC_T for each sample was normalized against control experiment or calibrator and expressed as $\Delta\Delta C_T$. Relative quantitation is given by $2^{-\Delta\Delta CT}$ to express the upregulation or downregulation of the target gene under the treatments compared to the control.

Six independent experiments were performed for each data point and three ΔC_T were measured for each experiment.

Statistical analysis

All quantitative results were presented as mean ± SE. Experimental data were analyzed using two-tailed Student's *t*-test assuming unequal variances. A P-value < 0.05 was considered significant.

RESULTS

Lack of GFAP-lacZ expression in non-GFAP-expressing cell lines

To investigate if there is any possible aberrant expression of the transgene, three non-GFAP expressing cell lines, HepG2, C3A and NIH/3T3, were transiently transfected with the circular transgene plasmid, using a CMV-*lacZ* plasmid as a positive control. After the transfected cells were grown in full DMEM medium free of selection agent for 2 days, all three cell lines were examined for the β-galactosidase expression by X-gal staining method. Approximately 10-30% of the cells in each of the three lines transfected with the ubiquitous CMV-*lacZ* showed positive blue color after 2 h of staining. In contrast, none of the three lines transfected with the GFAP-*lacZ* showed any blue staining, indicating a total lack of *lacZ* expression driven by the GFAP promoter in these cell lines. Representative images from two independent experiments for all three cell lines are shown in Figure 2. After 24 h of X-gal staining, a few cells from the HepG2 line displayed blue color in both the transfected and

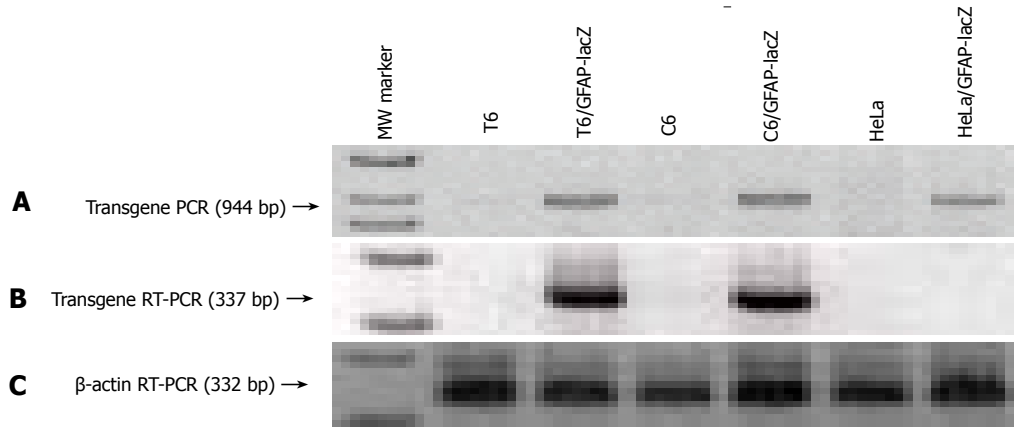

Figure 3 Verification of GFAP-*lacZ* transgene integration into host genome and legitimate expression in appropriate cell types. **A**: PCR of genomic DNA isolated from the transfected T6 (lane 3), C6 (lane 5) and HeLa (lane 7) showed the presence of a transgene band at 944 bp, and the absence of such band from the nontransfected T6 (lane 2), C6 (lane 4), and HeLa (lane 6); **B**: RT-PCR of total RNAs demonstrated that transgene only expressed in the transfected T6 (lane 3) and C6 (lane 5), but not in the transfected HeLa (lane 7); **C**: RT-PCR of β-actin as a reference ensuring equal sample loading. Lane 1 is the 1-kb ladder marker.

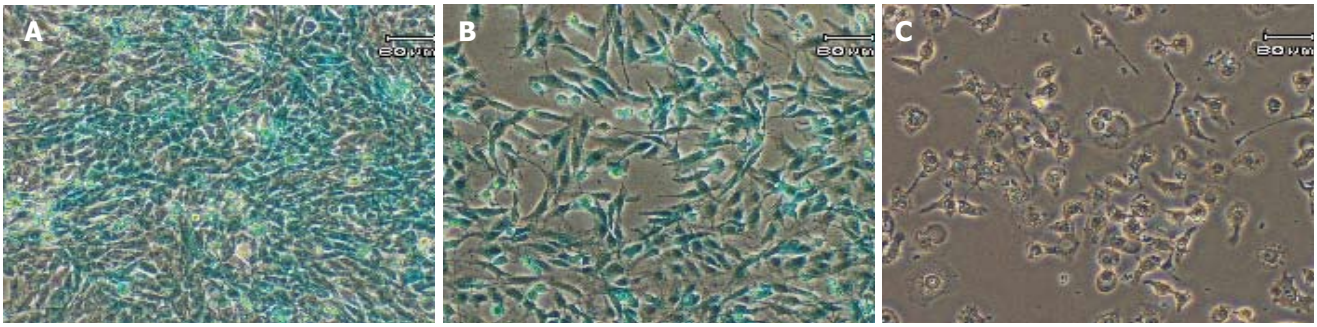

Figure 4 X-gal staining of stable transfectants. The GFAP-*lacZ* transgene expressed in the rat hepatic stellate cell line T6 (**A**), and the positive control rat astrocytic cell line C6 (**B**), but not in the non-GFAP-expressing cell line HeLa (**C**). Scale bar = 60 μm.

nontransfected groups alike (data not shown), most likely due to endogenous galactosidase-like activity.

Specific expression of GFAP-lacZ in GFAP-expressing cell line T6

The 2.2-kb hGFAP promoter has been consistently shown to direct gene expression specifically in the neural stellate cells-astrocytes[20,21,24]. It was completely unknown whether a GFAP promoter can direct gene expression specifically in the HSC type. Since stellate cells of both neural and hepatic origins have been reported to share many morphological, immunocytochemical and even developmental features[7-9,25], we decided to test this possibility by stably transfecting a rat HSC line T6[22] with the GFAP-*lacZ* transgene, using the rat astrocytic C6 cell line as a positive and the human HeLa cell line as a negative control. After selection in 250-500 mg/mL Zeocin for at least 6 wk, several independent stable cell clones were obtained for each cell line. Subsequently, PCR analysis of genomic DNA isolated from the stable clones confirmed the transgene integrity and integration into the host genome, as evidenced by the presence of an anticipated product size of 944 bp (Figure 3A). To check for transgene expression, total RNA was isolated from the stable transfectants and RT-PCR was performed to verify for the *lacZ* transcript. The presence of a specific band with a predicted size of 337 bp indicated the *lacZ* transcription in the positive control C6 line, and more importantly in our target T6 line as well, but not in the negative control line HeLa (Figure 3B). The β-actin sample is shown in Figure 3C as a control for equal RNA loading. Next, one clone from each of the three cell lines was randomly chosen for X-gal staining for 1 h. As expected, both the C6 and the T6 were positive and the HeLa was negative in blue staining (Figure 4). Therefore for the first time, a GFAP-based reporter gene was shown to specifically express in a HSC line. The representative staining results are shown in Figure 4. From this point onward, a T6 cell clone stably transfected with the 2.2 kb hGFAP-*lacZ* transgene (designated as T6/lacZ/C1 clone) was used for further experiments below. It was worth noting that the T6 cells transiently transfected with the GFAP-*lacZ* transgene stained positive for X-gal reaction as well.

Time- and dose-dependent induction of transgene by TGF-β1 in T6/lacZ/C1 cells

TGF-β1 has been reported to exert broad biological effects on the cells, including the regulation of GFAP in astrocytes[26-29], and the fibrogenesis in the

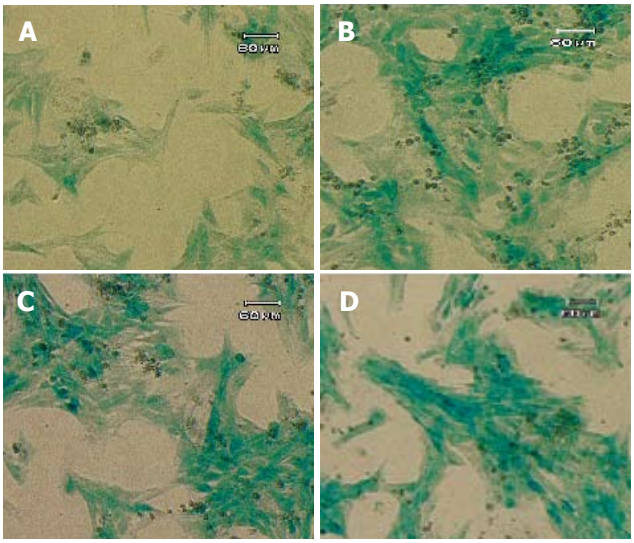

Figure 5 Induction of GFAP-*lacZ* transgene expression by TGF-β1. The T6/*lacZ*/C1 cells were cultured and incubated in full DMEM supplemented with 0 (**A**), 0.1 (**B**), 1 (**C**), and 10 (**D**) ng/mL of TGF-β1 for 3 days, and stained with X-gal substrate for 1 h. Cells treated TGF-β1 displayed more intense blue staining and more activated morphology (thicken processes). Scale bar = 60 μm.

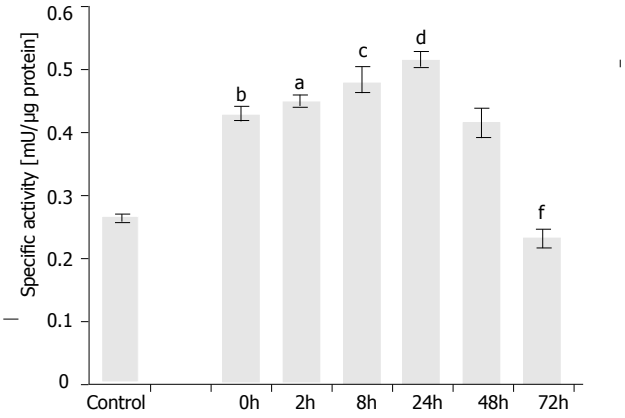

Figure 6 Time-dependent induction of GFAP-*lacZ* gene by TGF-β1 in the T6/*lacZ*/C1 cells. Cells were grown in full DMEM was assayed for basal *β*-galactosidase activity (control). Cells adopted in low serum DMEM for 12 h were supplemented with 1 ng/mL of TGF-β1 for various times (0, 2, 8, 24, 48, and 72 h), and assayed for enzymatic activity. When compared to the basal level, cells cultured in low serum DMEM had elevated level of transgene expression ($^{b}P < 0.001$). When compared the 0 h, transgene expression level was significantly induced at 2 h ($^{a}P < 0.05$), 8 h ($^{c}P < 0.005$), and 24 h ($^{d}P < 0.001$), but significantly reduced at 72 h ($^{f}P < 0.001$) compared with 0 h. Data presented as mean ± SE.

HSC[30-33]. However, very little was known about the potential regulation of GFAP (and GFAP-based transgene) by the potent pro-fibrogenic TGF-β1 in HSC. To obtain a first hint, the T6/lacZ/C1 cells were treated with TGF-β1 at various concentrations (0, 0.1, 1, and 10 ng/mL) in the full DMEM medium for 72 h. The cells were then stained with X-gal for one hour and photographed. When compared to the untreated cells, cells cultured with TGF-β1 displayed a more intense blue staining and activated morphology (thicken cellular processes), as shown in Figure 5.

To examine time-dependent induction, the T6/*lacZ*/C1 cells stimulated with 1 ng/mL of TGF-β1 for

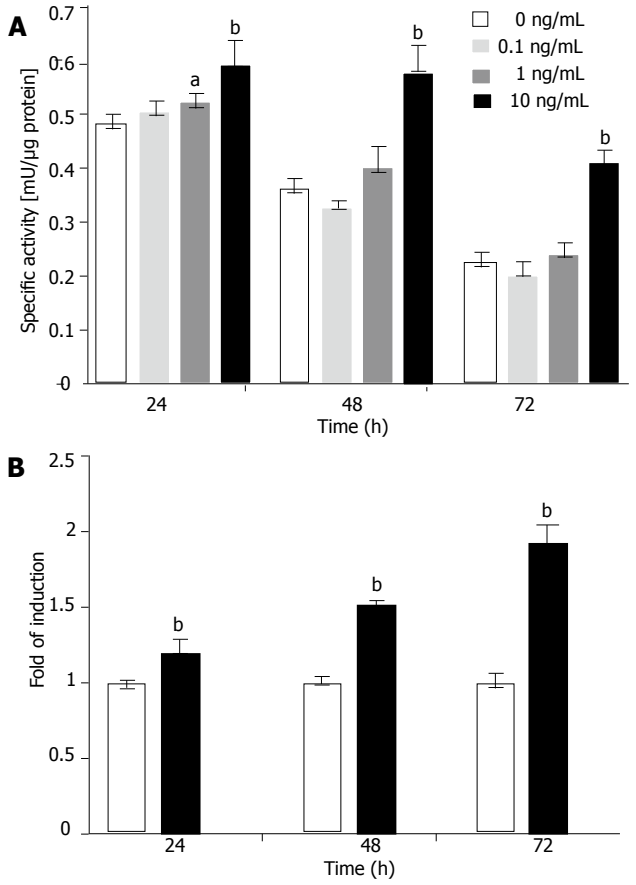

Figure 7 Dose-dependent induction of transgene. (**A**) The T6/*lacZ*/C1 cells cultured in low serum DMEM supplemented with different concentrations of TGF-β1 (0, 0.1, 1, and 10 ng/mL) for various times (24, 48, and 72 h) were assayed for β-galactosidase activity. The transgene was significantly induced by 10 ng/mL of TGF-β1 at all time points. (**B**) Data extracted from (A) illustrated that the fold of induction increased with times, despite the drop in absolute enzymatic activity. Data presented as mean ± SE. $^{a}P < 0.05$, $^{b}P < 0.001$ compared with no cytokine treatment.

various times (0, 2, 8, 24, 48, and 72 h) were assayed for β-galactosidase activity. Firstly, it was interesting to note that the transgene expression was significantly increased ($P < 0.001$) from 0.29 mU/μg to 0.42 mU/μg (or a 24% increase) by simply lowering the serum concentration from 10% to 0.5% and culturing for 12 h. This acute and transient increase in the transgene expression could be a stress response for cells adopted from full- to lower-serum medium. Secondly, under 1 ng/mL of TGF-β1 the transgene was rapidly induced when measured at 2 h ($P < 0.05$) and 8 h ($P < 0.005$), and eventually peaked at 24 h ($P < 0.001$), with a specific activity being 0.52 mU/μg. At 48 h, the expression level dropped to the same level as at 0 h. After 72 h, the level slipped to 58% of the value at 0 h ($P < 0.001$). The assay results are depicted in Figure 6.

To investigate dose-dependent induction, the T6/*lacZ*/C1 cells were grown in low serum DMEM with various concentrations of TGF-β1 (0, 0.1, 1, and 10 ng/mL) for various times (24, 48, and 72 h) and assayed for β-galactosidase activity. Significant transgene induction was observed for the 10 ng/mL TGF-β1 treatment at all time points ($P < 0.001$). However the only other (less) induction was seen with 1 ng/mL of TGF-β1 at 24 h ($P < 0.05$). The assay results are plotted in Figure 7A. It is

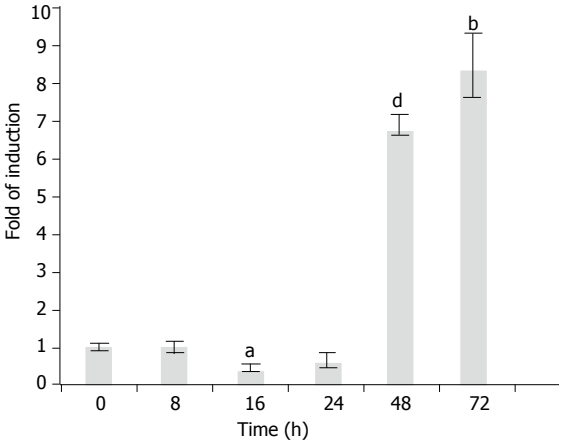

Figure 8 Time-dependent induction of the endogenous GFAP gene by TGF-b1. T6/GFAP-*LacZ* cells were incubated with 1 ng/mL of TGF-b1 in low serum DMEM for various times (0, 8, 16, 24, 48, and 72 h), and the endogenous rat GFAP mRNA was quantified with real-time RT-PCR. Data were presented as mean ± SE. $^{b}P < 0.01$, $^{a}P < 0.005$ and $^{d}P < 0.001$, when compared to treatment at 0 h.

interesting to note that when the values of control groups (for 10 ng/mL) were normalized to 1 and the highest induction (nearly two fold) was seen at 72 h, though the absolute enzymatic activity (0.43 mU/μg) was the lowest among the three time points (Figure 7B).

Induction of endogenous GFAP expression by TGF-β1

The GFAP, along with other HSC markers desmin, SMAA, and vimentin, was known to express in the HSC-T6 cells[22]. In order to investigate whether the endogenous GFAP expression is also subject to TGF-β1 regulation as the transgene, the T6/*lacZ*/C1 cells treated with TGF-β1 (1 ng/mL) for various times (0, 2, 8, 16, 24, 48, and 72 h), and the rat GFAP transcripts were quantified using a real-time RT-PCR method. When compared to the basal level (normalized to 1) at 0 h, the GFAP mRNA level remained relatively steady within the first 24 h (except a brief but significant 0.5-fold suppression at 16 h, $P < 0.005$), then sharply elevated to 7- and 8.5-folds of the basal level at 48 and 72 h, respectively (Figure 8).

DISCUSSION

Cell specificity of transgene expression

To our best knowledge, this is the first report demonstrating that a GFAP promoter can drive HSC-specific expression of a reporter gene, in a similar fashion as the endogenous GFAP does. The current findings also provided molecular biology support to the early immunocytochemical observations on the GFAP staining on HSC[7-11,36,37]. Based on our transfection studies of multiple cell types, the 2.2 kb hGFAP promoter is both necessary and sufficient to confer HSC-specific expression *in vitro*, which was reminiscent to the conclusion made on cultured astrocytes using the same promoter[20]. Several key *cis*-acting elements, especially the AP-1 site (for binding fos/jun family transcription factors), have been mapped to the so-called B box in the human GFAP promoter from position-1612 to-1489, and proved to be critical for mediating astrocyte-specific transgene expression[20,38,39]. We therefore attempted to speculate that the rat T6 cells should also express a similar set of relevant transcription factors as the astrocytes do, to permit selective GFAP-*lacZ* expression in HSC. On the contrary, the non-GFAP expressing cell lines (HepG2, C3A, HeLa, and NIH/3T3) may lack appropriate *trans*-acting factor profile, leading to the complete null expression of the transgene, as judged by RT-PCR and prolonged X-gal staining.

GFAP promoter elements for mediating the induction by TGF-β1

Our quantitative data showed that the 2.2 kb hGFAP-*lacZ* transgene, as well as the endogenous rat GFAP, were induced by TGF-*β1* in a time- and dose-dependent manner in the T6/*lacZ*/C1 cells. This observation on the HSC was similar to earlier reports on the astrocytes[28,40]. The molecular mechanism underlying the TGF-β1 induction has been extensively studied. A sequence motif resembling the NF-1 (nuclear factor-1) site located in the near upstream (-106 to -153) of the rat GFAP promoter was shown to confer a full response to TGF-β1 induction in cultured astrocytes[40]. Similarly, two NF-1-like sites were mapped to the B (-1612 to -1489) and D (-132 to -57) boxes in the human GFAP promoter[20]. It was also known that some factor(s) in the nuclear extracts from astrocytes could bind to the NF-1 sites and possibly mediates the TGF-β1 induction process.

The endogenous GFAP gene displayed a similar trend in response to TGF-β1 as the 2.2 kb GFAP-*lacZ* transgene did. However, different responding dynamics were noted between the endogenous GFAP gene and the artificial GFAP-*lacZ* transgene. First, the transgene induction was obvious within the first 2 h of TGF-β1 stimulation, while the endogenous gene did not show any significant induction until day 2 of the TGF-β1 treatment. The difference in induction time line could be partially due to assay detection sensitivities, presumably with the enzymatic assay (with amplification capability) being more sensitive, therefore showing an earlier induction. An earlier detection of a similar 2.2-kb hGFAP-*lacZ* transgene was documented in a transgenic mouse model during brain development, in which the transgene showed up several days ahead of the endogenous GFAP[21]. In addition, unknown regulatory elements residing outside the 2.2-kb fragment could also contribute to the different responding profiles between the transgene and the endogenous GFAP. Nevertheless, both the endogenous and transgene at least displayed the same upward trend in responding to the same TGF-β1 cytokine, legitimizing the use of GFAP-*lacZ* transgene as a surrogate for the endogenous GFAP in studying TGF-β1 stimulation.

Relationship between HSC activation and GFAP (and GFAP-lacZ) expression

Early studies on the rat HSC showed that GFAP expression decreased as quiescent HSCs were activated or transdifferentiated to myofibroblast-like during fibrosis[7-9]. However, the notion of an inverse relationship between the GFAP expression and the HSC activation may not be

as simple and straightforward. For example, whether the activated HSCs and myofibroblasts in vivo represented different activation stages of the same cell lineage, or two distinct cell types of different origins remained controversial[41]. It is worthy noting that some recent data showed that activated HSCs expressed higher level of GFAP[11,34,37]. Our current data clearly showed that both the endogenous GFAP and the hGFAP-lacZ transgene were upregulated in a time- and dose-dependent manner in the rat HSC-T6 cells activated by TGF-β1.

Our data demonstrated for the first time that the 2.2 kb hGFAP promoter is capable of expressing the *lacZ* reporter specifically in the rat HSC-T6 line and responding to TGF-β1 activation by re-regulation, in a similar manner as the rat astrocytic cell line C6. Furthermore our data also suggested that the molecular and cellular mechanisms (relevant *trans*-acting factors and signaling pathways) underlying the specific GFAP expression and induction are likely conserved in HSC line T6 and in the neural stellate cell line C6 alike. The current findings should provide a valuable genetic tool for manipulating HSCs towards the development of anti-fibrotic therapies.

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S- Editor Guo SY **L- Editor** Elsevier HK **E- Editor** Cao L

PO Box 2345, Beijing 100023, China
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World J Gastroenterol 2006 February 7; 12(5):731-738
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

Variable expression of cystatin C in cultured trans-differentiating rat hepatic stellate cells

Axel M Gressner, Birgit Lahme, Steffen K Meurer, Olav Gressner, Ralf Weiskirchen

Axel M Gressner, Birgit Lahme, Steffen K Meurer, Olav Gressner, Ralf Weiskirchen, Institute of Clinical Chemistry and Pathobiochemistry, RWTH Aachen University, D-52074 Aachen, Germany
Supported by the Federal Ministry of Education and Research of Germany (Network of Excellence in Viral Hepatitis *Hep-Net*) and the Deutsche Forschungsgemeinschaft (SFB-6542, TPA9) to RW and AMG
Correspondence to: Axel M Gressner, Institute of Clinical Chemistry and Pathobiochemistry, RWTH Aachen University, D-52074 Aachen, Germany. gressner@rwth-aachen.de
Telephone: +49-241-8088678/9 Fax: +49-241-8082512
Received: 2005-07-01 Accepted: 2005-07-28

Abstract

AIM: To study the expression of cystatin C (CysC), its regulation by transforming growth factor-β1 (TGF-β1) and platelet-derived growth factor (PDGF) and the potential interference of CysC with TGF-β1 signaling in this special cell type.

METHODS: We evaluated the CysC expression in cultured, profibrogenic hepatic stellate cells and trans-differentiated myofibroblasts by Northern and Western blotting and confocal laser scanning microscopy.

RESULTS: CysC was increased significantly in the course of trans-differentiation. Both TGF-β1 and PDGF-BB suppressed CysC expression. Furthermore, CysC secretion was induced by the treatment with TGF-β1. Although CysC induced an increased binding affinity of TGF-β receptor type III (beta-glycan) as assessed by chemical cross-linking with [^{125}I]-TGF-β1, it did not modulate TGF-β1 signal transduction as shown by evaluating the Smad2/3 phosphorylation status and [CAGA]-MLP-luciferase reporter gene assay. Interestingly, the shedding of type III TGF-β receptor beta-glycan was reduced in CysC-treated cells. Our data indicated that CysC expression was upregulated during trans-differentiation.

CONCLUSION: Increased CysC levels in the serum of patients suffering from liver diseases are at least partially due to a higher expression in activated hepatic stellate cells. Furthermore, TGF-β1 influences the secretion of CysC, highlighting a potentially important role of cysteine proteases in the progression of hepatic fibrogenesis.

Key words: Cystatin C; TGF-β; Hepatic stellate cells; Trans-differentiation; Beta-glycan

Gressner AM, Lahme B, Meurer SK, Gressner O, Weiskirchen R. Variable expression of cystatin C in cultured trans-differentiating rat hepatic stellate cells. *World J Gastroenterol* 2006; 12(5): 731-738

http://www.wjgnet.com/1007-9327/12/731.asp

INTRODUCTION

Cystatin C (CysC), belonging to the type II cystatin gene superfamily, is the most abundant extracellular inhibitor of cysteine proteinases[1]. Mature CysC is a 13-kD, positively charged, secreted protein composed of 120-122 amino acids expressed by many cell types. It is present in large quantities in cerebrospinal fluid, seminal plasma, serum and other body fluids. One of the most prominent functions of CysC is related to the inactivation of the cathepsin family members of cysteine proteinases, which are involved in the cleavage of membrane and extracellular matrix proteins among others and thus in disease-related tissue remodeling. The diagnostic value and prognostic significance of CysC determination have been reported for several diseases[2,3], and correlations have been found between CysC expression, mutations, and clinical status of patients with autoimmune disease, cerebral amyloid angiopathy, hereditary brain hemorrhage, atherosclerosis, aortic aneurysms, and multiple sclerosis[4-8].

A further highly relevant function of CysC has been recently reported, which is concentrated on the inhibitory effect on transforming growth factor-β (TGF-β)-signal transduction in normal and malignant cells[9]. The TGF-β antagonizing mechanism is at least partially due to the interference of ligand binding to the type II TGF-β receptor. *In vitro*, TGF-β1 is a potent inducer of CysC secretion in vascular smooth muscle cells[8]. Furthermore, expression of CysC mRNA in astrocyte precursor cells is directly linked to the activity of TGF-β[10]. TGF-β is known to be the fibrogenic master cytokine in human and experimental liver fibrosis due to its ability to stimulate the expression and inhibition of degradation of extracellular matrix (ECM) proteins and to promote the trans-differentiation of profibrogenic hepatic stellate cells (HSCs) to ECM-producing hepatic myofibroblasts (MFBs)[11]. The phenotypic and functional trans-differentiation of

HSCs is initiated in acute and chronic inflammatory liver diseases, ultimately leading to organ fibrosis and cirrhosis. Significantly elevated serum concentrations of CysC have been recently described in chronic liver diseases showing a strong correlation between the degree of elevation and the severity of disease[12,13]. Based on these new findings, we tried to figure out the potential functional relevance of CysC in the profibrogenic liver cell subtype, HSCs. Therefore, we studied in cultured rat HSCs, the expression of CysC, its regulation by TGF-β1 and platelet-derived growth factor (PDGF) and the potential interference of CysC with TGF-β signaling in this special cell type. The results showed that CysC expression in HSCs was modulated by TGF-β and platelet-derived growth factor-BB (PDGF-BB) depending on the trans-differentiation status of the cells. However, CysC had no direct effect on TGF-β signaling itself.

MATERIALS AND METHODS

Cell isolation and culture

HSCs, Kupffer cells (KCs), and sinusoidal endothelial cells (SECs) were isolated from male Sprague-Dawley rats by the pronase-collagenase method[14]. HSCs were further purified by a single-step density gradient centrifugation as previously described[15]. KCs and SECs were enriched by centrifugal elutriation[16] and RNA was directly isolated without cell cultivation. HSCs were seeded in Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker Europe, Verviers, Belgium) supplemented with 100 mL/L fetal calf serum (FCS) (Hyclone Fetal Bovine Serum, Perbio), 4 mL glutamine (PAA Laboratories GmbH, Linz, Austria), 100 IU/mL penicillin and 100 μg/mL streptomycin (PAA Laboratories). Hepatocytes (PCs) were isolated following the collagenase method of Seglen[17]. The medium was renewed one day after initial plating and then every day. Cultures were maintained at 37 °C in a humidified atmosphere containing 50 mL/L CO_2. Prior to treatment with recombinant human CysC (rhCysC), TGF-β1, and PDGF-BB (R&D Systems, Wiesbaden, Germany), the cells were starved for 16 h in 0.2% FCS.

RNA isolation and Northern blot analysis

Total RNA was purified using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After resuspension in water, the concentration was determined by UV absorbance. Northern blot analysis was carried out as previously described[18].

cDNA preparation, RT-PCR, real time PCR

To generate a probe for Northern blot analysis of CysC expression, total RNA (2 μg) from rat HSCs was reverse transcribed using the Omniscript transcriptase (Qiagen) and random hexamer primers (Invitrogen, Karlsruhe, Germany). The reaction was allowed to proceed at 37℃ for 60 min and cDNA products were stored at -20 °C until use. For amplification of rat CysC, aliquots of first strand cDNAs were subjected to PCR with primers 5'-AGT ACA ACA AGG GCA GCA ACG ATG-3' and 5'-AGG AGA AGA GAA CCA GGG GAC AGC-3', dNTPs (each 10 mmol/L dATP, dCTP, dGTP, dTTP) in 1× PCR reaction buffer and 2.5 U *Taq* DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany) resulting in the amplification of a 454-bp CysC fragment[19]. PCR conditions were at 94 °C for 5 min (initial denaturation), at 94 °C for 60 s, at 60 °C for 60 s, at 72 °C for 3 min (40 cycles), and at 72 °C for 10 min (final extension). Aliquots of the reaction products were separated and visualized on 1% (w/v) agarose gels and the identity of the fragment was demonstrated by sequencing. Quantitative analysis of rat CysC mRNA was performed with a LightCycler (LC) System in 20 μL reaction volume using the LC-FastStart DNA Master SYBR Green I kit (Roche). Thermocycling was performed with 2 μL of cDNA products and 0.75 μmol/L of each primer specific for CysC. No-template controls were prepared by adding 2 μL PCR grade, sterile H_2O to 18 μL of master mix. Cycling conditions were one cycle of denaturation at 95 °C for 10 min, followed by 45 amplification cycles at 95 °C for 10 s, at 60 °C for 5 s, at 72 °C for 22 s. The PCR products were melted in a temperature transition procedure from 65 °C to 95 °C in steps of 0.1 °C/s and fluorescence was measured and plotted online against the temperature to obtain the fragment-specific melting points. Differences between crossing points were taken to estimate the relative concentrations.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

For immunoblotting, proteins from rat liver cells were separated under reducing conditions on 4-12% Bis-Tris gradient gels using MES running buffer (Invitrogen). The electroblotting onto 0.2-μm protran NC membranes (Schleicher & Schuell, Dassel, Germany) was carried out according to standard procedures. Blocking and incubation of the membranes with individual antibodies were performed as described previously[18] with slight modifications. Briefly, membranes were blocked with 5% (w/v) non-fat milk powder in Tris-buffered saline-Tween 20 (TBST). For Western blot analysis, the following primary antibodies were diluted in 2.5% (w/v) non-fat milk powder in TBST: clone asm-1 (Cymbus Biotech., Chandlers Ford, UK) against α-smooth muscle actin (α-SMA) (1:10 000), rabbit anti-human CysC rat/human CysC and its precursor (1:500) (Upstate Biotechnology, Lake Placid, USA), rabbit anti-phospho-Smad2 (Ser465/467) (1:1000) (New England Biolabs GmbH, Frankfurt/Main, Germany), rabbit anti-Smad2 (1:200) (Zymed Laboratories, San Francisco, CA, USA), goat anti-Smad2/3 (N-19), (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:100), mouse anti-β-actin (1:10 000) (Cymbos). The antiserum PS1 was raised against a phosphorylated C-terminal peptide of Smad1 and showed cross-reactivity with phosphorylated Smad3 (1:1 000)[20]. Primary antibodies were visualized using horseradish peroxidase (HRP)-conjugated anti-mouse, -rabbit or -goat IgG (Santa Cruz Biotechnology) or alkaline phosphatase (AP)-conjugated anti-rabbit or mouse IgG (Santa Cruz Biotechnology). Alternatively, the primary antibodies against CysC and Smad2/3 (N-19) were detected with swine anti-rabbit and rabbit anti-goat IgG biotin antibody followed by streptavidin-HRP conjugate (DAKO Diagnostics, Hamburg, Germany). Staining was

performed with the SuperSignal West Dura Extended chemiluminescent substrate (Pierce, Rockford, IL, USA) for HRP exposed in a Lumi-Imager™ (Roche) or a color staining using NBT/BCIP substrate (Perbio Science, Bonn, Germany).

Sequence analysis

All primers were obtained from MWG-Biotech (Ebersberg, Germany) and sequencing was done with the ABI PRISM BigDye® termination reaction kit (PE Applied Biosystems, Weiterstadt, Germany) as described[21].

Enzyme linked fluorescence cytological labeling assay (ELF 97 assay)

Ten thousand cells per well were cultured in black 96-well plates for different times under standard conditions and fixed with 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS) (pH 7.4). For generation of myofibroblasts (MFBs), HSCs cultured for seven days were trypsinized and subcultured for further 3 days. The cells were permeabilized on ice in 0.1% (w/v) Triton X-100/0.1% (w/v) sodium citrate, blocked in 1% (w/v) BSA/PBS pH 7.4 and incubated with anti-α-smooth muscle actin (1:500) or anti-CysC antibody (1:200). Non-immune IgGs were used as negative controls. The ELF-97 assay (Molecular Probes Europe; Leiden, NL) was performed according to an established procedure[22]. The fluorescence intensity was measured in a Multilabel counter (Victor, Wallac ADL GmbH, Freiburg, Germany) for the quantitative evaluation of α-SMA and CysC (excitation 365 nm, emission 515 nm). The staining intensity of negative controls was subtracted from the determined values and in parallel the fluorescence was also recorded in an inverted fluorescence microscope (Axiovert M135, Zeiss, Frankfurt, Germany). Subsequently, to normalize the fluorescence values (FU) of α-SMA or CysC, the DNA content of parallel cultures was measured fluorometrically (excitation 485 nm, emission 525 nm) and quantified against calf thymus DNA as standard using the SYBR Green I dye (Molecular Probes Europe).

Immunofluorescence staining and confocal microscopy

Forty thousand cells per well (1.8 cm^2) were cultured in 4 chamber polystyrene vessels (Becton, Dickinson, Franklin Lakes, NJ, USA) for different times under standard conditions and fixed with 4% (w/v) paraformaldehyde in PBS (pH 7.4). Permeabilization, blocking and CysC antibody (Upstate Biotechnology, 1:200) incubation were carried out as described above. After washing, a biotin-linked swine anti-rabbit IgG (DAKO) (1:300) was used as the secondary antibody followed by incubation with streptavidin-FITC (DAKO) (1:150). For the detection of α-SMA, a direct CY3-conjugated antibody (clone AC-40; Sigma, Taufkirchen, Germany) was used (1:200). Non-immune IgGs (Santa Cruz) served as negative controls. After washing and mounting with antifade, the cells were subjected to high-resolution confocal laser scanning microscopy in a LS 410 inverse microscope (Zeiss) using a standard objective (40×1.3 oil).

Adenoviral infection of HSCs and luciferase measurement

Adenoviral stocks of Ad $(CAGA)_9$-MLP-luciferase were prepared as previously described[23]. Two thousand cells per well were cultured in black 96 well plates. After infection at a multiplicity of infection (MOI) of 50, cells were serum starved at 0.2% FCS for 16 h, pre-incubated with or without rhCysC for 1.5 h, respectively, followed by cytokine stimulation as indicated. The luciferase activity was measured with the Steady-Glo luciferase assay substrate (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Affinity labeling and cross-linking of TGF-β receptors

Affinity labeling with [^{125}I]-TGF-β1 (Amersham Pharmacia Biotech, Freiburg, Germany) and cross-linking were performed as described previously[24]. Briefly, confluent monolayers of HSCs (5×10^5 cells/10 cm^2) were cultured for 4 days in medium containing 10% FCS. After aspiration of medium, monolayers were washed with Krebs-Ringer-HEPES binding buffer (128 mmol/L NaCl, 5 mmol/L KCl, 5 mmol/L $MgSO_4$, 13 mmol/L $CaCl_2$, 50 mmol/L HEPES) containing 0.5% (w/v) BSA (radioimmunoassay grade) and pre-incubated with or without CysC for 1 h at 37 °C. Cells were washed with ice-cold buffer and receptor binding was performed in the presence or absence of CysC. After equilibration for 10 min on ice, cells were incubated with 2.8 ng/mL [^{125}I]-TGF-β1 (1 621 Ci/mmol) with or without a 200-fold excess of unlabeled TGF-β1 (R&D Systems, Minneapolis, USA) for 3 h at 4 °C. As a further control, an antibody directed against the extracellular domain of betaglycan was used to suppress ligand binding. Bound ligand was cross-linked to its receptor in Krebs-Ringer-HEPES buffer lacking the BSA with disuccinimidyl suberate (Perbio Science) for 15 min at 4 °C. The reaction was stopped by aspiration of the supernatant followed by washing with STE buffer [10 mmol/L Tris-HCl pH 7.4, 250 mmol/L sucrose, 1 mmol/L ethylenediaminetetraacetic acid (EDTA)] including 1 mmol/L phenyl methyl sulfonyl fluoride (PMSF). Cross-linked [^{125}I]-TGF-β1 was extracted with TTE buffer (10 mmol/L Tris-HCl, pH 7.4, 10 mL/L (v/v) Triton X-100, 1 mmol/L EDTA) including proteinase inhibitor cocktail complete (Roche). The detergent soluble, cell debris-free fraction was precipitated by ice-cold 10% TCA (w/v) in the presence of 0.1 mg/mL sodium deoxycholate (DOC). The protein precipitates were subsequently washed with acetone, air dried, and dissolved in a small volume of LDS electrophoresis sample buffer (Invitrogen). Sample volumes corresponding to the normalized DNA contents were subjected to NuPAGE SDS-PAGE. For autoradiography, dried gels were exposed to KODAK X-OMAT AR films (Eastman Kodak Company, Rochester, NY, USA) using intensifying screens.

CysC secretion in HSCs

Serum-starved HSCs were treated with rhTGF-β1 (10 ng/mL) for 24 or 48 h and the cell-free supernatants were subjected to standard immunoprecipitation using goat antibody (sc-16989) directed against CysC (Santa Cruz). The resulting precipitates were washed and analyzed for CysC expression by Western blotting using a primary rabbit antibody directed against CysC (Upstate) and

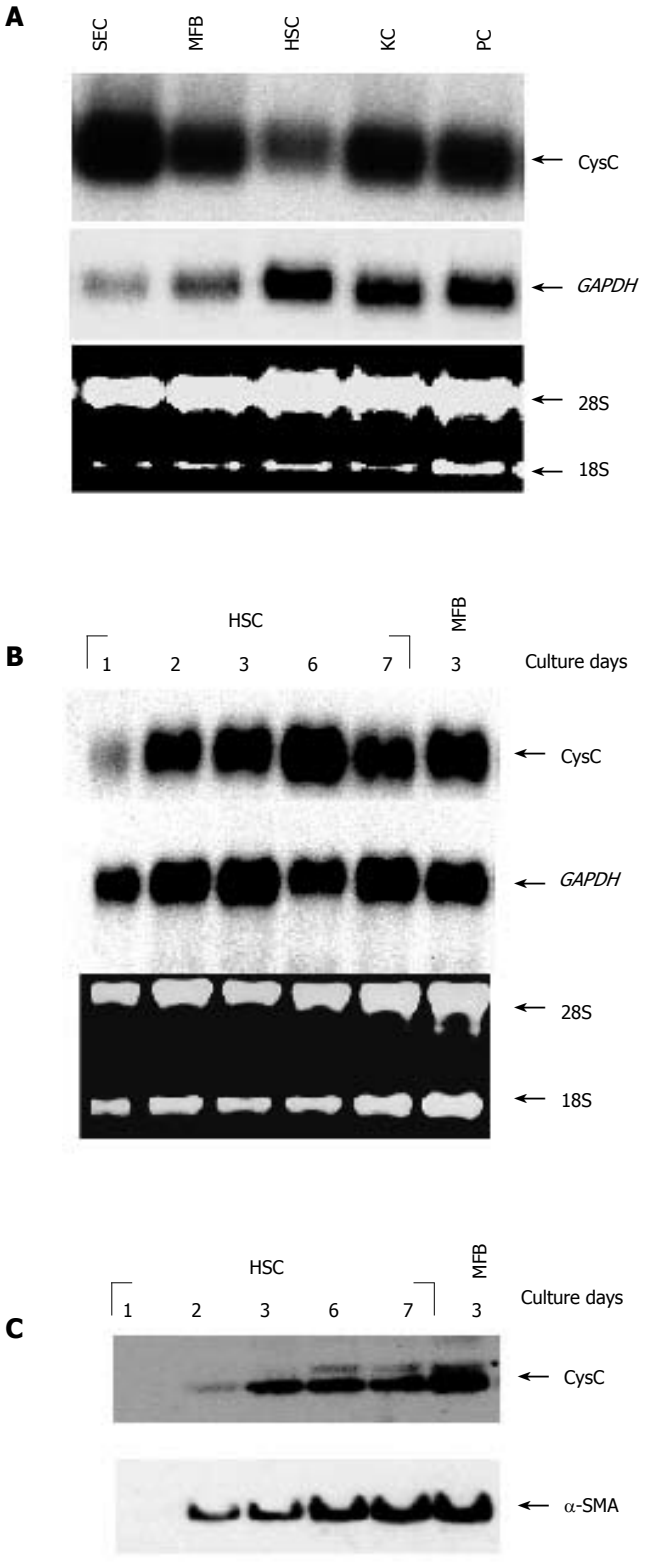

Figure 1 CysC mRNA expression in different rat liver cell types. **A:** Equal amounts of total RNA samples (10 μg) isolated from cultured SECs, MFBs, HSCs, KCs, and PCs were analyzed by Northern blot. The blot was hybridized with a ^{32}P-labeled probe specific for rat CysC; **B:** total RNA samples (5 μg/lane) of trans-differentiating HSCs/MFBs at indicated time intervals were analyzed for CysC expression by Northern blot analysis. As internal loading controls **(A and B)**, the 18S and 28S rRNA as well as signals obtained after hybridization with a *GAPDH*-specific probe are shown. The blots were exposed for 4 (GAPDH) or 24 h (CysC), respectively; **C:** protein extracts (20 μg/lane) from primary rat HSCs cultured for 1 to 7 days or rat MFBs cultured for 3 days were analyzed for CysC expression by Western blotting under reducing conditions (50 mmol/L DTT) using antibody P-14 (sc-16989). To verify cellular trans-differentiation, the blot was stripped, and tested for α-SMA expression using a mouse monoclonal antibody (clone asm-1).

visualized with a goat anti-rabbit IgG-HRP conjugate.

RESULTS

Expression of CysC in isolated liver cells

To analyze the expression of CysC in different liver cell subpopulations, we performed Northern blot analysis (Figure 1A). In agreement with the general assumption that CysC is a constitutive active gene expressed in all nucleated cells at a constant rate[25], we found a single CysC transcript of 0.8-0.9 kb in size in SECs, KCs, PCs, HSCs and their trans-differentiated myofibroblastic phenotype. However, compared to HSCs, we found a significant increase of CysC transcripts in their trans-differentiated phenotype. Subsequent analysis revealed that the activation of HSCs was accompanied with an upregulation of CysC expression during the trans-differentiation process (Figure 1B). In these analyses, the levels of mRNA encoding the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as well as the 28S and 18S rRNAs were used as internal quality and loading controls. To demonstrate CysC expression at the protein level, we further performed Western blot analysis using cellular extracts isolated from cells at different time points of trans-differentiation (Figure 1C). We found that the amount of CysC protein correlated with the amounts of transcripts. To illustrate and confirm the increase of CysC protein more quantitatively, we further performed confocal immunocytochemistry (Figure 2A) and ELF-assay (Figure 2B), respectively. Again, both visualizations of cellular CysC expression by confocal laser scanning microscopy and quantitative ELF-assay indicated that CysC expression was strongly increased during cellular activation and trans-differentiation.

CysC did not modulate TGF-β1 signal transduction in HSCs

A recent report demonstrated that CysC is able to antagonize TGF-β binding to its cell surface receptors thereby inhibiting TGF-β signaling[9]. To test if this antagonizing effect on TGF-β signaling was also present in HSCs, we monitored the changes in the TGF-β sensitive CAGA-MLP-luciferase reporter assay system[26]. Therefore, cultured HSCs were first infected with 50 MOI Ad-(CAGA)$_9$-MLP-luciferase[22], preincubated with different concentrations of rhCysC and then exposed to TGF-β1. The TGF-β-stimulated luciferase activity was compared to unstimulated control cells, which revealed that preincubation with CysC had no effect on the reporter assay (Figure 3A). Similarly, Western blot analysis further revealed that TGF-β-induced phosphorylation of Smad2 and Smad3 did not change in the presence of CysC, again demonstrating that this cysteine protease inhibitor could not antagonize TGF-β signaling in cultured HSCs (Figure 3B).

CysC promoted TGF-β binding to TGF-β type III receptor betaglycan

Based on a recent report demonstrating that CysC could dose-dependently inhibit the physical interaction of TGF-β to murine TGF-β type II receptor[9], we performed cross-linking experiments to test if CysC had the same

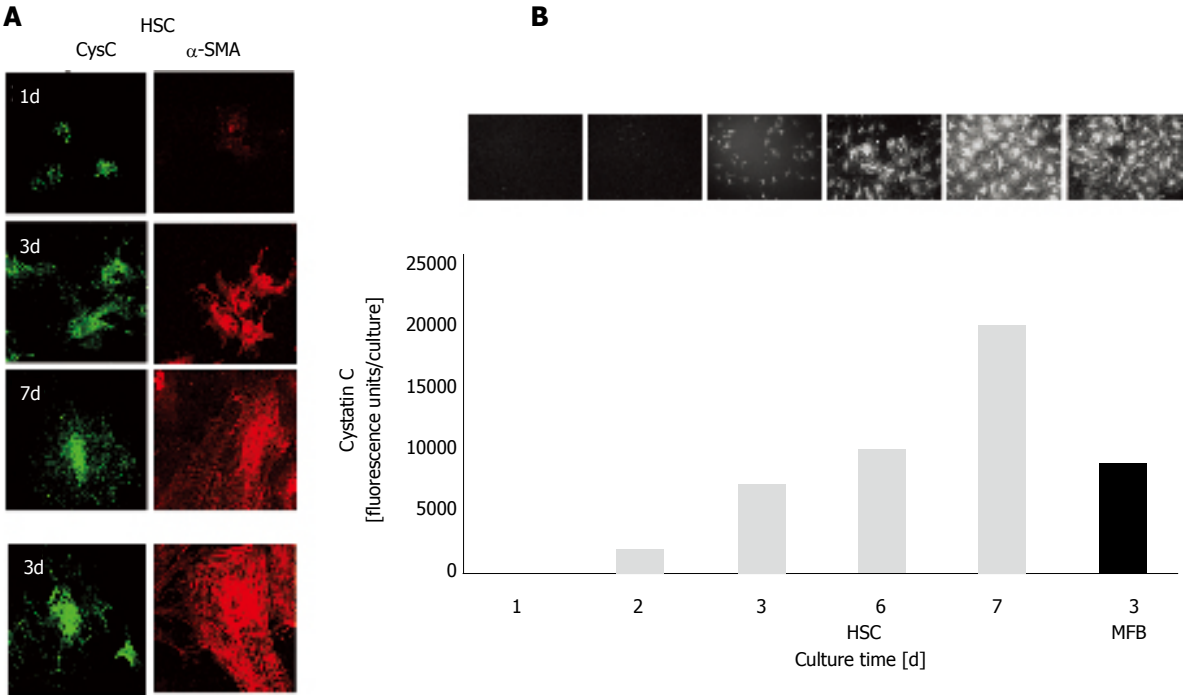

Figure 2 Confocal laser scanning microscopy and ELF assay for quantitative determination of CysC expression in HSCs and MFBs. **A:** HSCs/MFBs were seeded on glass cover slides for indicated time intervals. After fixation, cells were permeabilized and incubated with primary antibodies directed against CysC or α-SMA. Primary antibodies were visualized with a FITC-labeled secondary antibody (CysC, green fluorescence) or a Cy3-labeled secondary antibody (α-SMA, red fluorescence). Original magnification was ×400. Negative controls, using normal IgGs instead of primary antibodies showed no staining (data not shown); **B:** HSCs/MFBs cultured in 96-well plates for indicated time intervals were fixed and stained for CysC. The staining was visualized by fluorescence microscopy (original magnification ×200). Fluorescence intensities were measured quantitatively using a 96-well fluorescence reader (excitation 365 nm, emission ≥515 nm).

effects in the rat system. Interestingly, we found that the binding of TGF-β to the type II receptor was not affected in the presence of physiological concentration of CysC (data not shown). Since the transmembrane signaling by TGF-β was critically modulated in the presence of the type III receptor (betaglycan), which was expressed at high level in HSCs and in turn associated with the type II receptor in a second step, we next addressed the question if CysC was able to modify the binding of TGF-β to this receptor. Binding of TGF-β1 to betaglycan was analyzed by cross-linking experiments using iodinated TGF-β1. HSCs cultured for 4 days were first incubated with rhCysC. Thereafter, the cells were exposed to [^{125}I]-TGF-β1 and the iodinated ligand was chemically cross-linked to the receptors. Subsequently, betaglycan was separated by SDS-PAGE, and the amount of ligand bound to this receptor was visualized by autoradiography (Figure 4, lanes 1 and 2). Interestingly, we found that the binding to betaglycan was elevated after preincubation with CysC. The specificity for the betaglycan labeling was demonstrated by preincubation with a betaglycan-specific antibody (Figure 4, lanes 3 and 4).

TGF-β and PDGF-BB suppressed CysC expression in HSCs

Several reports demonstrated that CysC transcript expression could be stimulated by TGF-β1[9,10,27]. To test if CysC expression was induced in HSCs by TGF-β1, we performed Northern blot analysis with RNA isolated from HSCs that were treated with TGF-β1 (5 ng/mL) for 18 or 38 h, respectively (Figure 5A, lanes 2 and 5). Surprisingly, the contents of CysC transcripts were decreased in TGF-β-stimulated HSCs. Furthermore, the same effect was observed when PDGF-BB was applied causing a significant decrease of CysC transcript expression (Figure 5A, lanes 3 and 6). To confirm that CysC expression in HSCs was indeed suppressed by TGF-β and PDGF-BB, we repeated our experiments at the protein level. As shown in Figure 5B, both TGF-β1 and PDGF-BB significantly suppressed the synthesis of CysC in HSCs.

TGF-β increased secretion of CysC in HSCs

To further explore the impact of TGF-β on CysC regulation, we tested if TGF-β1 could modify CysC secretion. Therefore, we treated serum-starved HSCs with TGF-β1 (10 ng/mL) for 24 or 48 h and determined the amount of CysC in the cell-free supernatants. Compared to control cells, the amount of secreted CysC was markedly increased in cultures stimulated with TGF-β1 (Figure 6). Furthermore, we detected two different CysC protein bands with an approximate molecular mass of 13 and 18 ku most likely representing proteins with a different N-glycosylation status[28].

DISCUSSION

The response to chronic liver injury is wound healing and subsequently fibrosis representing the enhanced ECM production and deposition ending in cirrhosis. In the last decades a growing body of evidence indicates that HSCs and their trans-differentiated phenotype, the MFBs, as

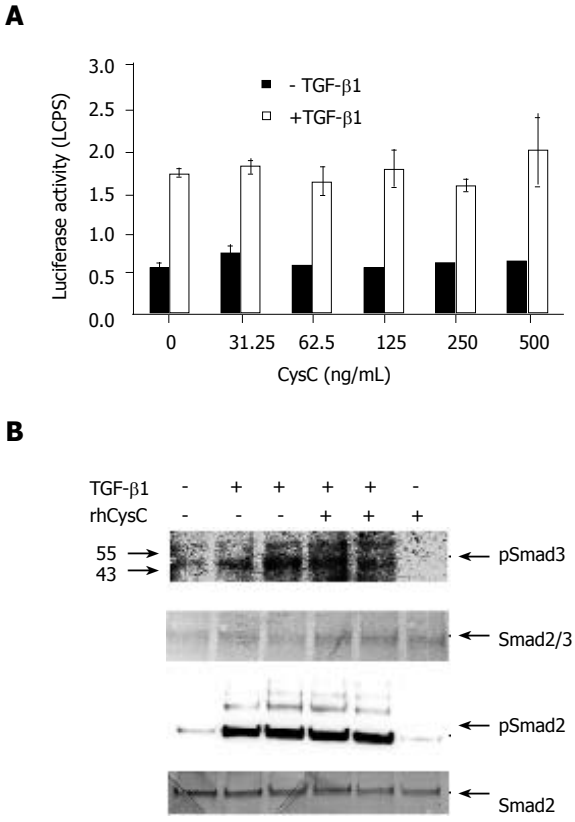

Figure 3 Effect of rhCysC on TGF-β1-induced reporter gene activity and Smad2/3 phosphorylation. **A:** Cultured HSCs were infected with 50 MOI Ad-$(CAGA)_9$-MLP-luciferase followed by 1 h incubation with the indicated doses of rhCysC prior to exposure with TGF-β1 (1 ng/mL) for 4 h or left untreated. The mean ± SD of the measured luciferase activities (n = 4) was given; **B:** phosphorylated Smad2 (pSmad2) and Smad3 (pSmad3) were detected by immunoblotting of whole cell protein lysates prepared after treatment of HSCs with or without 500 ng/mL rhCysC for 1 h followed by incubation with 1 or 5 ng/mL TGF-β1, respectively for an additional hour. The amount of total Smad2/3 or Smad3 was used as internal loading control.

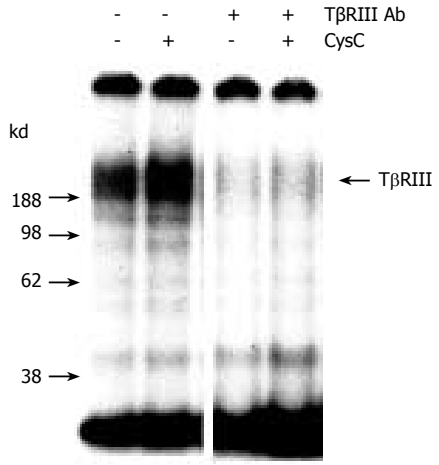

Figure 4 Effect of rhCysC on TGF-β1 binding to beta-glycan. Cultured HSCs were incubated for 1 h with or without rhCysC or an antibody specific for the extracellular domain of beta-glycan followed by the application of [^{125}I]-TGF-β1. The ligand was chemically cross-linked to the receptor and subsequently TβRIII was immunoprecipitated, separated by SDS-PAGE, and visualized by autoradiography. Control experiments including a 200-fold excess of unlabeled TGF-β1 showed no receptor labeling (data not shown).

well as TGF-β1 and PDGF-BB are central cell types and mediators in this wound healing process. In healthy livers, the balance between synthesis and degradation of ECM is tightly controlled. During injury and fibrosis progression, there is increased expression of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) resulting in an imbalance in the turnover of ECM and a conversion of the low-density subendothelial matrix into matrix-rich interstitial collagens. Additionally, the activity of collagenolytic cathepsins is elevated in experimental and clinical hepatic fibrogenesis[29,30] and elevated serum concentrations of CysC are found in patients suffering from chronic liver diseases[12,13]. Therefore, it is possible that an imbalance between cathepsins and CysC contributes to liver fibrosis. The demonstration that members of the cystatin superfamily can physically interact with metalloproteinases thereby protecting them from autolytic activation, further suggesting that there is a direct linkage between the MMP/TIMP and the cathepsin/cystatin enzyme systems[31]. Although all these studies have clearly shown that elevated serum CysC concentrations are clinically important for the progression of liver fibrogenesis, the exact cellular source and the regulation of CysC within different liver cell subpopulations have not been explored.

In the present study, all liver cell types tested were able to synthesize CysC, CysC expression was up-regulated in activated HSCs and trans-differentiated MFBs, TGF-β1 and PDGF-BB suppressed CysC expression at the RNA and protein level, CysC did not modulate TGF-β1 signal transduction in HSCs but induced affinity of betaglycan for TGF-β1, TGF-β1 induced secretion of this inhibitor of cysteine proteinases.

The finding that all liver cells tested express CysC (Figure 1A) is not surprising since it has been well established that CysC behaves like a "housekeeping gene" expressed in all nucleated cells, in all tissues and cell types, although mRNA levels vary several-fold between and among the tissues[25]. However, the finding that CysC expression is strongly increased in activated HSCs and trans-differentiated MFBs is somewhat unexpected since previous studies have demonstrated that the CysC promoter is of constitutive nature[32]. This upregulation is consistent with the findings that patients with liver cirrhosis have an average three-fold greater serum CysC concentration which closely matches to the degrees of fibrosis and is elevated even in patients with mild fibrosis. Thus, increased CysC levels in serum of these patients are at least partially due to a higher expression of this protein inhibitor of cysteine proteinases. The observation that both TGF-β and PDGF-BB could suppress the expression of CysC is at first somewhat striking and unexpected since TGF-β1 and PDGF-BB are the main effectors during hepatic fibrogenesis and, moreover, TGF-β1 can stimulate CysC expression in murine embryo cells, uterine decidual and 3T3-L1 cells[9,10,27]. The conflict might be explained by the finding that only HSCs respond to TGF-β1, whereas fully trans-differentiated MFBs are insensitive to the treatment with TGF-β1[24]. Thus, in ongoing hepatic fibrogenesis, when HSCs become refractory against

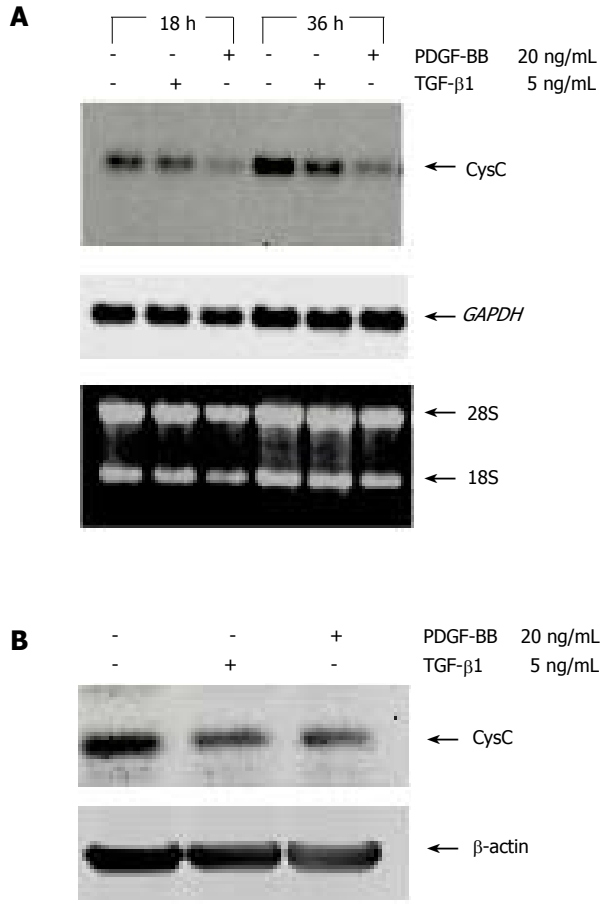

Figure 5 Effect of PDGF-BB and TGF-β1 on CysC expression in HSCs. **A:** Cultured HSCs were serum starved to 2 mL/L FCS followed by treatment with or without PDGF-BB or TGF-β1 for 18 and 36 h, respectively. Total RNA (5 μg/lane) was isolated and analyzed by Northern blot for CysC expression. As internal loading controls, the 18S and 28S rRNA as well as signals obtained after hybridization with a GAPDH-specific probe were shown; **B:** for CysC protein expression, cells were treated with or without PDGF-BB or TGF-β1 for 72 h and protein lysates (20 μg/lane) were subjected to Western blot analysis using an antibody specific for CysC. The expression of β-actin was used as internal protein loading control.

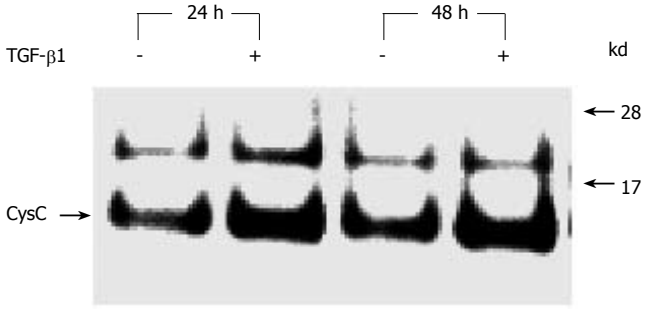

Figure 6 TGF-β1-induced CysC secretion in HSCs. Serum-starved HSCs were treated with TGF-β1 (10 ng/mL) for 24 or 48 h and the cell-free supernatants were subjected to immunoprecipitation using goat antibody P-14 directed against CysC. Subsequently, the precipitates were analyzed for CysC expression by Western blotting using an independent primary rabbit antibody directed against CysC and visualized with a goat anti-rabbit IgG-HRP conjugate.

TGF-β, they start to increase CysC expression potentially leading to the observed higher serum concentrations.

In HSCs, TGF-β signaling is not influenced by the presence of CysC. Although CysC has been found to increase the ligand binding to betaglycan, we observed that it had no effects on TGF-β receptor type II binding (data not shown) and TGF-β reporter gene assay or Smad2/3 phosphorylation. Possibly, CysC inhibits a specific proteinase required for shedding of betaglycan. In line with such a hypothesis, the shedding of betaglycan is mediated by membrane type MMP-1[33] and members of the cystatin superfamily can interact with MMPs and protect them from autolytic degradation[31]. The missing impact of CysC on TGF-β-binding to the type II receptor might be due to the absence of specialized spliced forms of the type II receptor, i.e. type IIb present in mice[34] but not in rat HSCs/MFBs (Meurer, Gressner, Weiskirchen, unpublished observation).

Finally, we found that TGF-β induced secretion of CysC in HSCs. This ability of TGF-β is also described in vascular smooth muscle cells[8], suggesting that it might be a general hallmark of smooth muscle cells. It is also conceivable that the observed decrease of cellular CysC content upon stimulation with TGF-β1 is a consequence of CysC release from intracellular storage sites. However, this effect might only in part contribute to the intracellular decrease of CysC since we observed a clear decrease of CysC mRNA in cells stimulated with TGF-β1.

In conclusion, the concentration of serum CysC is correlated with the severity of liver dysfunction. CysC expression is upregulated in the course of HSC activation and trans-differentiation into MFB, which gives a plausible explanation for the correlation of CysC concentrations with the progression of liver fibrosis.

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S- Editor Wang XL and Guo SY **L- Editor** Elsevier HK **E- Editor** Li HY

PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

World J Gastroenterol 2006 February 7; 12(5):739-746
World Journal of Gastroenterology ISSN 1007-9327

CLINICAL RESEARCH

Microproteinuria in patients with inflammatory bowel disease: Is it associated with the disease activity or the treatment with 5-aminosalicylic acid?

Androniki C Poulou, Konstantinos E Goumas, Dimitrios C Dandakis, Ioannis Tyrmpas, Maria Panagiotaki, Androniki Georgouli, Dimitrios C Soutos, Athanasios Archimandritis

Androniki C Poulou, Konstantinos E Goumas, Dimitrios C Dandakis, Ioannis Tyrmpas, Maria Panagiotaki, Androniki Georgouli, Dimitrios C Soutos, Gastroenterology Department of Red Cross Hospital of Athens, Athanasaki 1, 11521 Athens, Greece
Athanasios Archimandritis, 2nd Department of Internal Medicine, University of Athens Medical School "Hippokratio" General Hospital, 11521 Athens, Greece
Correspondence to: Dr Androniki Poulou, Red Cross Hospital of Athens, Spetson 25, Voula, 16673 Athens, Greece. ddandakis@yahoo.com
Telephone: +30-210-6414724 Fax: +30-210-6414635
Received: 2005-07-03 Accepted: 2005-07-20

Abstract

AIM: To investigate whether microproteinuria in patients with inflammatory bowel disease (IBD) is associated with the disease activity or the treatment with 5-aminosalicylic acid (5-ASA).

METHODS: We prospectively studied microproteinuria in 86 consecutive patients with IBD, 61 with ulcerative colitis (UC) and 25 with Crohn's disease (CD), before as well as 2 and 6 months after their inclusion in the study. Forty-six patients received 5-ASA for a period of 28.8 months (range 1-168 mo). Microalbuminuria (mALB) and urine levels of the renal tubular proteins β2-microglobulin (β2mGLB) and β-*N*-acetyl-D-glucosamidase (β-NAG) as well as the creatinine clearance were determined in a 12-h overnight urine collection. Tumor necrosis factor-α (TNF-α) serum levels were also measured.

RESULTS: A total of 277 measurements (194 in UC patients and 83 in CD patients) were performed. The prevalence of abnormal microproteinuria in UC and CD patients was 12.9% and 6.0% for mALB, 22.7% and 27.7% for β2mGLB, and 11.3% and 8.4% for β-NAG, respectively. mALB was not associated with IBD activity. β2mGLB and β-NAG urine levels were correlated to UC activity (UCAI: $P<0.01$; UCEI: $P<0.005$). mALB in UC patients and β-NAG urine levels in CD patients were related to TNF-α serum levels. An association was noticed between microproteinuria and smoking habit. Treatment with 5-ASA was not correlated to the severity of microproteinuria or to the changes of creatinine clearance.

CONCLUSION: Microproteinuria is mainly associated with UC and its activity but not affected by 5-ASA.

Key words: Inflammatory bowel disease; Microproteinuria; 5-aminosalicylic acid

Poulou AC, Goumas KE, Dandakis DC, Tyrmpas I, Panagiotaki M, Georgouli A, Soutos DC, Archimandritis A. Microproteinuria in patients with inflammatory bowel disease: Is it associated with the disease activity or the treatment with 5-aminosalicylic acid? *World J Gastroenterol* 2006; 12(5): 739-746

http://www.wjgnet.com/1007-9327/12/739.asp

INTRODUCTION

Complications of the urinary system are not uncommon in patients with inflammatory bowel disease (IBD). Their incidence has been reported to vary from 4% to 23% and is greater in patients with more severe and long-standing disease[1]. Apart from secondary complications, such as nephrolithiasis, hydronephrosis and amyloidosis, other associations between IBD and renal disorders have also been described, regardless of treatment[2-5]. Membranous glomerulonephritis, rapidly progressive glomerulonephritis, mesangiocapillary glomerulonephritis, IgA nephropathy, thin basement membrane disease and kidney granuloma are connected with IBD[6-10]. In addition, non-specific morphological changes in the glomeruli of patients with IBD, such as podocyte effacement and mesangial deposition of immunoglobulin and complement have also been well documented[11]. On the contrary, cases of interstitial nephritis are attributed to the nephrotoxic effect of aminosalicylates[11]. However, before the establishment of 5-ASA as a treatment of choice in IBD, studies have identified renal tubular lesions in a proportion of patients[12]. Till date, more than 37 cases implicating the drug in interstitial nephritis (mainly mesalazine but also sulfasalazine, balsalazide, and olsalazine) have been reported to the Committee on Safety of Medicines (CSM)[13].

Abnormal microproteinuria [microalbuminuria (mALB) and/or tubular proteinuria] has been reported both in patients with ulcerative colitis (UC) and in those with Crohn's disease (CD)[13-17]. Increased mALB, equivalent to an albumin excretion rate of 20-200 μg/min, is generally regarded as a sensitive indicator of glomerular disease and has been widely used as a clinical marker of incipient diabetic nephropathy[18]. Mahmud *et al*.[14] have found that there is a significant correlation between mALB and IBD activity. Increased urine levels of some tubular proteins, such as β-*N*-acetyl-D-glucosamidase (β-NAG), α_1-microglobulin, β_2-microglobulin (β_2mGLB), alkaline phosphatase and gamma-glutamyltransferase, have been reported to be reliable indirect indices of renal tubular dysfunction[19]. Moreover, Riley *et al*.[15] have also found that the incidence of elevated urinary β-NAG is low in patients with quiescent UC, which is irrelevant to the dose and duration of mesalazine treatment. Kreisel *et al*.[17] reported that there is a strong correlation between abnormal tubular proteinuria and the activity of UC but not the activity of CD. On the contrary, Schreiber *et al*.[20] showed that increased prevalence of tubular proteinuria is attributed to high dosages of 5-ASA.

The etiology of abnormal microproteinuria in patients with IBD remains ambiguous. We conducted the present 6-month prospective study in order to investigate the incidence of mALB and tubular proteinuria in patients with IBD, emphasizing on the possible relationship between microproteinuria and the disease activity or the treatment with 5-ASA.

MATERIALS AND METHODS

Patients and sample selection

A total of 86 out of 166 screened consecutive Caucasian patients with confirmed chronic IBD (61 with UC and 25 with CD), treated at the Gastroenterology Department of the Red Cross Hospital of Athens from June 1998 to April 2001, were prospectively included in this study. Patients with signs of urinary tract infection and a history of acute or chronic nephropathy, diabetes mellitus, hypertension, severe liver disease, cardiac failure, use of non-steroidal anti-inflammatory drugs (NSAIDs) or other nephrotoxic drugs, patients with a present or recent pregnancy, patients treated with olsalazine, and patients who refused to participate in this study were excluded. Fifty patients were male, 36 were female and their mean age was 42.7 (range 15-77 years) years. Fifty-nine were out-patients and 27 were in-patients. At the time of their inclusion in this study, the IBD was active in 38 patients (27 with UC and 11 with CD) and inactive in 48 patients. Thirty out of eighty-six patients were first diagnosed with IBD (23 with UC and 7 with CD). The mean duration of the disease was 69.8 months (range 0-576 months). The group of patients with UC consisted of 16 patients with total colitis and 45 with left sided colitis (two of them with rectal disease only). The group of patients with CD included 11 patients with ileo-colonic disease, 8 with small bowel disease only and 6 with colonic disease only. Due to the small number of patients, two subgroups of patients with CD were considered in the subsequent analysis, one consisted of the patients with ileo-colonic or colonic disease and the other consisted of those suffering from small bowel disease only. Extra-intestinal manifestations at present or during the last 2 months before the first meeting or during follow-up, were recorded in 29 patients (14 with UC, 15 with CD). Forty-six patients received mesalazine (Salofalk 2-3 g/day or Asacol 1.6-2.4 g/day) for a mean period of 28.8 months (range 1-168 months). During the last year, 20 of them were treated with prednisolone for a mean period of 1.9 months (range 1-12 months) and 6 with azathioprine for a mean period of 4.9 months (range 1-10 months).

According to the study design, all patients were evaluated at the time of their inclusion in the study (stage 1), two (stage 3) and six (stage 4) months afterwards. Twenty-seven patients (19 with UC, 8 with CD) with severely exacerbated disease at the time of their inclusion in the study were re-evaluated 10 days after intensive corticosteroid-based treatment (stage 2). A meticulous routine and more specific clinical and laboratory evaluation of the patients was conducted in each stage of the study, including determination of the hematocrit value, white blood cell and platelet count, serum levels of C-reactive protein (CRP) and tumor necrosis factor-alpha (TNF-α), usual urinalysis, levels of mALB and tubular proteinuria (β_2mGLB, β-NAG) and creatinine clearance. Measurements of microproteinuria and creatinine clearance were performed using a 12-h urine collection, which has been proved equivalent to a 24-h urine collection[21] or an 8-h urine collection[22]. The criterion of our choice was the better compliance of our patients. The clinical disease activity quantifications were performed on the day of urine collection, using reliable activity indices for UC[23] and CD[24]. All patients with UC underwent colonoscopy at the 1st, 3rd, and 4th stages of the study and an endoscopic index of the activity of UC (UCEI)[25] was calculated for these patients[25]. Finally, a detailed drug history was taken from all the patients to confirm the type, dose and duration of medication.

The blood samples for the measurement of serum levels of TNF-α after centrifugation were stored at -48 ° C. The urine samples for the determination of urine levels of microalbumin were stored at 2-8 °C for a time not exceeding 2 weeks, at -20 °C for β_2mGLB and at -48 °C for β-NAG, respectively.

Microproteinuria (mALB and tubular proteinuria) was correlated to patients' characteristics including sex, age, smoking habits; features of the diseases including activity, extent, duration, presence of extraintestinal manifestations; medication including type (mesalazine, corticosteroids, azathioprine) and duration. Furthermore, separate analyses of the courses of microproteinuria were conducted for patients with highly active disease at the time of their inclusion in the study and those with a first diagnosis of the disease, who did not receive any medication.

Analytical techniques (chemical analysis)

Urea and creatinine in serum as well as creatinine in urine were measured with standard techniques. Serum CRP concentrations were determined by nephelometry.

Urinary albumin concentrations were measured using a radioimmunologic assay (^{125}I radioimmunologic analysis, EURO/DPC, Glyn Rhonwy, UK), which could indicate

Table 1 Incidence of abnormal microproteinuria in patients with active and inactive IBD (%)

	Ulcerative colitis (n = 194)			Crohn's disease (n = 83)		
	Active (n = 49) UCAI ≥ 6	Inactive (n = 145) UCAI < 6	P	Active (n = 15) CDAI ≥ 150	Inactive (n = 68) CDAI < 150	P
Urine m-ALB (>18 μg/min)	16.3	11.7	NS	13.3	4.4	0.06
Urine β_2 mGLB (>120 μg/min)	40.8	16.6	<0.001	53.3	22.1	<0.05
Urine β-NAG (>5.9 U/L)	26.5	6.2	<0.001	13.3	7.4	NS

Table 2 Microproteinuria and creatinine clearance in active and inactive IBD (mean±SE)

	Ulcerative colitis (n = 194)			Crohn's disease (n = 83)		
	Active (n = 49)	Inactive (n = 145)	P	Active (n = 15)	Inactive (n = 68)	P
Urine m-ALB (μg/min)	11.89±3.19	9.0±1.03	NS	8.59±2.65	7.02±1.07	NS
Urine β_2mGLB (μg/min)	204±50.2	88.0±10.28	<0.01	359±130.9	92.2±16.26	<0.05
Urine β-NAG (U/L)	4.69±0.64	2.39±0.16	<0.001	3.07±0.90	2.36±0.25	NS
Urine Kcr (mL/min)	114.4±6.03	120.3±3.24	NS	135.6±8.34	127.7±3.96	NS

NS: Not significant.

subtle increases in albumin excretion above the normal range but below that was detected by standard "dipstick" analysis (equivalent to a urinary albumin excretion rate of 20-200 μg/min)[26].

Urinary β2mGLB concentrations were measured using a radioimmunologic assay (^{125}I radioimmunologic analysis, RADIM)[27].

Urinary β-NAG concentrations were determined colorimetrically using a commercially available kit (Boehringer-Mannheim, Germany). The optical density of the samples was measured at 580 nm (Cobas Bioanalyser, Roche). Results were calculated by reference to a standard curve[15,28].

Serum TNF-α was measured using a sandwich enzyme-linked immunosorbent assay (ELISA; Quantikine, R&D Systems Inc., Minneapolis, MN, USA: sensitivity 0.35 pg/mL)[29,30].

Quantification of disease activity

The activity of UC was quantified using the UC activity index (UCAI) introduced by Rachmilewitz[23]. A score greater than or equal to six was considered to be suggestive of active UC. The activity of CD was estimated according to the Best CD activity index (CDAI)[24]. CD was considered to be active in cases with CDAI greater than 150. Finally, the endoscopic activity of UC was also evaluated according to a reliable endoscopic index established by other researchers[25].

Statistical analysis

The Mann-Whitney *U*-test, the Wilcoxon rank-sum *W*-test and the Spearman's rank correlation test were used for statistical evaluation of the non-parametric data. Student's *t*-test was applied for statistical analysis of all parametric data. $P<0.05$ was considered statistically significant.

RESULTS

Eighty-two out of eighty-six patients participating in three stages of the study (time: 0, 2nd, and 6th month) completed the follow-up. Twenty-seven of them participated in one more stage (time: 10th day). Two out of eighty-six patients participated in two stages (time: 0, 10th day) and two in one stage only (time: 0). A total of 277 distinct evaluations were performed in 86 IBD patients, 194 in patients with UC and 83 in patients with CD. The IBD was active in 38 out of 86 patients at the 1st stage, in none out of 27 patients at the 2nd stage, in 7 out of 82 patients at the 3rd stage and in 6 out of 82 patients at the 4th stage of the study.

Abnormal urine levels of albumin (>18 μg/min), β2mGLB (>120 μg/min) and β-NAG (>5.9 U/L) were found in 12.9%, 22.7%, and 11.3% of the patients with UC and in 6%, 27.7%, and 8.4% of those with CD, respectively, more frequently in active than in inactive disease (Table 1). The mean values of mALB, tubular microproteinuria and creatinine clearance in active and inactive IBD are shown in Table 2. No differences in albuminuria were found between active or inactive UC and CD. In contrast, significantly higher urine levels of β_2-mGLB were detected both in patients with active UC and in those with active CD. Urine levels of β-NAG were

Table 3 Correlation of microproteinuria and creatinine clearance of IBD patients after 5-ASA treatment (mean±SE)

	Ulcerative colitis ($n=194$) 5-ASA treatment		P	Crohn's disease ($n=83$) 5-ASA treatment		P
	No ($n=37$)	Yes ($n=157$)		No ($n=15$)	Yes ($n=68$)	
Urine m-ALB (μg/min)	8.99±2.10	9.90±1.29	0.794	8.08±2.57	7.13±1.08	0.562
Urine β_2mGLB (μg/min)	163±61.16	107±12.07	0.852	274±129.2	111.0±20.15	0.083
Urine β-NAG (U/L)	4.22±0.82	2.67±0.18	0.118	2.79±0.91	2.42±0.26	0.845
Urine Kcr (mL/min)	117±7.25	119±3.11	0.987	130±7.62	129±4.05	0.713

Table 4 Correlation between microproteinuria in patients with ulcerative colitis and disease activity parameters ($n=194$ measurements)

		Ulcerative colitis activity parameters					
		UCAI	UCEI	CRP	ESR	Platelets	Serum TNF-α
Urine m-ALB (μg/min)	r_S	0.0283	0.0420	0.0232	0.0091	0.0826	0.1665
	P	0.6954	0.5612	0.7479	0.9001	0.2520	0.0203
Urine β_2mGLB (μg/min)	r_S	0.1957	0.1037	0.1059	0.0033	0.0540	0.0123
	P	0.0062	0.1501	0.1414	0.9634	0.4543	0.8648
Urine β-NAG (U/L)	r_S	0.2633	0.2335	0.1438	0.1224	0.2336	0.1057
	P	0.0002	0.0010	0.0455	0.0891	0.0010	0.1426

r_S: Spearman's correlation coefficient; r_P: Pearson's correlation coefficient.

higher in active UC, but not in active CD.

Serum creatinine and its clearance

The levels of serum creatinine were normal in all the patients. Although creatinine clearance values varied widely, its mean values did not differ significantly between the patients with active and those with inactive UC (114.4 ±6.03 mL/min *vs* 120.3±3.24 mL/min, $P>0.10$) as well as between the patients with active and those with inactive CD (135.6±8.34 mL/min *vs* 127.7±3.96 mL/min, $P>0.10$). Our male IBD patients showed higher creatinine clearance values than female IBD patients (UC: 134±3.19 mL/min *vs* 95±4.07 mL/min, $P<0.001$; CD: 137.1±5.16 mL/min *vs* 118.8±4.22 mL/min, $P<0.05$). Creatinine clearance values were inversely correlated with the age of UC patients (ulcerative colitis: $r_P=-0.374$, $P<0.001$; CD: $r_P=-0.164$, $P>0.10$).

In addition, creatinine clearance values were higher in smokers with UC (smokers: 127±4.04 mL/min; non-smokers: 114±3.81 mL/min, $P<0.05$) and in those with CD (smokers: 137.3±4.50 mL/min; non-smokers: 113.1±4.50 mL/min, $P<0.001$). Finally, no correlation of creatinine clearance values with 5-ASA treatment was observed (UC patients treated with and without 5-ASA: 119±3.11 mL/min and 117±7.25 mL/min, respectively, $P=0.987$; CD patients treated with and without 5-ASA: 129±4.05 mL/min and 130±7.62 mL/min, respectively, $P=0.713$).

Microproteinuria and IBD patients' parameters

No correlation was found between microproteinuria (mALB or tubular proteinuria) and the age or the sex of our IBD patients (data not shown). However, albuminuria was significantly lower in smokers with UC than in non-smokers (smokers: 8.22±2.16 μg/min; non-smokers: 10.6 ±1.23 μg/min, $P=0.021$) and the urine levels of β-NAG were significantly lower in non-smokers with CD than in smokers (non-smokers: 1.62±0.19 U/L; smokers: 2.93± 0.37 U/L, $P=0.023$).

Microproteinuria and IBD activity

In our patients with UC, urinary concentrations of β-NAG were significantly correlated with the UCAI ($r_S=0.26$, $P<0.0005$), the UCEI ($r_S=0.23$, $P<0.005$) and the serum CRP levels ($r_S=0.14$, $P<0.05$). Similarly, in these patients urinary levels of β_2 mGLB were significantly associated with the UCAI ($r_S=0.2$, $P<0.01$) but not with the UCEI or the serum CRP levels (Table 4). On the contrary, the patients with CD did not show any correlation of tubular microproteinuria with the parameters of their disease activity (Table 5).

No significant relationship was found between the levels of mALB and the disease activity in patients with UC neither in those with CD (Tables 4 and 5). A significant association between mALB and serum levels of TNF-α was noticed only in UC patients. Finally, a history of recent or present extra-intestinal manifestations did not influence the levels of mALB and tubular microproteinuria in IBD patients (data not shown).

Microproteinuria and IBD duration and extent

A significant inverse relationship was found between the levels of mALB and the duration of UC ($r_S=-0.24$, $P<0.001$). In contrast, a tendency towards a positive association was noticed between the severity of mALB and the duration of CD ($r_S=0.19$, $P=0.078$). No significant differences were found between the levels of microproteinuria (mALB and tubular microproteinuria)

Table 5 Correlation between microproteinuria in patients with Crohn's disease and disease activity parameters ($n=83$ measurements)

		Crohn's disease activity parameters				
		CDAI	CRP	ESR	Platelets	Serum TNF-α
Urine m-ALB (μg/min)	r_S	0.1186	0.0326	0.0352	0.1483	0.1537
	P	0.2856	0.7686	0.7522	0.1810	0.1654
Urine β$_2$mGLB (μg/min)	r_S	0.0813	0.0187	0.0624	0.1727	0.2100
	P	0.4648	0.8669	0.5751	0.1184	0.0568
Urine β-NAG (U/L)	r_S	0.1106	0.0319	0.0947	0.0527	0.2676
	P	0.3196	0.7744	0.3347	0.6359	0.0145

r_S: Spearman's correlation coefficient; r_P: Pearson's correlation coefficient.

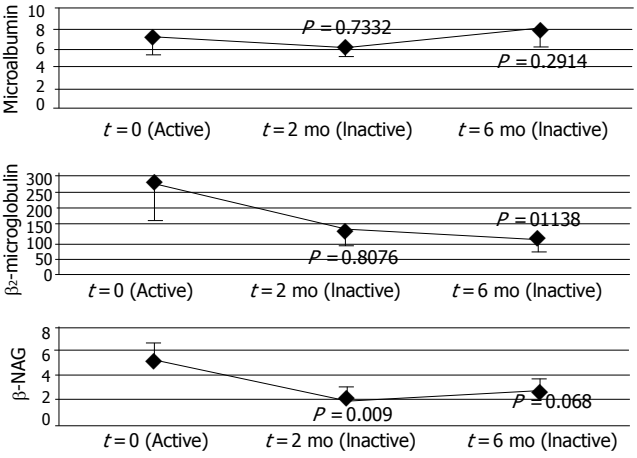

Figure 2 Changes of microproteinuria in the first-diagnosed patients with active IBD ($n=22$) before and after inducing remission.

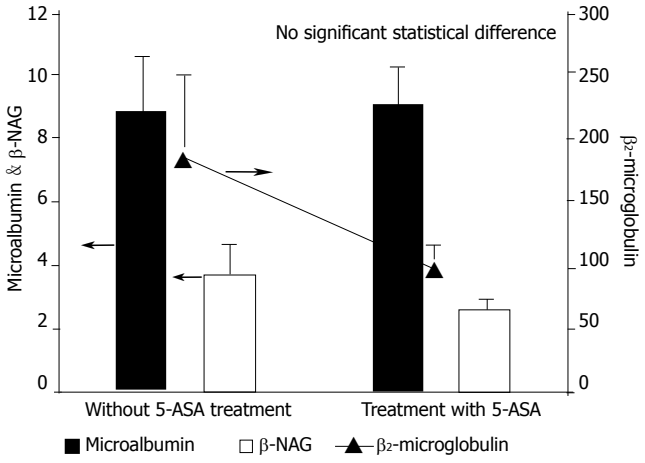

Figure 1 Correlation of IBD patients' microproteinuria (mALB, β_2mGLB, β-NAG) with 5-ASA treatment.

and the extent of UC (total *vs* left side colitis) as well as the extent of CD (small intestine+colon *vs* small intestine only).

Microproteinuria and IBD therapy

No differences were found in the levels of mALB and tubular microproteinuria between the IBD patients who received or did not receive 5-ASA therapy (Table 3 and Figure 1). Moreover, no differences were found between the duration of 5-ASA treatment and the levels of mALB (UC: $r_s=-0.06$, $P=0.43$; CD: $r_s=0.05$, $P=0.70$), the urinary levels of β_2mGLB (UC: $r_s=0.06$, $P=0.42$; CD: $r_s=0.17$, $P=0.16$) and β-NAG (UC: $r_s=-0.02$, $P=0.77$; CD: $r_s=0.02$, $P=0.88$). The levels of microproteinuria were also similar in the patients treated and those not treated with corticosteroids and/or azathioprine (data not shown).

Microproteinuria in the first-diagnosed patients with active IBD

Among the 30 IBD patients with a first diagnosis of the disease at the time of their inclusion in this study, 22 had active IBD (17 had UC, 5 had CD). The 6-month course of mALB and tubular microproteinuria in the patients with an initially active IBD never treated with 5-ASA, exhibited a particular interest (Figure 2). In these patients, microproteinuria showed a tendency of gradual reduction by the time when IBD remission was achieved, although this reduction was statistically significant for β-NAG only.

Microproteinuria in patients with severely active IBD

Twenty-seven of the patients (19 with UC, 8 with CD) were admitted to our hospital due to severely active disease. In these patients, we also evaluated the levels of microproteinuria in one more stage 10 days after the initiation of an intensive therapy (including corticosteroids). An early (10 days after) reduction of mALB (10.56 ± 2.27 μg/min *vs* 6.13 ± 1.43 μg/min, $P<0.01$) as well as urine levels of β-NAG (5.60 ± 1.10 U/L *vs* 3.17 ± 0.60 U/L, $P<0.05$) and β_2mGLB (241.2 ± 96.82 μg/min *vs* 99.6 ± 33.33 μg/min, $P>0.10$) was observed.

DISCUSSION

Although the incidence of renal complications is relatively low in patients with IBD, none of our patients developed clinically significant renal disease. No significant changes in serum creatinine and its clearance were recorded during the 6-month follow-up period. Furthermore, creatinine clearance was not correlated with the type, activity or the extent of IBD, which is in accordance with the majority of the published studies[13-17].

We observed a moderately abnormal mALB in 12.9% and 6% of our patients with UC and CD, respectively, which is in agreement with Fraser *et al*[13], Riley *et al*[15], Bonnet *et al*[31] and Zehnter *et al*[32]. Increased mALB reliably predicts diabetic nephropathy and an increased incidence of cardiovascular-related morbidity and mortality in diabetic and hypertensive patients as well as in the elderly[18,33,34]. Moreover, mALB has been described as a non-specific marker for acute illness[35], most probably as a result of the acute phase response to inflammatory

mediators. It has been suggested that mALB in IBD patients may result from increased renal microvascular permeability in response to increased circulating cytokines[14,30,36]. This hypothesis is compatible with our finding of a significant relationship between mALB and serum TNF-α levels in our UC patients ($P < 0.05$), a thesis which is also supported by other researchers[13,15,31,32].

We did not find any significant correlation between the levels of mALB and the IBD activity, which is in agreement with many published studies[13,15]. In contrast, Mahmud *et al*.[14,37] found that increased mALB in the vast majority of IBD patients is related to both clinical and histopathological activities of the disease. Differences between these studies may be attributed to the varied disease activity. In our study, a relatively small proportion of the patients had a considerable disease activity.

Abnormal tubular microproteinuria, reflecting subclinical tubular damage, is a quite common finding among IBD patients[15,17]. However, its incidence varies widely[13-17,31,32,38], from 0% in the studies by Biddle *et al*[38] (β-NAG and β2mGLB) and Mahmud *et al*[14] (glutathione-*S*-transferase) to 75% in the study by Bonnet *et al*[31] (γ-glutamyl transferase and alkaline phosphatase). Hitherto, it has been difficult to prove whether tubular microproteinuria results from the effect of 5-ASA or is an extra-intestinal manifestation of the IBD itself[13]. Its structural similarity to other salicylates and non-steroidal anti-inflammatory drugs has led to the speculation that 5-ASA may cause tubulointerstitial damage of the kidney[11].

A significant correlation between tubular microproteinuria and IBD activity was found only in our patients with UC. This might be due to the fact that our patients with severely active CD were much fewer than those with UC. Kreisel *et al*.[17] have observed an increased urinary β-NAG excretion in 28% of the patients with UC and in 19% of those with CD, especially higher in active disease. Schreiber *et al*.[28] acknowledged their inability to determine whether high levels of tubular microproteinuria are attributable to the severity of the disease's activity or 5-ASA treatment (or both), since 11% of their patients not receiving 5-ASA also had a tubular microproteinuria. Fraser *et al*.[13] found that urinary excretion of β-NAG and α_1-microglobulin was increased in 48% and 52% of patients with IBD at diagnosis, respectively. A positive relationship between the urine levels of several tubular proteins and the IBD activity has been proposed by the majority of the inv-estigators[13-17,31,39]. In contrast, Zehnter *et al*.[32] showed that increased urine levels of β_2-mGLB, alanine aminopeptidase and β-NAG in IBD patients were not correlated with the disease activity.

In this study, 22 first-diagnosed patients with active IBD had increased levels of tubular microproteinuria at diagnosis, which decreased after treatment with corticosteroids and 5-ASA. Moreover, 27 IBD patients with severely active disease manifested an early significant decrease of both microalbuminuria and tubular microproteinuria after being treated with corticosteroids. In addition, we found that there was a positive relationship between microalbuminuria and serum TNF-α levels, as well as between tubular microproteinuria and serum CRP and TNF-α levels, suggesting that microproteinuria in IBD patients may be related to a systemic inflammatory response and that it is an extra-intestinal manifestation of the active disease rather than a consequence of a subclinical renal injury due to 5-ASA treatment[13,15,17,20,31,32,37]. However, We did not find any differences in the severity of microproteinuria between the IBD patients with and those without a recent or present extra-intestinal manifestation of the disease, which is in agreement with Kreisel *et al*[17] and Mahmud *et al*[30].

Activation of the intestinal immune system, production of inflammation mediators or imbalance between activating and suppressing mediators play a pivotal role in the pathogenesis of IBD. In particular, TNF-α may play an important role in the inflammatory cascade of IBD and increased serum TNF-α levels have been reported in active IBD[40]. It has been reported that interleukin-1 and TNF-α inhibit the synthesis and induce a shedding of cell surface glycosaminoglycans[41], which might lead to an increased microvascular permeability and tissue damage[36,42].

No relationship was found between the severity of microproteinuria and the extent as well as the duration of IBD in our study, which is in concordance with other studies[14,20,32,43]. It is worth emphasizing on our finding of a significant correlation between microproteinuria and the smoking habit, which was negative in UC and positive in CD, because smoking alters the colonic mucosal blood flow[44] and decreases its permeability. Epidemiologic data suggest that smoking exerts a protective effect against UC[45] and is harmful to CD[45,46]. Consequently, differences in microproteinuria between smokers with UC and those with CD may be related to these pathophysiologic and epidemiologic data.

In this study, mALB was not correlated with the IBD treatment as it is almost universally accepted[13,15,31,32,37]. In addition, using sensitive markers of renal tubular toxicity[19], we were unable to find any evidence that treatment with mesalazine, corticosteroids or azathioprine was related to an increased tubular damage. However, this lack of correlation does not necessarily exclude nephrotoxicity. A potential nephrotoxicity of aminosalicylates, although rare, remains an existent possibility[11].

Riley *et al*[15] showed that there was no evidence of nephrotoxicity in a cohort of patients with quiescent UC receiving mesalazine or sulfasalazine. Furthermore, Kreisel *et al*[17] have demonstrated that there is no positive relationship between the cumulative dose of 5-ASA and the severity of tubular microproteinuria. In accordance to these studies, we observed that tubular microproteinuria in patients with active IBD at diagnosis decreased after inducing remission of the disease, even though the patients received the treatment with 5-ASA and corticosteroids. Fraser *et al*[13] have demonstrated that both the prevalence and degree of tubular microproteinuria in patients with a new diagnosis of IBD are relatively unaffected by 5-ASA treatment, which is in agreement with a number of studies[14,38,43,47]. In contrast, Schreiber *et al*[20] have reported that there is a positive relationship between tubular microproteinuria and cumulative 5-ASA exposure, but they questioned whether their study could provide a definite answer to the possible impact of chronic inflammation on

microproteinuria. A similar positive relationship between tubular microproteinuria and 5-ASA treatment has also been reported[31,32].

In conclusion, abnormal mALB and tubular microproteinuria are quite frequent in IBD patients. Subclinical renal tubular and/or glomerular damage are related to the IBD itself, representing an extra-intestinal manifestation of the disease. Moreover, treatment with 5-ASA does not affect microproteinuria in IBD patients, microproteinuria in IBD patients does not predict an adverse renal response to 5-ASA.

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S- Editor Wang XL and Guo SY **L- Editor** Elsevier HK **E- Editor** Li HY

PO Box 2345, Beijing 100023, China
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World J Gastroenterol 2006 February 7; 12(5):747-754
World Journal of Gastroenterology ISSN 1007-9327

CLINICAL RESEARCH

Prospective, randomized, and active controlled study of the efficacy of alginic acid and antacid in the treatment of patients with endoscopy-negative reflux disease

I-Rue Lai, Ming-Shiang Wu, Jaw-Town Lin

I-Rue Lai, Department of Surgery, National Taiwan University Hospital, National Taiwan University College of Medicine, Taipei, Taiwan, China
Ming-Shiang Wu, Jaw-Town Lin, Division of Gastroenterology, Department of Internal Medicine, National Taiwan University Hospital, National Taiwan University College of Medicine, Taipei, Taiwan, China
First author: I-Rue Lai
Correspondence to: Dr Ming-Shiang Wu, Division of Gastroenterology, Department of Internal Medicine, National Taiwan University Hospital, No. 7, Chun-San South Road, Taipei, Taiwan, China. stanley@ha.mc.ntu.edu.tw
Telephone: +886-2-23123456 extn. 6651
Received: 2005-05-04 Accepted: 2005-06-09

Abstract

AIM: To assess the efficacy and safety of a compound containing alginic acid plus antacid (Topaal®) compared to equal-strength antacid (Nacid®) in patients with endoscopy-negative reflux disease (ENRD).

METHODS: A total of 121 patients with ENRD were randomized to receive Topaal® (65 patients) or Nacid® (56 patients) for 6 weeks, with a consultation every 3 weeks. The primary end-point assessment was the change in the severity of heartburn as evaluated using a visual analog scale (VAS) at 6 weeks. The secondary end-point assessments were the VAS at 3 weeks, the change of frequency of the reflux symptom, the change of quality of life and the adverse effects.

RESULTS: Demographics of randomized subjects in each treatment group were comparable except that the Topaal® group included more males. The baseline characteristics between the groups were similar. After 6 weeks of treatment, the reduction of VAS of heartburn was more prominent in the Topaal® group (-6.29 cm *vs* -4.11 cm). At the 3rd week, Topaal® group showed greater reduction of VAS for heartburn ($P = 0.0016$), regurgitation ($P = 0.0006$), vomiting ($P = 0.0373$), and belching ($P < 0.0001$). The patients of the Topaal® group had lower frequency of heartburn ($P = 0.0015$) and pain ($P = 0.0163$) at the end of the 6-week treatment period. From the doctor's point of view, the Topaal® group also showed significant reduction in the severity of heartburn ($P = 0.0020$), regurgitation ($P = 0.0081$), vomiting ($P = 0.0182$), and belching ($P = 0.0018$) at the end of the treatment. The improvement of the quality of life was more remarkable in the Topaal® group at the end of the 6-week treatment period ($P < 0.0001$). For the adverse effect, there was no difference in both the groups.

CONCLUSION: Topaal® is more effective than Nacid® for the treatment of symptoms presented by patients with ENRD.

Keywords: Alginic acid; Endoscopy-negative reflux disease

Lai IR, Wu MS, Lin JT. Prospective, randomized, and active controlled study of the efficacy of alginic acid and antacid in the treatment of patients with endoscopy-negative reflux disease. *World J Gastroenterol* 2006; 12(5):747-754

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INTRODUCTION

The incidence of gastroesophageal reflux disease (GERD) in Taiwan has been increasing. According to the endoscopic surveillance reports, the rate of erosive esophagitis in Taiwan has increased from 2.4% to 14.5% in the past 20 years[1,2].

GERD is a disorder in which the gastric contents are refluxed into the esophagus, causing irritation and injury to the esophageal mucosa[3]. The typical symptoms of GERD include heartburn and regurgitation. Less common symptoms or symptoms suggestive of more aggressive reflux disease include dysphagia, odynophagia, or gastrointestinal bleeding. Atypical complaints include chest pain, hoarseness, sore throat, chronic cough, and asthma[3]. It has been shown that defective lower esophageal sphincter (LES) is the major mechanism underlying in most patients with reflux[4].

Endoscopy is the most common diagnostic tool to grade and to define GERD. However, only one-third of patients with GERD have esophageal mucosal erosion or ulceration[3]. Patients who experience typical heartburn despite that no evident mucosal lesions found at endoscopy are defined to have endoscopy-negative reflux disease (ENRD)[5]. Compared with patients who have reflux-related erosive esophagitis, those with ENRD

are more likely to be younger, of lower body weight, and without a hiatal hernia[6]. Approximately 50% of those with ENRD have abnormal intra-esophageal acid exposure[6]. Although persons with ENRD experience similar decrements in their quality of life as those for individuals with erosive esophagitis, their complaints were often ignored if the diagnosis and treatment are based only on endoscopic finding. It has been proposed that the analysis of symptoms is probably the most useful method for the diagnosis of GERD[7]. The pharmacological treatment of ENRD comprises two therapeutic classes: (1) local antacids or anti-secretory drugs including histamine receptor and proton pump inhibitors (PPI)[8], which act by decreasing the acidity of the reflux contents; (2) alginates which act by decreasing the gastric reflux into the esophagus and by protecting the esophageal mucosa[9]. PPI were shown to provide symptom relief in patients with ENRD[8,10] but these treatments are not curative and not cheap. A cohort study of patients with ENRD has shown that after a median time of 10 years following the original diagnosis, 75% of patients are on prolonged antisecretory therapy because of recurrent symptoms/lesions of gastro-esophageal reflux disease[11]. The study confirms that ENRD is a protracted disease, which requires long-term medical therapy in most of the patients. Besides, Taiwan's reimbursement system covers the PPI only when patients have endoscopic evidence of erosive esophagitis. Local antacid and alginates are more convenient and cheap options for patients with ENRD[9]. However, there were few studies comparing the efficacy of these two classes of drug on the treatment of ENRD. We therefore carried out a prospective, randomized trial comparing the efficacy and tolerance of sodium alginate (Topaal®) vs antacid (Nacid®) in the treatment of patients with ENRD.

MATERIALS AND METHODS

Selection of patients

Disease definition: All patients who presented with the classic symptoms including heartburn and/or regurgitation, but who did not have either Barrett's esophagus or definite endoscopic esophageal mucosal breaks (esophageal mucosal erosion or ulceration) were referred to have ENRD[7,10].

Entry procedures: The patients prior to the participation in this study signed the informed consent document, which has been approved by an ethical review board of National Taiwan University Hospital.

This study enrolled outpatients of both sexes, who were aged between 18 and 75 years, and were diagnosed to have ENRD. The included patients were asked to discontinue antacid, metoclopramide, cisapride, H_2 blocker and PPI for at least 3 days before entering the study. Those patients who had a history of intolerance or allergy to alginic acid and antacid, endoscopic evidence of esophagitis, history of partial or total gastrectomy, or had esophageal stricture, pregnancy or lactation were excluded from the enrollment.

Study endpoints

Primary efficacy endpoint: The primary efficacy endpoint was the change in the severity of heartburn as evaluated by using a visual analog scale (VAS) at the 6th week of treatment compared to baseline in the intention-to-treat (ITT) population. The VAS[12,13] is a 100-mm straight line with anchors (0 cm indicated no symptom and 10 cm indicated terrible) placed at both poles. Patients were asked to place a mark somewhere along the line that best described the actual status of their symptoms. The ITT population was defined as randomized patients who received at least one dose of study medication, had a baseline value and at least one post-baseline efficacy assessment. In this analysis, data from the Topaal® group were compared with data from Nacid® group.

Secondary efficacy endpoints: There were three secondary endpoints evaluated in this trial: (1) the change in the severity of reflux symptoms including heartburn, regurgitation, dysphagia, epigastric pain, nausea, vomiting and belching by VAS at the 3rd week of treatment compared to baseline; (2) the change in the frequency of heartburn, regurgitation, pain, and sleeping disturbance according to patient's diary; and (3) the change in the quality of life from doctor's point of view. All of these secondary efficacy endpoints were assessed in the ITT population.

During the 7th day before the first visit and the entire study period, each patient had to record the frequency (number of episodes per day) on the patient's diary. Each patient was asked to record the symptom of reflux disease at the moment it happened. At least a 4-d record in a week was necessary for this evaluation.

The patient's quality of life judged by the investigator's point of view was graded by modified visick grading[11] on each visit as the following scores: no symptoms (= 1), mild symptoms easily controlled (= 2), moderate symptoms not controlled but not interfering daily life (= 3), moderate symptoms interfering daily life (= 4), and symptoms as bad or worse (= 5).

Safety endpoint: The incidence of adverse drug reaction (ADR), whether reported spontaneously, elicited by questioning, or observed by the investigators, of both Topaal® and Nacid® groups was calculated in the safety population. Safety population was defined as patients who received at least one dose of study medication after randomization. The ADR was recorded and graded according to the WHO definition[14]. The investigators also assessed the causal relationship of any adverse event to the study medication by using the ADR probability scale which was generated from Naranjo *et al* [15].

All routine laboratory variables (hematology and serum chemistry) were recorded at each visit. Every assessment was compared with baseline values.

Allocation of patients

This was a prospective, randomized, open-label and active-controlled study. Doctors prescribed the test drugs according to the random number sheet, which is generated by SAS. Eligible patients were allocated the next sequential patient number based on preprinted numbers on the study drug labels.

Patients with symptomatic GERD will underwent endoscopy. Randomization occurred within 7 days of the baseline endoscopy. Patients with ENRD were enrolled into the study and then randomly allocated to treat with

Table 1 Demographics between groups- (safety populations)

	All (134)	Topaal (69)	Nacid (65)	*P*
Sex				
Male:Female	36:98	25:44	11:54	0.0186[1]
Age (yr)	41.9 ± 13.4	41.6 ± 14.8	42.4 ± 11.8	0.7300[2]
Body mass index	23.1 ± 3.8	23.4 ± 3.8	22.8±3.8	0.3547[2]
(kg/m^2)	75.2 ± 6.8	75.3 ± 7.0	75.1±6.7	0.9075[2]
Pulse rate (bpm)	117.0 ± 14.1	119.7 ± 14.2	114.1 ± 13.5	0.0211[2]
SBP (mmHg)	75.0 ± 1.01	76.6 ± 9.5	73.2 ± 10.6	0.0516[2]
DBP (mmHg)				0.9658[3]
History of heartburn (%)	75 (56.4)	39 (56.5)	36 (56.3)	
6–12 mo	45 (33.8)	23 (33.3)	22 (34.4)	
1–5 yr	13 (9.8)	7 (10.1)	6 (9.4)	
>Section 15 yr				0.4011[1]
Smoking (%)	14 (10.5)	9 (13.0)	5 (7.7)	
Yes	120 (89.5)	60 (87.0)	60 (92.3)	
No				1.0000[1]
Drinking (%)	5 (3.7)	3 (4.4)	2 (3.1)	
Yes	129 (96.3)	66 (95.6)	63 (96.9)	
No				0.0881[1]
Coffee (%)	28 (20.9)	10 (14.5)	18 (27.7)	
Yes	106 (79.1)	59 (85.5)	47 (72.3)	
No				
VAS (cm)				0.8388[2]
Heartburn	7.5 ± 3.4	7.6 ± 3.0	7.4 ± 3.7	
Regurgitation	8.2 ± 3.0	8.1 ± 3.1	8.2 ± 2.9	
Dysphagia	1.7 ± 3.2	1.4 ± 3.0	2.0 ± 3.5	
Epigastric pain	5.4 ± 4.1	4.6 ± 4.1	6.2 ± 3.9	
Nausea	3.5 ± 3.8	3.3 ± 3.8	3.7 ± 3.8	
Vomiting	1.1 ± 2.7	1.2 ± 2.7	1.0 ± 2.6	
Belching	6.2 ± 4.0	5.9 ± 3.9	6.4 ± 4.1	
Quality of life (%)				0.8489[3]
Mild symptoms	4 (3.0)	3 (4.4)	1 (1.5)	
Moderate w/o interfering	33 (24.6)	15 (21.7)	18 (27.7)	
Moderate with interfering	66 (49.3)	36 (52.2)	30 (46.2)	
Symptom as bad/worse	31 (23.1)	15 (21.7)	16 (24.6)	

[1]Fisher's exact test; [2]ANOVA model; [3]Mantel–Henszel test; VAS: visual analog scale.

either Topaal® or Nacid®. No other medical treatment for reflux esophagitis such as other antacid, metoclopramide, cisapride, H_2 blocker and PPI was allowed.

The duration of treatment was 6 wk and each patient returned for assessment every 3 wk. At randomization (visit 1) and visit 2, all patients will received one plastic bottle of study medication. Every bottle contains 200 chewable tablets of Topaal® or Nacid®, including 4-day supply in the event of a delay in the scheduled appointment. The dosage of Topaal® and Nacid® is two chewable tablets t.i.d. and h.s.

At the initial assessment, the medical history, past history, life-style (smoking, drinking, coffee consumption) and baseline demographic data, hematologic and serum biochemistry were recorded. A physical examination will be by performed.

Treatment medications

Drug information: Topaal® contains 200 mg alginic acid, 30 mg colloidal aluminum hydroxide and 40 mg magnesium hydrocarbonate per tablet. Nacid® contains 500 mg $Mg_6Al_2(OH)_{16}CO_3$ $4H_2O$ per tablet.

Topaal® is an alginate-based raft-forming formulation[16] comprising alginic acid and antacid. In the presence of gastric acid, alginates precipitate and form a gel[16]. The bicarbonate inside is converted to carbon dioxide, which is then entrapped within the gel precipitate, converting it into foams floating on the surface of the gastric contents, much like a raft on water. The "raft" can act as a physical barrier to reduce reflux episodes.

Table 2 The change in the VAS of heartburn (ITT population)

Unit: cm	Topaal Mean (SE)	Nacid Mean (SE)	Difference	*P*
Visit 1/baseline				
Observed data	7.52 (0.42)	7.43 (0.45)	0.09	0.8774
Visit 3/week 7				
Observed data	1.15 (0.30)	3.06 (0.34)	–1.91	<0.0001
Change from baseline	–6.39 (0.28)	–4.44 (0.31)	–1.95	<0.0001

ANCOVA model: Dependent variable = (baseline value) + treatment.

Statistical analysis

Comparisons of frequency employed the χ^2 test of Fisher's exact test, the test of linear trend for the ordered variables, Student's *t*-test of non-parametric Wilcoxon test, and variance analysis for the repeated VAS, all adjusted to the initial values. Two-sample *t* test was used to compare the continuous data such as height, weight, age, the number of heartburn, the number of sleeping disturbance etc., and the 95%CI for the difference were calculated. Statistical significance was assessed at the 5% level. The principle analysis was carried out on an ITT basis.

A listing of patients with withdrawal as well as those with premature termination of study drugs, along with the date and reasons for the termination was provided.

RESULTS

Disposition of patients

The trial was conducted from June 10, 2003 (first patient screened) to December 24, 2004 (last patient completed). Figure 1 gives an overview of the disposition of the patient population for the treatment phases. A total of 134 patients were randomized to the treatment phase; 69 patients were randomized to Topaal® and 65 were randomized to Nacid®. Of the 134 randomized patients, a total of 112 patients completed the study.

The safety population included 134 patients (69 in the Topaal® group, 65 in the Nacid® group) who received at least one dose of study medication after randomization. The ITT population consisted of all randomized subjects who received at least one dose of study treatment and who had a baseline and at least one post-baseline efficacy assessment. Three of the sixty-five patients in Nacid® group dropped out for safety/efficacy reason; six in Nacid® group and four in the Topaal® group dropped out for administrative reasons (e.g., lost to follow-up, moved out of the area, etc.). Therefore, 65 patients in the Topaal® group and 56 patients in the Nacid® group were included in the efficacy population.

Table 3 Summary of the changes in the VAS at 3 weeks (ITT population)

Unit: cm	Topaal (65)		Nacid (56)		Difference	*P*-value
	Mean	(SE)	Mean	(SE)		
Heartburn						
Observed data	2.98	0.37	4.43	0.40	–1.44	0.0091
Change from baseline	–4.52	0.31	–3.03	0.34	–1.49	0.0016
Regurgitation						
Observed data	3.52	0.37	5.32	0.40	–1.80	0.0012
Change from baseline	–4.55	0.33	–2.84	0.36	–1.72	0.0006
Dysphagia						
Observed data	0.65	0.27	1.25	0.29	–0.60	0.1326
Change from baseline	–0.97	0.19	–0.68	0.20	–0.28	0.3138
Epigastric pain						
Observed data	2.08	0.42	3.88	0.45	–1.80	0.0039
Change from baseline	–2.93	0.33	–2.10	0.36	–0.84	0.0925
Nausea						
Observed data	1.25	0.32	1.89	0.34	–0.65	0.1705
Change from baseline	–2.10	0.25	–1.51	0.27	–0.59	0.1137
Vomiting						
Observed data	0.31	0.18	0.61	0.20	–0.30	0.2662
Change from baseline	–0.92	0.12	–0.55	0.13	–0.37	0.0373
Belching						
Observed data	2.54	0.40	5.14	0.43	–2.60	<0.0001
Change from baseline	–3.64	0.32	–1.40	0.34	–2.23	<0.0001

ANCOVA model: Dependent variable = (baseline value) + treatment.

Demographics and baseline characteristics

Demographic characteristics of randomized subjects in each treatment group are summarized in Table 1. The mean age of the patients in both treatment groups was about 42 years. There were more female than male patients participating in the study in both groups. Although there were significant differences in gender ratio between groups (36.23% male in the Topaal® group *vs* 16.92% male in the Nacid® group), no evidence showed that the gender difference would have an effect on the pharmacokinetics or efficacy of study medications. The body mass index (BMI), pulse rate and diastolic blood pressure were all comparable between treatment groups. Although the patients in the Topaal® group seem to have higher systolic blood pressure than the patients in the Nacid® group (119.7 ± 14.2 mmHg in the Topaal® group *vs* 114.1 ± 13.5 mmHg in the Nacid® group), those values were all clinically considered to be within normal limits.

Table 1 also summarizes patients' baseline symptom analysis that included VAS, the reflux symptoms from doctor's point of view, and quality of life from doctor' s point of view. More than 56% of the patients in each group had suffered from the reflux symptom of heartburn for at least 6-12 mo. No significant difference in other characteristics including life-style was noted. The baseline characteristics of all randomized subjects were compatible between the two treatment groups, and no statistically significant difference between treatment groups was detected, except for the mean VAS of epigastric pain ($P = 0.0218$).

EFFICACY EVALUATION

The change in the severity of the heartburn as evaluated using a VAS at 6 wk, using LOCF data from ITT population, was the primary efficacy endpoint of this trial. At baseline, the mean VAS of heartburn was 7.52 cm in the Topaal® group and 7.43 cm in the Nacid® group. At the end of the 6-week treatment period, the mean VAS of heartburn was 1.20 cm in the Topaal® group and 3.36 cm in the Nacid® group. Overall, the mean change from baseline in VAS of heartburn over the 6-weeks treatment was -6.29 cm (-6.85 to -5.74 cm) in the Topaal® group, and -4.11 cm in the Nacid® group (-4.71 to -3.51 cm). The mean difference between the two treatment groups was -2.19 cm (-3.01 to -1.37 cm) and the difference was statistically significant ($P < 0.0001$) as detailed in Table 2.

The first secondary endpoint was to compare the change of VAS at 3 wk (Table 3). There were significant differences in the mean change of VAS for heartburn ($P = 0.0016$), regurgitation ($P = 0.0006$), vomiting ($P = 0.0373$) and belching ($P < 0.0001$). No significant differences were found in the mean change of VAS for nausea, epigastric pain, and dysphagia. The results suggested that Topaal® is superior to Nacid® for the improvement of the predominant symptoms of ENRD such as heartburn and regurgitation.

The second secondary endpoint was to compare the frequency of heartburn, regurgitation, pain, and sleep disturbance according to patient's diary every week during treatment period (Figure 2). Patients taking Topaal® showed consistently and significantly lower frequency of heartburn than those who were taking Nacid® throughout the entire 6-week period ($P = 0.0015$). Patients taking Topaal® also had fewer episodes of pain than those who were taking Nacid® from the 4th week to the end of the study. Both Topaal® and Nacid® decreased the frequency of regurgitation during the study period compared to baseline, but the difference between treatments was not significant. No difference was observed for the change in the frequency of sleeping disturbance.

The third and fourth secondary endpoints were to

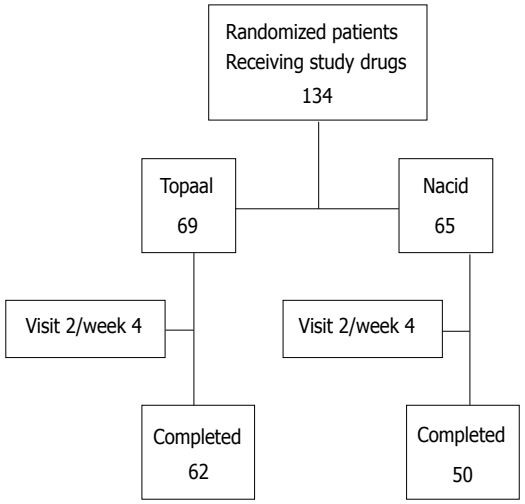

Figure 1 Disposition of patients in the treatment phase.

compare the change in the severity of the reflux symptoms (Table 4) and the quality of life from doctor's point of view (Table 5). There were no significant differences in the severity of the reflux symptoms and the quality of life from doctor's point of view at baseline. At the end of the 6-week treatment period, patients who took Topaal® showed better improvement in the severity of heartburn ($P=0.0020$), regurgitation ($P=0.0081$), vomiting ($P=0.0182$), and belching ($P=0.0018$) than those who took Nacid®. There were no significant differences in the improvement of dysphagia ($P=0.7551$), epigastric pain ($P=0.2648$), and nausea ($P=0.0577$).

For the quality of life from doctor's point of view, there were no significant differences at baseline. About 50% of the patients in each group suffered from moderate reflux symptoms interfering with the quality of life before the treatment. At the end of treatment period, patients who took Topaal® had greater alleviations of symptoms ($P<0.0001$) and greater reduction of symptom score from baseline ($P<0.0001$) than those who took Nacid®. The results suggested that Topaal is more efficient than Nacid for improving the quality of life of ENRD patients.

SAFETY EVALUATION

The ADRs of Topaal® and Nacid® were the safety end-points of this trial. In this analysis, data from the Topaal® group were compared with data from Nacid® group.

Adverse events which occurred during the course of the study were recorded. Seven patients had at least one adverse event after entering the study, three (4.35%) patients in the Topaal® group and four (6.15%) patients in the Nacid® group. The most commonly adverse events reported by Topaal®-treated patients were constipation (2 patients, 2.90%). For Nacid® group, two patients (3.08%) experienced diarrhea and two patients (3.08%) reported constipation. No statistical significant difference was found between the two groups in the incidence of adverse event. All of these adverse events were resolved and graded as mild or moderate in severity. None of them was considered by the investigators to be definitely related to treatment. No dosage modification of medication was applied for the three adverse events in Topaal®-treated patients, whereas two of the four adverse events in Nacid® -treated patients led to dose reduction of medication. No serious adverse event was reported during the entire study period. The results suggest that Topaal® had a similar safety profile as Nacid®.

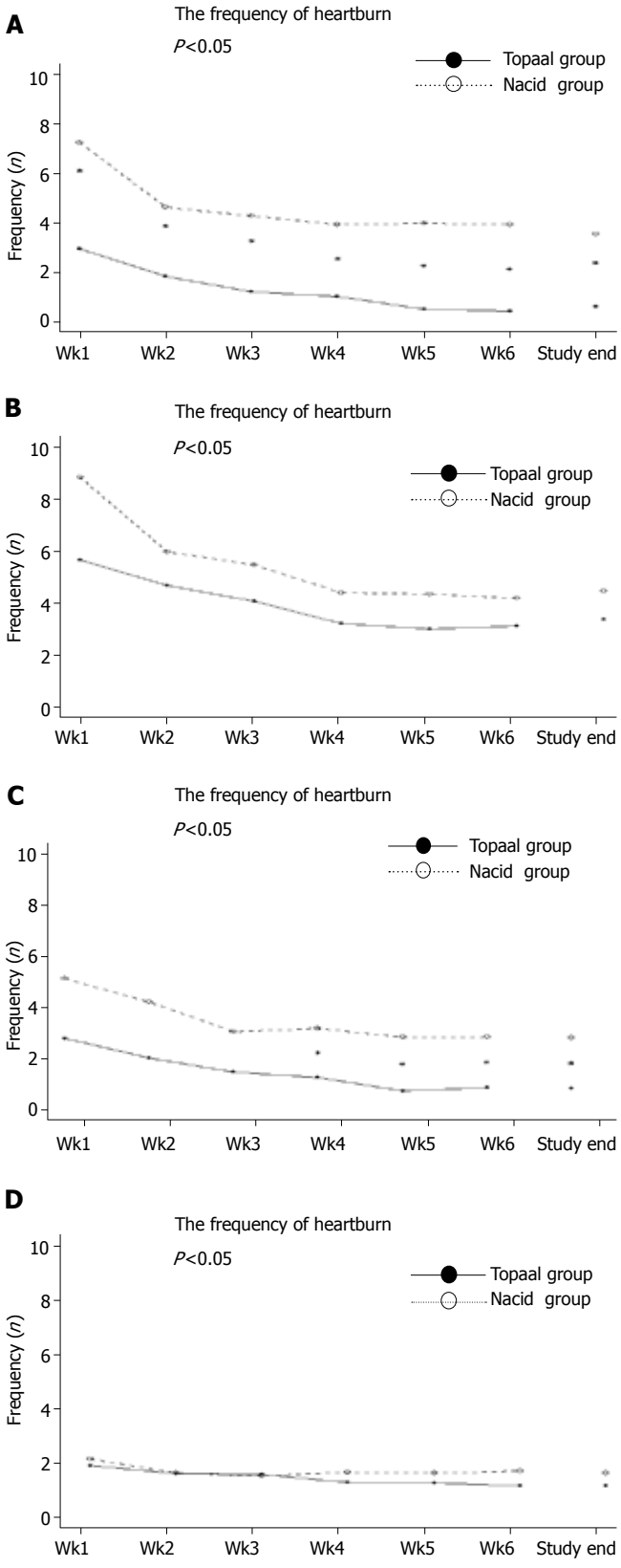

Figure 2 The frequency of heartburn, regurgitation, pain and sleep disturbance.

Table 4 The change in the severity of the reflux symptom

Population: intent-to-treat	Overall (%)	Topaal (%)	Nacid (%)	*P*-value
Heartburn				0.0019[1]
Change from baseline				
–1	4 (3.3)	1 (1.5)	3 (5.4)	
0	28 (23.1)	8 (12.3)	20 (35.7)	
1	42 (34.7)	21 (32.3)	21 (37.5)	
2	30 (24.8)	23 (35.4)	7 (12.5)	
3	17 (14.1)	12 (18.5)	5 (8.9)	
Regurgitation				0.0625[1]
Change from baseline				
–1	2 (1.6)	1 (1.5)	1 (1.8)	
0	30 (24.8)	12 (18.5)	18 (32.1)	
1	42 (34.7)	21 (32.3)	21 (37.5)	
2	36 (29.8)	21 (32.3)	15 (26.8)	
3	11 (9.1)	10 (15.4)	1 (1.8)	
Dysphagia				0.2559[1]
Change from baseline				
–1	4 (3.3)	2 (3.1)	2 (3.8)	
0	90 (74.4)	50 (79.6)	40 (71.4)	
1	16 (13.2)	5 (7.5)	11 (19.6)	
2	10 (8.3)	7 (10.8)	3 (5.3)	
3	1 (0.8)	1 (1.5)	0 (0.0)	
Epigastric pain				0.2517[1]
Change from baseline				
–2	3 (2.5)	1 (1.5)	2 (3.8)	
–1	4 (3.3)	3 (4.6)	1 (1.8)	
0	57 (47.1)	30 (46.2)	27 (48.2)	
1	33 (27.3)	15 (23.1)	18 (32.1)	
2	19 (15.7)	11 (16.9)	8 (14.3)	
3	5 (4.1)	5 (7.7)	0 (0.0)	
Nausea				0.2210[1]
Change from baseline				
–1	1 (0.8)	0 (0.00%)	1 (1.8)	
0	70 (57.9)	33 (50.8)	37 (66.1)	
1	23 (19.0)	15 (23.1)	8 (14.3)	
2	25 (20.7)	15 (23.1)	10 (17.9)	
3	2 (1.7)	2 (3.1)	0 (0.0)	
Vomiting				0.1888[1]
Change from baseline				
–1	1 (0.8)	0 (0.0)	1 (1.8)	
0	100 (82.6)	50 (79.6)	50 (81.3)	
1	9 (7.4)	6 (9.2)	3 (5.4)	
2	9 (7.4)	7 (10.8)	2 (3.6)	
3	2 (1.7)	2 (3.1)	0 (0.0)	
Belching				0.0366[1]
Change from baseline				
–1	8 (6.6)	2 (3.1)	6 (10.7)	
0	48 (39.7)	21 (32.3)	27 (48.2)	
1	42 (34.7)	25 (38.5)	17 (30.4)	
2	19 (15.7)	13 (20.0)	6 (10.7)	
3	4 (3.3)	4 (6.2)	0 (0.0)	

$\chi 2$ test.

DISCUSSION

The design of this study is based on symptomatic analysis. This is an original, though not blind, trial that compares the therapeutic effects of an alginate to an antacid on ENRD patients. For the best interest of the patients, a placebo-controlled study was not considered in our design. The investigators' and the patients' assessments of efficacy over the study period are similar and in favor of the alginate. After 6-week treatment, Topaal® was found to be more effective than Nacid® in reducing VAS of five symptoms including heartburn, regurgitation, vomiting, and belching in patients with ERND. Patients in Topaal® group had fewer episodes of heartburn and epigastric pain at the end of treatment. Also, from doctor's point of view, Topaal® is more effective than Nacid® in reducing symptoms of reflux and in improving the quality of life in patients with ERND. Our result is consistent with that of other trials that compared the effects of alginate-based formulation with antacids on patients with reflux esophagitis[17] and volunteers[18],though their patient groups were more heterogenous.

According to our data, the symptom-relieving effect in the Topaal® group was faster than that of Nacid®. Patients

Table 5 Summary of the change of quality of life

ITT population	Overall (%)	Topaal (%)	Nacid (%)	*P*-value
Visit 1/baseline				
Observed data				0.9298
No symptom	0 (0.0)	0 (0.0)	0 (0.0)	
Moderate symptom	4 (3.3)	3 (4.6)	1 (1.8)	
Moderate symptom w/o interfering	29 (24.0)	13 (20.0)	16 (28.6)	
Moderate symptom with interfering	61 (50.4)	35 (53.9)	26 (46.4)	
Symptom as bad/worse	27 (22.3)	14 (21.5)	13 (23.2)	
Study end				
Observed data				<0.0001
No symptom	24 (19.8)	19 (29.2)	5 (8.9)	
Moderate symptom	51 (42.2)	34 (52.3)	17 (30.4)	
Moderate symptom w/o interfering	27 (22.3)	8 (12.3)	19 (33.9)	
Moderate symptom with interfering	15 (12.4)	3 (4.6)	12 (21.4)	
Symptom as bad/worse	4 (3.3)	1 (1.5)	3 (5.4)	
Decrease from baseline				<0.0001
–1	1 (0.8)	0 (0.0)	1 (1.8)	
0	24 (19.8)	7 (10.8)	17 (30.4)	
1	36 (29.8)	16 (24.6)	20 (35.7)	
2	36 (29.8)	22 (33.9)	14 (25.0)	
3	16 (13.2)	13 (20.0)	3 (5.4)	
4	8 (6.6)	7 (10.8)	1 (1.8)	

[1]Mantel–Henszel $\chi 2$ test.

taking Topaal® had major reduction in VAS scores of heartburn and regurgitation from the 1st week of study, and patients taking Nacid® did not feel the difference in symptoms until the 2nd week of study. Alginic acid is different from antacids in the mechanism of efficacy. The alginic acid is converted to sodium alginate by the small amount of antacid contained in the formulation. This salt floats atop of the esophagogastric junction and provides a barrier when reflux occurs[16]. The physical property of Topaal® might explain why it worked faster and better than Nacid® in relieving the symptoms of reflux. On the other hand, our results did not show differences in improving the sleep disturbance in patients of both groups. We did not assess the incidence of night refluxer or asthma in the enrolled patients who might have greater disturbance in sleeping. We deduced that the incidence of night refluxer maybe low because both study groups had low VAS score of sleep disturbance at the baseline. Though alginate products have been shown to be effective in relieving symptoms of reflux, it has not been proved that the alginates promote healing of erosive esophagitis. In this way, alginate-based formulation is a reasonable option for patients with ENRD.

Goves *et al*[19] compared the effectiveness of PPI, with alginates for heartburn relief in dyspeptic patients. This design showed that PPI was superior to alginates in relieving the heartburn of patients at 4 weeks of treatment. This is not surprising for they included patients with severe esophagitis, and omeprazole had the dramatic antisecretory actions. However, considering ENRD is a protracted disease, alginate is a better option for long-term and on-demand use in these patients for cost-effectiveness. For adverse events, Topaal® group had a compatible safety profile as Nacid® group. Alginates have been shown to be safe when used in pregnant women[20] and children[21] In our patients treated with Topaal®, the most commonly reported adverse events were constipation (2.90%).

In conclusion, Topaal® is more effective than Nacid® in symptomatic control of patient with ENRD. For the safety measures, the results supported that Topaal® group had a compatible safety profile as Nacid® group.

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S- Editor Guo SY **L- Editor** Elsevier HK **E- Editor** Wu M

PO Box 2345, Beijing 100023, China
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World J Gastroenterol 2006 February 7; 12(5):755-759
World Journal of Gastroenterology ISSN 1007-9327

CLINICAL RESEARCH

Clinical application of self-expanding metallic stent in the management of acute left-sided colorectal malignant obstruction

You-Ben Fan, Ying-Sheng Cheng, Ni-Wei Chen, Hui-Min Xu, Zhe Yang, Yue Wang, Yu-Yao Huang, Qi Zheng

You-Ben Fan, Zhe Yang, Yue Wang, Yu-Yao Huang, Qi Zheng, Department of Surgery, Sixth People's Hospital, Shanghai Jiaotong University, Shanghai 200233, China
Ying-Sheng Cheng, Department of Radiology, Sixth People's Hospital, Shanghai Jiaotong University, Shanghai 200233, China
Ni-Wei Chen, Hui-Min Xu, Department of Surgery, Sixth People's Hospital, Shanghai Jiaotong University, Shanghai 200233, China
Correspondence to: Dr. Qi Zheng, Department of Surgery, Sixth People's Hospital, Shanghai Jiaotong University, Shanghai 200233, China. sshosp@public.sta.net.cn
Telephone: +86-21-64368920 Fax: +86-21-64701361
Received: 2005-07-05 Accepted:2005-09-19

Key words: SEMS; Acute left-sided colorectal malignant obstruction

Fan YB, Cheng YS, Chen NW, Xu HM, Yang Z, Wang Y, Huang YY, Zheng Q. Clinical application of self-expanding metallic stent in the management of acute left-sided colorectal malignant obstruction. *World J Gastroenterol* 2006; 12(5): 755-759

http://www.wjgnet.com/1007-9327/12/755.asp

Abstract

AIM: To summarize our experience with the application of self-expanding metallic stent (SEMS) in the management of acute left-sided colorectal malignant obstruction.

METHODS: A retrospective chart review of all patients undergoing placement of SEMS between April 2000 and January 2004 was performed.

RESULTS: Insertion of SEMS was attempted in 26 patients under fluoroscopic guidance with occasional endoscopic assistance. The sites of lesions were located in splenic flexure of two patients, left colon of seven patients, sigmoid colon of eight patients and rectum of nine patients. The intended uses of SEMS were for palliation in 7 patients and as a bridge to elective surgery in 19 patients. In the latter group, placement of SEMS allowed for preoperative systemic and bowel preparation and the following one-stage anastomosis. Successful stent placement was achieved in 22 (85%) of the 26 patients. The clinical bowel obstruction resolved 24 hours after successful stent placement in 21 (95%) patients. Three SEMS-related minor complications occurred, two stents migrated and one caused anal pain.

CONCLUSION: SEMS represents an effective and safe tool in the management of acute malignant colorectal obstruction. As a bridge to surgery, SEMS can provide time for systematic support and bowel preparation and obviate the need for fecal diversion or on-table lavage. As a palliative measure, SEMS can eliminate the need for emergent colostomy.

INTRODUCTION

Acute left-sided colorectal malignant obstruction represents a frequent surgical emergency. It is the first symptom in approximately 15-20% of colorectal cancers[1-2]. Single-stage surgery (subtotal colectomy with primary anastomosis or partial colectomy with intraoperative lavage) appears to be gaining acceptance as a good therapeutic option in such cases. However, this treatment option is not feasible in all patients, and it is often necessary to use a two-stage procedure (Hartmann's procedure) or to perform permanent colostomy in advanced stages of the disease[3]. Smothers *et al.*[4] found that the overall surgical morbidity and mortality are significantly higher in patients with colon cancer undergoing emergency surgery than in those undergoing selective surgery. Some other factors such as advanced age as well as stage and volume of the disease are closely correlated with emergency surgery, and emergency surgery has a strong independent negative influence on immediate surgical morbidity and mortality. If emergency surgery could be transferred to elective surgery after a simple effective therapy, complications would be significantly decreased in patients with acute left-sided colorectal malignant obstruction.

SEMS[5-7] has been used to relieve obstruction in different situations. Application of metallic stents in the treatment of acute malignant colonic obstruction was first reported by Dohmoto[8] in 1991 and has become a promising treatment option, though the number of reported cases is only about 600 in the world[9,10]. Placement of SEMS across the obstructing lesion can relieve obstructive symptoms and avoid emergency surgery. In most cases, a radical colectomy with primary

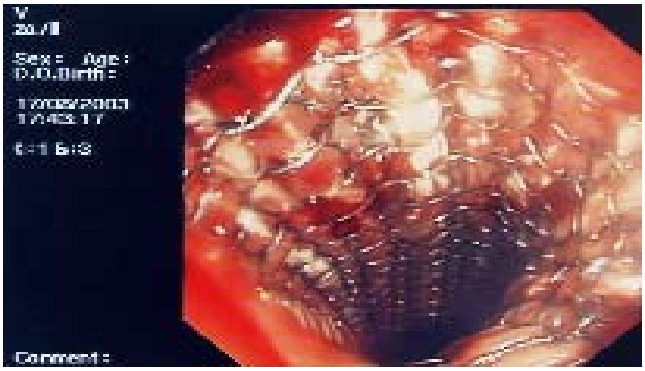

Figure 1 Endoscopic view of partially expanded stent immediately after the stent placement.

anastomosis can be subsequently performed in 6-9 days. Colostomy is the only surgical option, and implantation of stent is the best final palliative treatment in some cases with advanced stages of the disease[11-23]. This report describes the experience with the application of SEMS in the management of acute malignant left-sided colorectal obstruction.

MATERIALS AND METHODS

Patients

All patients had clinical symptoms and signs of acute bowel obstruction. From April 2000 to January 2004, a total of 26 patients (16 men, 10 women; mean age: 63.23 years; range: 38-85 years) were selected to receive decompressive therapy by implanting SEMS. Patient selection criteria included the site of obstruction from the middle rectum to the descending colon and absence of bowel necrosis and perforation. Patients with more proximal obstructions were excluded on the basis of poor site accessibility and low emergency surgery risk. Age, general condition, and tumor stage were not used as exclusion criteria.

All patients received plain radiography of the abdomen and emergency contrast-emphasized computed tomography. The sites of obstruction were located in rectum of nine patients, sigmoid colon of eight patients, descending colon of seven patients and splenic flexure of two patients. Informed consent was obtained from all patients or from their relatives after the risks and benefits of treatment were fully explained.

Procedure

All procedures were performed at the interventional radiology by endoscopy (Figure 1) and fluoroscopy (Figure 2). Neither analgesia nor sedation was administered during the procedure. There was no routine administration of antibiotics.

Once the guide wire passed through the lesion, the catheter was advanced to the most proximal point, and contrast medium was injected to evaluate tumor length and exclude the possibility of perforation. The superstiff 0.038-inch guide wire was then inserted, and a stent was placed using the delivery system under fluoroscopic control (Figure 2).

Several silver clips were clipped around the lesion during colonoscopy. Then the guide wire inserted through the lesion under direct vision was confirmed to arrive at the splenic flexure under fluoroscopic monitor.

The stent used in all patients was esophageal endoprosthesis (Nanjing Microinvasive Corporation, China), 20 or 25 mm in diameter and 70-100 mm in length. After the placement of the stent, a catheter was inserted over the superstiff guide wire until the tip projected a few centimeters beyond the stent. Contrast medium was injected to ensure that the stent was placed properly across the tumor and to assess the stent patency.

When one stent was not long enough to span the lesion, a second stent was inserted to overlap the first. Balloon dilation was not performed in any of the patients either before or after the implantation of the stent.

Patients were transferred to the surgical ward for observation after the procedure. Abdominal plain radiography was performed to assess the stent expansion and patency as well as possible complications in 24 h. Once symptoms of obstruction remitted, further tumor staging and clinical evaluation were carried out to determine which patients should receive selective surgical therapy and which patients should undergo stent implantation as the definitive palliative treatment.

RESULTS

Successful stent placement was achieved in 22 (85%) of the 26 patients. The procedure could not be completed in four patients. The sites of lesions were located in splenic flexure of one patient, descending colon of two patients and sigmoid colon of one patient. The successful rate was 50% in splenic flexure (1/2), 71% in the descending colon (5/7), 89% in sigmoid colon (7/8), 100% in rectum (9/9), respectively. The obstruction could not be relieved in three patients even though the guide wire and catheter were advanced successfully to the edge of the lesion. Though the guide wire was successfully advanced proximally, the stent could not be implanted in the remaining patient because of the tortuosity of the rectosigmoid region. Emergency laparotomy was performed in these four patients, and colostomy or Hartmann's procedure was completed.

A total of 25 self-expanding metallic stents were implanted. One stent was placed in 19 patients and two stents in 3 patients. The mean fluoroscopy time of the procedure was 46 min (range: 15-120 min).

The clinical bowel obstruction resolved in 21 patients (21/22, 95%) within 24 h after successful stent placement, with expansion and patency observable in all radiographic films. Clinical failure occurred in one patient and symptom of incomplete obstruction persisted for 24 h following the successful placement of SEMS. Emergency laparotomy revealed vegetable residues proximal to the lesion and Hartmann's procedure was performed. Complications of SEMS placement occurred in three patients. Stent migration occurred in two patients after obstruction disappeared but no re-obstruction was found after stent migration. Mild anal pain persisted for one month in the remaining one because the stent was close to the anus. The

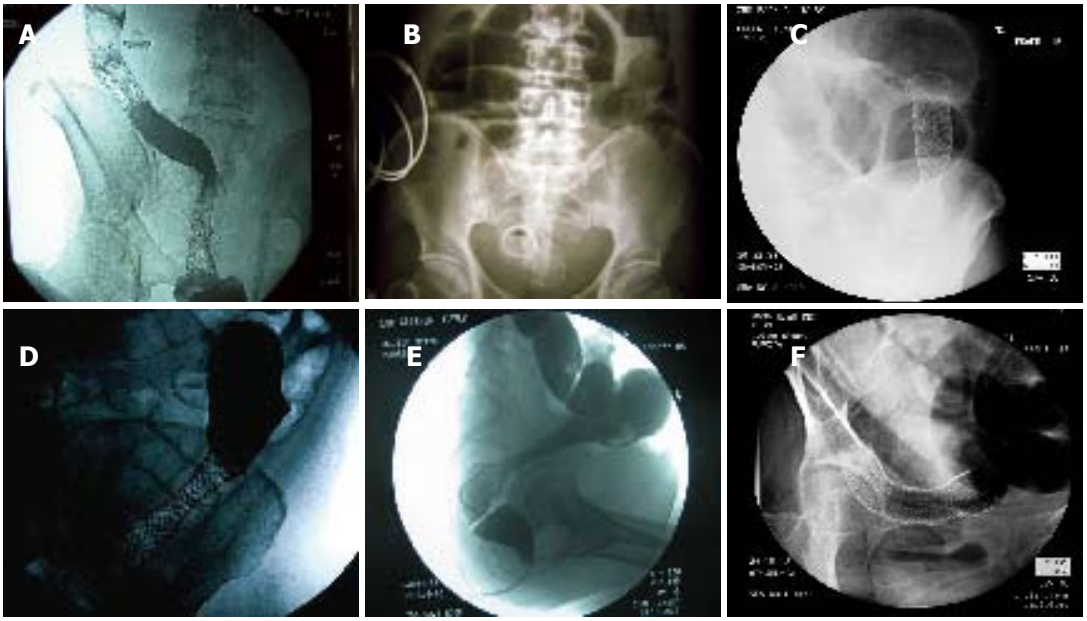

Figure 2 Good patency of the second stent coaxially placed covering the entire malignant stricture (**A**), a patent and nearly full in the upper rectum (**B**), SEMS with a waist shape in descending colon cancer (**C**), SEMS bridging the descending colon (**D**), a patent stent after SEMS placement in the rectum (**E**) and a bent stent in sigmoid cancer (**B**).

pain resolved after analgesic was administered (Table 1).

Surgery

Patients with operable colorectal carcinoma were considered as candidates for selective surgery. The mean time between stent placement and surgery was 8 ± 3 days (range: 5-11 days). These patients underwent adequate bowel preparation and adjuvant systematic therapy. The stent did not interfere with or prolong the surgery in any of these patients. The stents were fully expanded as well as in good position and afforded sufficient tumoral coverage (except for two patients with stent migration) and adequate cleaning of the colon in all patients during surgical exploration. Left colectomy, sigmoidectomy, and (low) anterior resection were performed in five patients, respectively.

Palliation

Stent placement was considered as the definitive palliative treatment of colonic obstruction in seven patients. Lesions were located at the sigmoid of three patients and rectum of four patients. Two patients suffered from gastric carcinoma with sigmoid or rectal seeding and three patients had primary sigmoid or rectal carcinomas with inoperable liver metastases. One patient with sigmoid carcinoma failed in stenting and underwent Hartmann's procedure. The other six patients received adjuvant chemotherapy, or radiation therapy, or both.

DISCUSSION

Usually, it is not difficult to detect acute left-sided colorectal obstruction by abdominal radiography. CT can help find the site and etiology of colon stenosis and the nature of acute colonic occlusion. Barium enema for the diagnosis and treatment of colonic obstruction is not recommended when doctors plan to implant SEMS[12].

SEMS for colorectal obstruction was performed in the past either under radiological or endoscopic control. We introduced the guide wire under radiological and endoscopic guidance in most patients. The guide wire could be inserted under direct vision, radiation exposure could be reduced, and biopsies for histology could be taken during the procedure.

Endoscopic placement of SEMS is advantageous over radiological placement because the accessibility to colorectal tumor sites is greater and stents can directly pass through the working channel of the endoscope[13]. These advantages are especially obvious when the obstruction is proximal to the rectosigmoid. De Gregorio *et al.*[14] have reported that the guide wire cannot be progressed under fluoroscopic guidance but reaches the neoplastic stricture under endoscopic guidance. Interventional radiologists are much more experienced in passing the guide wires through obstructive lesions and deploying SEMS[12,13]. In our study, fluoroscopy in combination with endoscopy improved SEMS deployment. Even then, stent was still rather difficult to implant in the descending colon and splenic flexure compared with that in the rectum and sigmoid (6/9, 67%; 16/17, 94%; $P < 0.05$).

We treated 26 patients with acute malignant colorectal obstruction with SEMS placement with a successful rate of 85% (22/26). Only one patient (5%, 1/22) failed to relieve obstruction due to vegetable residues. If we had used colonoscopy to detect and get out the vegetable residues, the patient could have the obstruction relieved and emergency colostomy could not have been performed (Hartmann's procedure). After successful stent placement and proper bowel preparation, 15 patients achieved elective radical colorectal surgery and anastomosis without severe complication. Six patients with inoperable malignancy obviated emergency colostomy after successful stent placement. Although 85% of successful stent placement was a little lower than 90-95% of some foreign reports, we believe that success rate of SEMS placement would be further increased with more refined Chinese instruments and improved manipulation skill.

It was reported that SEMS placement can be used as a palliative treatment for colonic obstruction. In Cristina' s study, in 17 (of 43) patients with Dukes D tumor (40%),

Table 1 Details of patients with colorectal obstruction after SEMS placement

Patient No. (gender/ age yr)	Diagnosis	Site of obstruction	Nature of treatment	Stent insertion	Stent number	Subsequent treatment	Complication
1 (F/45)	Primary Ca descending	Splenic flexture	Temporizing	Failed	–	Colostomy	No
2 (F/48)	Primary Ca rectum, ascites, liver metastasis	Middle rectum	Palliative	Successful	2	Chemo-therapy	No
3 (F/73)	Primary Ca rectum	Middle rectum	Temporizing	Successful	1	LAR	No
4 (F/38)	Primary Ca stomach	Sigmoid	Palliative	Successful	2	Chemo-radio therapy	No
5 (M/61)	Primary Ca descending	Descending upper	Temporizing	Successful	2	Left colectomy	No
6 (M/83)	Primary Ca rectum	Rectum	Temporizing	Successful	1	AR	No
7 (M/72)	Primary Ca descending	Descending	Temporizing	Failed	–	Colostomy	No
8 (M/76)	Primary Ca sigmoid, liver metastasis	Sigmoid	Palliative	Successful	1	Chemo-therapy	No
9 (F/44)	Primary Ca rectum	Upper and middle rectum	Temporizing	Successful	1	LAR	No
10 (F/74)	Primary Ca sigmoid	Sigmoid	Palliative	Failed	–	Hartmann	No
11 (M/45)	Primary Ca descending	Descending	Temporizing	Successful	1	Left colectomy	Migration
12 (F/70)	Primary Ca rectum	Upper rectum	Temporizing	Successful	1	AR	No
13 (M/48)	Recurrent Ca rectum	Upper rectum	Palliative	Successful	1	Chemo-radio therapy	Anal pain
14 (M/72)	Primary Ca descending	Splenic flexture	Temporizing	Successful	1	Left colectomy	No
15 (F/63)	Primary Ca sigmoid	Sigmoid	Temporizing	Successful	1	Sigmoidectomy	Migration
16 (M/40)	Primary Ca rectum	Upper rectum	Temporizing	Successful	1	LAR	No
17 (M/72)	Primary Ca descending	Descending	Temporizing	Successful	1	Hartmann	Food residue obstruction
18 (F/79)	Primary Ca rectum Liver metastasis	Middle rectum	Palliative	Successful	1	Chemotherapy	No
19 (M/45)	Primary Ca descending	Descending	Temporizing	Successful	1	Left colectomy	No
20 (M/85)	Primary Ca descending	Descending	Temporizing	Successful	1	Left colectomy	No
21 (M/41)	Gastric Ca rectum seeding	Rectum	Palliative	Successful	1	Chemo-therapy	No
22 (M/69)	Primary Ca descending	Descending	Temporizing	Failed	–	Hartmann	No
23 (M/74)	Primary Ca sigmoid	Sigmoid	Temporizing	Successful	1	Sigmoidectomy	No
24 (M/85)	Primary Ca sigmoid	Sigmoid	Temporizing	Successful	1	Sigmoidectomy	No
25 (M/75)	Primary Ca sigmoid	Sigmoid	Temporizing	Successful	1	Sigmoidectomy	No
26 (F/67)	Primary Ca sigmoid	Sigmoid	Temporizing	Successful	1	Sigmoidectomy	No

the stent was considered as a definitive palliative treatment. In only one case the abdominal computed tomography scan failed to detect peritoneal implants of tumor, and the metastatic disease was identified at the time of laparotomy. As a result, 17 (94%) out of 18 unnecessary operations were avoided[24].

It was also reported that patients who were not operated have a shorter hospital stay and lower incidence of stoma after SEMS placement[25].

Binkert *et al.*[26] showed that SEMS placement can reduce the cost due to a shorter hospital stay and a lower complication rate. Morino *et al.*[27] have proposed a new minimally invasive therapeutic strategy for the management of acute malignant colonic obstructions: emergency endoscopic stenting followed by elective laparoscopic one-stage resection. They reported that four patients with malignant colonic obstruction had an immediate restored bowel functions after SEMS deployment within a period that varied from 4 to 5 days, then underwent a one-stage laparoscopic resection and were discharged 5-7 days later

without any complications.

Preoperative stent insertion for obstructive colorectal cancer can result in satisfactory postoperative outcome with good prognoses[28]. Perforation is the most severe complication of SEMS placement. Neoplastic strictures of the colon are generally soft. Acute neoplastic occlusion may be caused by neoplastic edema or stool residue or both. If the guide wire can be passed through neoplastic strictures, stent placement without balloon dilation should be easy for experienced physicians. The incidence of perforation was 2% (12/493) in the non-balloon dilatation group and 10% (10/105) in the dilatation group, suggesting that balloon dilation should not be recommended[9].

Some minor SEMS-placement-related complications including stent migration, mild bleeding, pain, and reobstruction may occur. Stent migration occurred in two patients of our group possibly due to abated edema and/or inadequate stent. Because of the limited cases and short survival time, we did not find reobstruction.

In conclusion, colorectal stenting procedure is effective and safe and can be used for acute left-sided colorectal malignant obstruction both as a temporary relief before selective resection and as a definitive treatment in palliative cases.

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S- Editor Wang XL **L- Editor** Elsevier HK **E- Editor** Li HY

PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

World J Gastroenterol 2006 February 7; 12(5):760-764
World Journal of Gastroenterology ISSN 1007-9327

CLINICAL RESEARCH

Objective assessment of the antispasmodic effect of Shakuyaku-kanzo-to (TJ-68), a Chinese herbal medicine, on the colonic wall by direct spraying during colonoscopy

Masato Ai, Taketo Yamaguchi, Takeo Odaka, Kanae Mitsuhashi, Tadayuki Shishido, Jun Yan, Ayaka Seza, Hiromitsu Saisho

Masato Ai, Taketo Yamaguchi, Takeo Odaka, Kanae Mitsuhashi, Tadayuki Shishido, Jun Yan, Ayaka Seza, Hiromitsu Saisho, Department of Medicine and Oncology, Graduate School of Medicine, Chiba University, Japan
Co-first-author: Masato Ai
Co-correspondent: Taketo Yamaguchi
Correspondence to: Taketo Yamaguchi, MD, Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan. yama.take@faculty.chiba-u.jp
Telephone: +81-43-226-2083 Fax: +81-43-226-2088
Received: 2005-07-14 Accepted: 2005-08-26

Abstract

AIM: To objectively evaluate the effect of TJ-68 on colonic spasms during colonoscopy.

METHODS: One hundred and one patients subjected to screening colonoscopy were randomly assigned to two groups: TJ-68 in 51 subjects and saline as the control in 50. The endoscope was inserted into the sigmoid colon, then a spastic region was identified and the tip of the colonoscope was positioned at a distance of about 10 mm from the spastic region. The endoscopic view was recorded before and after direct spraying of the TJ-68 solution or warm saline. The intraluminal area of the spastic region was serially measured using a computer image analyzer and expressed as pixel counts. The area under the curve (AUC) was calculated from the pixel curve. Statistical significance was assessed by Wilcoxon's test and Mann-Whitney *U* test.

RESULTS: The mean AUC of the spastic region before and after TJ-68 spraying was 29 128 and 121 943 pixels, respectively, while with saline, it was 31 635 pixels and 48 617 pixels, respectively. Thus, the AUC significantly increased after TJ-68 spraying compared with the spraying of saline ($P<0.001$).

CONCLUSION: Direct spraying of TJ-68 on the colonic mucosa suppressed colonic spasm and it may be useful during colonoscopy when anticholinergic agents are contraindicated.

Key words: Colonoscopy; Antispasmodic effect; Objective assessment

Ai M, Yamaguchi T, Odaka T, Mitsuhashi K, Shishido T, Yan J, Seza A, Saisho H. Objective assessment of the antispasmodic effect of Shakuyaku-kanzo-to (TJ-68), a Chinese herbal medicine, on the colonic wall by direct spraying during colonoscopy. *World J Gastroenterol* 2006; 12(5): 760-764

http://www.wjgnet.com/1007-9327/12/760.asp

INTRODUCTION

In colonoscopy, it is often difficult to insert the endoscope and observation tends to be hampered because of colonic spasms. In patients with colonic spasm sometimes excess air is used to get a good view of the colonic mucosa causing discomfort and pain in the patient, and resulting in an unsuccessful colonoscopy.

Hyoscine *n*-butyl bromide (Hyoscine) is usually and widely used as an antispasmodic agent in colonoscopy and it is administered by intramuscular or intravenous injection. However, systemic use of Hyoscine sometimes causes many adverse reactions; namely palpitation, dry mouth, urinary retention, orthostatic hypotension, anaphylactic reactions, and temporary visual impairment. Hyoscine, in addition, often causes serious complications in patients with heart disease and it is contraindicated in such cases. For this reason, a safer means to obtain the necessary antispasmodic effect is required for colonoscopy. In this regard, peppermint oil and warm water have been investigated by directly spraying them onto the colonic wall, however, there are some limitations for their clinical use[1,2].

Shakuyaku-kanzo-to (TJ-68), a Chinese herbal medicine, is traditionally used in China and Japan, to treat patients with convulsions, spasms, a pain in the limb, etc. Recently, the analgesic and sedative effects of TJ-68 as a premedication have been reported in endoscopy[3]. Although the antispasmodic effects of TJ-68 during barium enema examination was studied, the usefulness of TJ-68 as an antispasmodic agent in colonoscopy has not been examined[4].

The aim of this study was to objectively evaluate

Table 1 Background characteristics of the patients

	TJ-68	Control
No. of cases	51	50
Male/female	34/17	35/15
Age (yr) (mean±SD)	60.0±14.8	61.0±11.7
Indication of colonoscopy		
Polyp surveillance	15	13
Post EMR surveillance	0	3
Fecal occult blood positive	13	6
Abdominal pain	3	6
Constipation	4	3
Diarrhea	2	5
Anemia	1	0
Screening	5	5
Hematochezia	4	6
Bloody stool	4	2
Soft stool	0	1

[1]NS *vs* control (Mann-Whitney *U* test), [b]$P<0.001$ *vs* before (Wilcoxon's test), [d]$P<0.001$ *vs* control after (Mann-Whitney *U* test), [2]NS *vs* before (Wilcoxon's test).

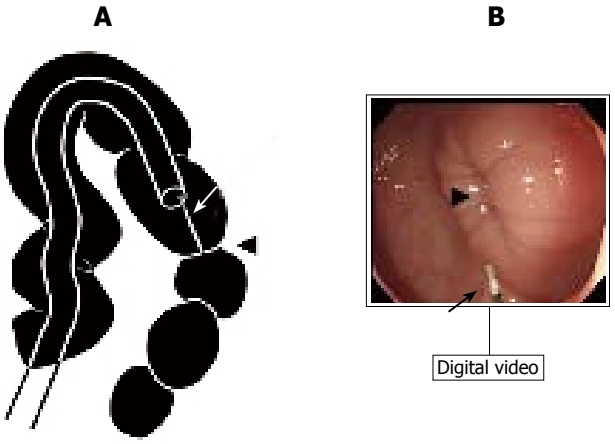

Figure 1 Illustration of the procedure (**A**) and actual endoscopic image (**B**). A distance of about 10 mm was kept between the spastic region (arrow head) and the tip of the colonoscope. The distance was objectively measured using a wire (arrow) which was inserted through the working channel.

the antispasmodic effect of TJ-68 by direct intraluminal spraying of TJ-68 solution during colonoscopy.

MATERIALS AND METHODS

Patients

A total of 531 patients were registered for endoscopy between July 2002 and March 2004. Exclusion criteria were as follows: suspected intestinal bleeding, severe abdominal pain accompanied by acute colitis and inflammatory bowel disease, history of abdominal surgery, and habitual user of drugs which may affect bowel motility. Of these, 110 patients who fulfilled the eligibility criteria were allocated at random to either the TJ-68 group or the control (saline) group. Of them, 9 patients - 5 in the TJ-68 group and 4 in the control group - were excluded from this study: due to loss of sight of the endoscopic view because of influx of residual irrigation solution from the oral side. The background characteristics of all the subjects are shown in Table 1. There was no significant difference in gender, mean age, or the reason for colonoscopy between the two groups.

Methods

This study was a prospective, randomized trial performed from July 2002 to March 2004 to compare the effects of TJ-68 solution (Tsumura Co., Tokyo, Japan) with those of saline, administered by intraluminal spraying on a colonic spastic area during colonoscopy. Colonoscopy was performed by one endoscopist (MA) using a standard colonoscope (CF-Q240I; Olympus Optical Co., Ltd, Tokyo). All patients were given a routine bowel preparation including 59 g of magnesium citrate in 250 mL (Magcorol P, Horii Pharmaceutical Ind., Ltd, Osaka, Japan) on the day before the procedure and ingestion of 2 L of a balanced solution of electrolytes with 137.155 g of polyethyleneglycol (Niflec, Ajinomoto Pharma Co., Ltd, Tokyo) before the procedure. The TJ-68 solution contained; 0.5 g of TSUMURA Shakuyaku-kanzo-to Extract Granules for Ethical Use dissolved in 50 mL saline (10^{-2} g/mL) at 36 °C. Normal saline was also prepared at 36 °C. In our experimental observations, we found that this concentration of TJ-68 solution significantly relaxed guinea pig colonic smooth muscle (unpublished), therefore we used TJ-68 at this concentration in the present study. During the procedure, we did not use any sedative agents.

With the patient in the left decubitus position, the endoscope was advanced to about 20-25 cm from the anal canal into the sigmoid colon and then, a spastic region was identified. With the tip of the colonoscope positioned at about 10 mm from the surface of the spastic area, endoscopic views were recorded for 6 min on digital videotape before and after spraying TJ-68 solution or saline through a working channel. During the examination, no air was used. Distance between the region and the tip of the endoscope was confirmed regularly using a measured guide-wire (Figure 1).

Informed consent was obtained from all the patients and the protocol was reviewed and approved by the Institutional Review Board of our hospital.

Measurement of the intraluminal spastic area

On the personal computer (VAIO PCG-GR5F, Sony), 12 spot images (3.0, 2.5, 2.0, 1.5, 1.0, and 0.5 min before and after spraying the solution) were captured every 30 s to assess luminal changes using a computer software (DVgate Still, Sony) from the recorded digital videotapes. The intraluminal spastic area was measured using a computer image analyzer (Adobe Photoshop 7.0, Adobe Systems Co., San Jose, CA, USA) and expressed as pixel counts (Figure 2). Then the mean pixel counts were used to construct a pixel curve in each group. The mean area under the curve (AUC) was calculated from the pixel curve obtained before and after spraying the TJ-68 solution or saline in each group.

Statistical analysis

Normally distributed continuous data are presented as the mean plus standard deviation and differences between the

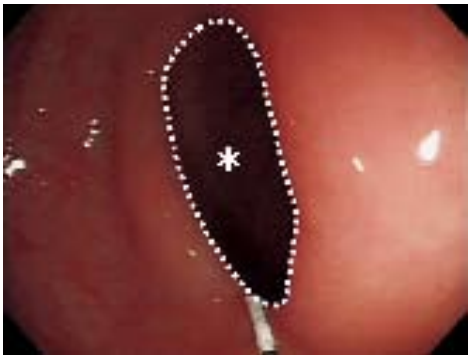

Figure 2 The white broken line demarcates the intraluminal spastic region and the area of the region was measured using a computer image analyzer (*).

Table 2 Comparison of the mean AUC between the TJ-68 and control groups before and after spraying of TJ-68 or saline

	Mean AUC (pixels × min)	
	Before	After
TJ-68	29 127[1]	121 942[b,d]
Control	31 635	4 8617[2]

[1]NS vs control (Mann–Whitney U test), [b]P<0.001 vs before (Wilcoxon's test), [d]P<0.001 vs control after (Mann–Whitney U test), [2]NS vs before (Wilcoxon's test).

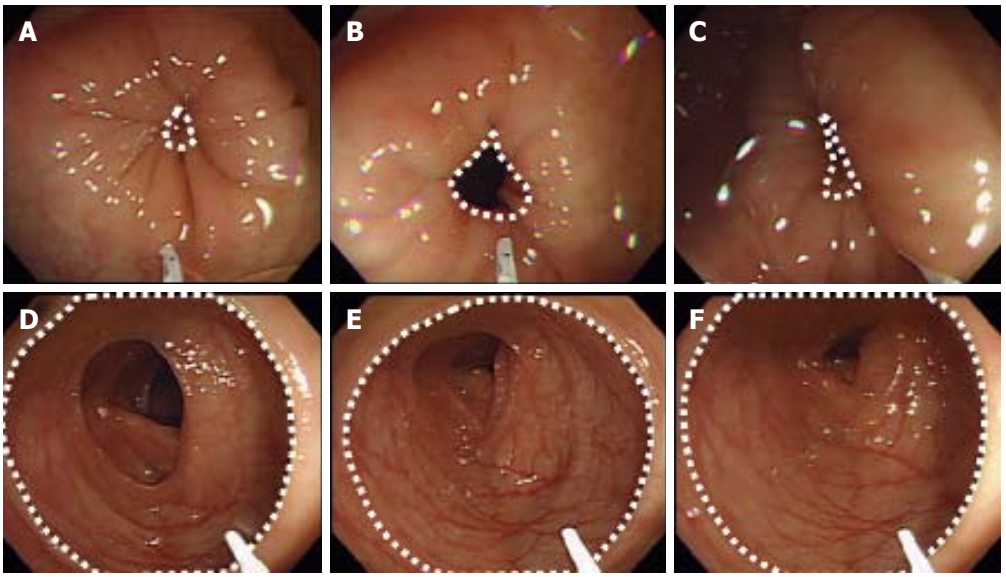

Figure 3 Endoscopic view in the TJ-68 group. Serial endoscopic views of the spastic region at the sigmoid colon before and after spraying of the TJ-68 solution are shown (A-F). The white broken line demarcates the area of the spastic region. The spastic region started to relax 0.5 min after spraying of the TJ-68 solution and remained open for 3 min.

groups were evaluated by Student's t test. Categorical data were compared with the χ^2 test and Fisher's exact test.

Wilcoxon's test was used to compare the AUCs before and after spraying the TJ-68 solution or saline in each group. Mann-Whitney U test was used to compare the AUCs before or after spraying, A P value of <0.01 was considered statistically significant. Data were analyzed using a statistical software package (Stat Mate III, ATMS Co., Ltd, Tokyo).

RESULTS

Representative endoscopic photographs of the spastic area in the sigmoid colon taken from one patient in each group before and after spraying of TJ-68 solution and normal saline are shown in Figures 3 and 4.

Antispasmodic effects of the TJ-68 solution

The mean changes of intraluminal spastic area before and after spraying of the agents are shown in Figure 5. In the TJ-68 group, the intraluminal area varied from 8 954 to 13 181 pixels at each point during 3 min before spraying, showing no difference, but after spraying, an obvious increase was observed. The intraluminal area rapidly increased 1 min after spraying and the mean area 3 min after it was 56 063 pixels. The mean AUC before and after TJ-68 spraying was 29 127 and 121 942 pixels/min, respectively, showing significant difference (P<0.001).

The mean intraluminal spastic area in the control group varied from 10 696 to 15 246 pixels at each point during 3 min before spraying, showing no difference. Spraying of saline did not significantly affect the mean intraluminal spastic area, and there was no significant increase of the mean AUC after spraying: 31 635 and 48 617 pixels/min, respectively. Thus, the AUC significantly increased after TJ-68 spraying compared with the spraying of saline (P<0.001, Table 2).

No complication was encountered in this study.

DISCUSSION

The TJ-68 solution caused the relaxation of the spastic colonic region as early as 0.5 min after spraying, and the relaxation was maintained for 3 min, or more. This phenomenon was sought to be as the local effects of the TJ-68 on the colonic wall. While in the control, the spastic area did not increase significantly after spraying of saline. Judging from the increase in the number of pixels in the control, this increase seemed to be simply attributable to a volume load. While the increase in the TJ-68 group was attributable to the active relaxing effect of TJ-68, and relaxation of the spastic area was observed within 0.5 min after spraying. We think there is a close correlation between the intraluminal movement of a spastic region

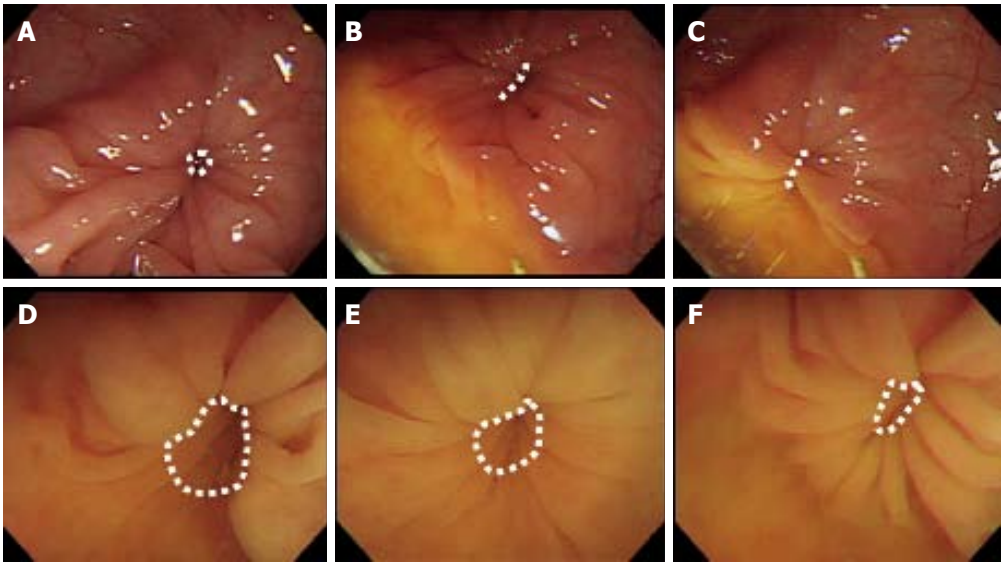

Figure 4 Endoscopic view in the normal saline group. Serial endoscopic views of the spastic region at the sigmoid colon before and after spraying of normal saline are shown (**A-F**). The white broken line demarcates the area of the spastic region. The region showed only little relaxation and did not open significantly during the study.

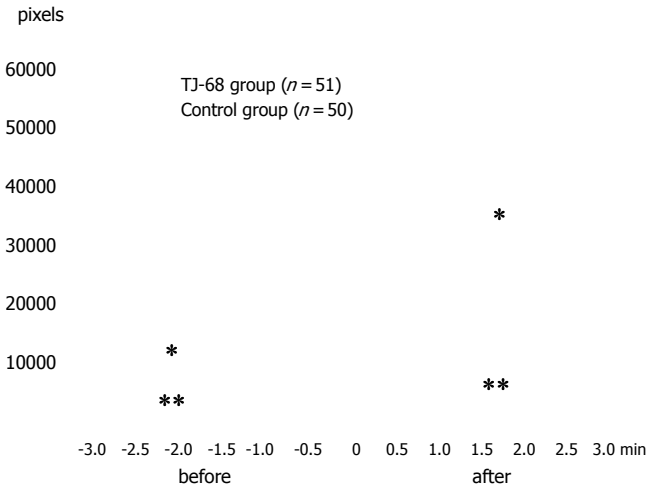

Figure 5 Pixel curve and area under the curve (AUC) before and after the administration of the TJ-68 solution and normal saline. The graph shows the mean AUC of the two groups before and after spraying of TJ-68 (*) and normal saline (**): the mean AUC in the TJ-68 group increased significantly from 29 127 to 121 942 pixels, but the control group did not show a significant increase.

and the intraluminal pressure. Actually, Asao *et al.* reported that the changes of the endoscopic findings by the intraluminal administration of the peppermint oil solution is correlated well with the intraluminal pressure measured by the Barostat[1]. Based on this phenomenon, we assume that TJ-68 will permeate and be absorbed through the colonic wall, and directly act on the myenteric plexus.

Since TJ-68 solution has a light brown color, blinding of the study is thought to be difficult in the strict sense. But selection of the patients or assignment of the agents to each group was performed in a strictly blind fashion. Furthermore, as relaxation of spasms was evaluated objectively, we believe that discernment of the agent after spraying had only a little influence on the outcome.

TJ-68 is an extract powder composed of Shakuyaku (*Paeoniae radix*) and Kanzo (*Glycyrrhizae radix*) combined at a ratio (g) of 1:1. TJ-68 inhibits acetylcholine-induced contraction and the contractile machinery of the smooth muscle by Kanzo and that of neurogenic contraction by Shakuyaku in guinea pig ileum[5,6]. Furthermore, the two components of TJ-68 exert synergistic effects[5].

Peppermint oil and warm water were examined in a way, analogous to our study, for their relaxing effect on the colonic wall after direct spraying[1,2]. Peppermint oil was reported to have a direct relaxing effect on the colonic smooth muscle when used in endoscopy or barium enema examination[1,7,8]. We also studied the antispasmodic effect of peppermint oil on the colonic wall using our method of evaluation and compared it to that of TJ-68. As a result, TJ-68 was found to have an antispasmodic effect similar or stronger than that of peppermint oil (data not shown). Peppermint oil is not sold via a regular medical distribution system. Furthermore, it is sometimes difficult to get and prepare the peppermint oil solution[1]. On the other hand, TJ-68 is available in any medical facility in Japan, because it is a regular medical prescription sold via a regular medical distribution system. In addition, the preparation of TJ-68 solution is easy and simple: only mixing TJ-68 with saline.

Church reported that warm water irrigation significantly reduced discomfort related to colonoscopy, but there was no difference regarding insertion time, total duration of colonoscopy, dose of midazolam administered, or frequency of severe spasm. Decrease of the patient's discomfort was the parameter of effectiveness of warm water in their study. But their study had some limitations because they used a sedation drug (midazolam) as premedication[2]. Moreover, warm water has basically no direct antispasmodic effect on the colonic wall.

Colonic relaxation by antispasmodic drugs has been evaluated using various parameters: pain score, colonic spasm score, difficulty of the procedure scored by an endoscopist, and dose of drugs used for sedation[1,2,9-14]. However, these parameters were considered to be subjective, moreover, most studies were performed under sedation[2,9-14]. In the present study, we evaluated colonic relaxation quantitatively. In addition, we did not use any drugs to induce sedation. But the clinical relevance of TJ-68 in colonoscopy should be investigated in subsequent studies, although our preliminary study showed significant

differences between the TJ-68 group and the control group in total colonoscopy time and patient's pain score (data not shown).

Contrary to the ordinary use of antispasmodic agents, we sprayed TJ-68 directly on the colonic wall. Antispasmodic agents are generally administered intramuscularly or intravenously, but systemic administration sometimes prevents the insertion of the endoscope because the muscle tone of the colon is excessively reduced[13]. Colonoscopy is difficult to carry out at the spastic region but not every spasm affects the examination. Ideally, the examiner expects to obtain effective relaxation in only some of them. In this respect, direct spraying of the antispasmodic agent TJ-68 is one of the meaningful ways of administration. But administration of this agent by spraying differed from the common oral administration. Since this was the first attempt to spray this agent directly onto the colonic wall, we had essentially no idea about the adverse effects. But the total amount of TJ-68 used in this study was no more than 2.5 g, that is to say one third of the oral dose: 7.5 g. In fact, no complication was experienced in this study.

In this study, we set the concentration of the TJ-68 solution at 10^{-2} mg/dL. Significant relaxation of the guinea pig distal colon was obtained with this concentration of TJ-68 in our preliminary experimental study; thus this concentration was thought to be effective enough to depress colonic spasm during colonoscopy.

In conclusion, we objectively demonstrated that intraluminal direct spraying of TJ-68 solution on the colonic mucosa could lower the colonic spasm. Additional studies are needed to determine the time required for colonoscopy or to assess patient's compliance before TJ-68 can be definitively recommended as a useful antispasmodic agent for use in colonoscopy.

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S- Editor Guo SY **L- Editor** Elsevier HK **E- Editor** Li HY

PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

World J Gastroenterol 2006 February 7; 12(5): 765-767
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Surgical treatment of patients with intermediate-terminal pancreatic cancer

Yu-Bin Liu, Liang Huang, Zhi-Yong Xian, Zhi-Xiang Jian, Jin-Rui Ou, Zi-Xian Liu

Yu-Bin Liu, Liang Huang, Zhi-Yong Xian, Zhi-Xiang Jian, Jin-Rui Ou, Zi-Xian Liu, Department of Hepatobiliary Surgery, Guangdong Provincial People's Hospital, Guangzhou 510080, Guangdong Province, China
Supported by the Medical Science Technology Foundation of Guangdong Province, No. WSTJJ2002102521010270100 8182
Correspondence to: Dr Yu-Bin Liu, Department of Hepatobiliary Surgery, Guangdong Provincial People's Hospital, Guangzhou 510080, Guangdong Province, China. benson_lau@hotmail.com
Telephone: +86-13826288388 Fax: +86-20-83827812-60920
Received: 2005-07-22 Accepted: 2005-08-15

Abstract

AIM: To investigate the surgical treatment of patients with intermediate–terminal pancreatic cancer.

METHODS: A retrospective analysis was made of the clinical data of 163 patients with intermediate–terminal pancreatic cancer who were surgically treated between August 1994 and August 2003.

RESULTS: A total of 149 patients underwent palliative surgery. The mortality rate of those who underwent cholecystojejunostomy alone was 14.2%, the icterus or cholangitis recurrence rate was 61.9% with an average survival period of 7.1 mo. The mortality rate for those who received hepatic duct-jejunostomy (HDJS) was 5.7%, the icterus or cholangitis recurrence rate was 6.8% with an average survival period of 7.1 mo. But 31.8% of the patients developed duodenum obstruction within 6 mo after the surgery, six of seven patients with severe pain were given peri-abdominal aorta injection with absolute alcohol and their pain was alleviated. The other patients underwent percutaneous transhepatic cholangial drainage (PTCD) and their icterus index returned to normal level within 40 d with an average survival period of 7.5 mo.

CONCLUSION: Roux-en-y HDJS combined with prophylactic gastrojejunostomy is recommended for patients with intermediate–terminal pancreatic cancer, and biliary prosthesis can partly relieve biliary obstruction in a short term.

Key words: Pancreatic cancer; Bile duct conduction; Gastrointestinal conduction; Biliary prosthesis

Liu YB, Huang L, Xian ZY, Jian ZX, Ou JR, Liu ZX. Surgical treatment of patients with intermediate-terminal pancreatic cancer. *World J Gastroenterol* 2006; 12(5): 765-767

http://www.wjgnet.com/1007-9327/12/765.asp

INTRODUCTION

Generally speaking, not until the patient has developed terminal pancreatic cancer can a diagnosis be made. The non-clinical survival time is not more than half a year and the post-operative survival rate is still lower than 20%[1]. At present, surgical treatment is the most common method for patients with intermediat-terminal pancreatic cancer. The hospital received 163 patients with intermediate–terminal pancreatic cancer between August 1994 and August 2003.

MATERIALS AND METHODS

Basic data

A total of 107 male and 56 female patients aged 28-79 years with an average age of 43.2 years were included in the study.

Clinical symptoms

The most common symptoms were upper-abdominal spasm and gastric bulge without any motivation. One hundred and twelve patients (68.7%) suffered from anorexia and only 51 patients (31.2%) developed icterus. Among these 163 patients, 124 were not in the hospital until they had icterus. All the 163 patients were diagnosed as intermediate–terminal pancreatic cancer by B-ultrasound, CT, MR and explorative operation.

Operation procedures

One hundred and forty-nine patients underwent palliative surgery. Among them, 28 patients received cholecystojejunostomy (CJS), 53 hepatic duct-jejunostomy (HDJS) and 61 biliary enteric anastomosis plus gastrojejunostomy, 7 terminal peri-abdominal aorta injection with absolute alcohol. The other 14 patients did not undergo surgery but biliary prosthesis placement for PTCD.

RESULTS

Among the 149 patients who received palliative surgical treatment, 10 died 30 days after the surgery. The estimated

mortality rate of those who underwent CJS, HDJS, and biliary enteric anastomosis plus gastrojejunostomy was 14.2% (4/28), 5.7% (3/53), and 4.9% (3/61), respectively. Among the 21 patients who underwent CJS, 13 developed icterus or cholangitis, the recurrence rate was 61.9% with an average survival period of 7.1 mo. Among the 44 patients who underwent hepaticocholedochojejunostomy, only 3 developed icterus or cholangitis, the recurrence rate was 6.8% with an average survival period of 10.3 mo. Likewise, the recurrence rate of icterus or cholangitis among the 51 patients who underwent biliary enteric anastomosis plus gastrojejunostomy was 5.9% (3/51), but the average survival period was 13.9 mo.

Fourteen out of the forty-four patients who were followed up after HDJS developed duodenal obstruction, the rate was 31.8%. Among them, 11 patients who received an additional gastrojejunostomy did not survive for more than 3 mo. Fifty-one patients who received biliary enteric anastomosis in combination with gastrojejunostomy needed no additional surgical treatment for duodenal obstruction.

The icterus index of 14 patients became normal within 40 days after biliary prosthesis placement, 11 followed-up cases had an average survival period of 7.5 mo. Seven patients were given absolute alcohol injection and their pain was relieved with an average survival period of 3.4 mo.

DISCUSSION

Surgical treatment of patients with intermediate–terminal pancreatic cancer

Most patients have already developed terminal pancreatic cancer when a confirmed diagnosis was made. Palliative surgical treatment is the first choice of treatment for these patients. The purposes of the treatment are to relieve biliary tract and duodenal obstruction, to control and alleviate the symptoms in order to prolong the post-operative survival time[2].

Common treatment approaches for intermediate–terminal pancreatic cancer include biliary enteric anastomosis, biliary prosthesis, and Roux-Y loop, etc. Biliary enteric anastomosis including cholecystojejunostomy (CJS) and HDJS is the most commonly used approach today.

CJS is easy to perform and does not incur severe trauma, but has certain disadvantages. For example, 10% of the patients have the opening of the cystic duct on the lower part of the common bile duct, thus the patients are liable to develop duodenal obstruction due to tumors and metastatic lymph nodes. Since it is difficult to make sure whether the bile duct is blocked during surgery, cholangiography is needed, thus the surgery is time-consuming. Such a surgery is inappropriate in the presence of cholecystitis, gallstones or ill-function. Post-operative icterus and cholangitis are more likely to occur. Rosemurgy *et al*[4] demonstrated that the success rate of CJS and HDJS to relieve icterus is 89% and 97%, respectively, and the incidence rate of icterus and cholangitis is 20% and 8%, respectively. In our patients, the recurrence rate of icterus or cholangitis was 61.9%, much higher than that after HDJS (6.8%). The average survival period (7.1 mo) was far lower than that after HDJS (10.3 mo), suggesting that HDJS is a better therapeutic approach than CJS.

It was reported that Roux-en-Capital Y anastomosis can be performed for patients with pancreatic cancer, because the suturing position of HDJS is relatively higher and less liable to be assaulted by tumors and suturing point is larger[4]. On the other hand, Roux-en-y anastomosis can significantly reduce the incidence of reflux cholangitis and hepatitis[6]. In addition, HDJS incurs direct hepatobiliary drainage, thus effectively reducing the incidence of icterus as well as the recurrence rate of icterus or cholangitis[6]. We believe that it is suitable for patients whose potential survival period is relatively longer. However, the common bile duct in the superior border of the duodenum should be cut off leaving the distal end blocked, and the proximal end should be anastomosed to the duodenum to avoid tumor-incurred icterus. The common bile duct should be sutured with only one layer of stitches and the passage above the stitches should be unobstructed.

Biliary tract drainage or percutaneous transhepatic biliary drainage (PTBD) can effectively reduce icterus. But loss of bile seriously unbalances the electrolytes.

Pre-emptive application of gastrojejunostomy and pancreaticojejunostomy

One of the most important problems in the treatment of pancreatic cancer is whether the surgeon can resolve the duodenal obstruction during the biliary enteric looping process. It was reported that apart from direct tumor assaults, gastrointestinal dysfunction caused by visceral nerves is also a reason for duodenal obstruction[7]. When duodenal obstruction occurs, GJS cannot improve the gastric drainage[8]. Therefore pre-emptive GJS is not recommended. But 13-34% of the patients undergoing biliary enteric anastomosis suffer from duodenal obstruction[6]. An additional GJS can prolong the survival period significantly but does not increase the mortality rate. Gastrointestinal conduction can assist the treatment by providing nutrition. The data in this series also showed that compared to biliary enteric anastomosis only, biliary enteric anastomosis combined with GJS resulted in a sharp increase of mortality rate but prolonged the post-operative survival time. Besides, biliary enteric anastomosis alone tended to cause duodenal obstruction, while the combination reduced its occurrence, suggesting that GJS should be normally accepted as an approach for unresectable patients. This is because terminal pancreatic cancer patients are more liable to develop duodenal obstruction, the mortality rate is high and the survival time is short when GJS is performed after the diagnosis of duodenal obstruction. Biliary enteric anastomosis in combination with GJS does not increase the risk of operative deaths, while prolongs the post-operative survival time.

It was reported that biliary enteric anastomosis combined with pancreatoenterostomy can prevent duodenal or pancreatic obstruction, relieve the accompanying pain caused by dilation and indigestion, and reduce the mortality rate[10]. We believe that patients with apparent indigestive symptoms and pancreatic dilation confirmed by ultrasonic and CT examinations can undergo pancreatoenterostomy, since three anastomoses can be made in one surgery and there is no need for subsequent surgeries.

Application of biliary prosthesis

Biliary prosthesis is an advanced technique and the trauma caused by it is small, thus, patients can recover quickly. In our study, 14 patients with terminal pancreatic cancer underwent PTCD and their icterus index returned to normal level within 40 d, suggesting that this operation is applicable to terminal pancreatic cancer patients or very old patients in bad physical conditions. But this new technique leads to some complications, such as a relatively high failure rate and no long-term effect on reducing icterus. It is suggested that surgical treatment can reduce icterus in patients and prolong post-surgery survival[2].

Treatment of persistent pain

Persistent pain is one of the major symptoms of intermediate-terminal pancreatic cancer patients, 30-40% of the patients suffer from abdominal and back pain due to cancer-afflicting post-peritoneum nerve plexus or chronic pancreatitis. In addition, high pressure of biliary tract, cholangitis or incomplete duodenal obstruction can also be a factor. Pain can be relieved or partly relieved by biliary or gastrointestinal by-pass surgery for some patients. But they have no effect on pain caused by post-peritoneum nerve plexus affliction. Some scholars have proposed that using a dose of 20 mL alcohol (50%) or lidocaine (1%) as a blockage injection to post-peritoneum nerve plexus near the tumor can alleviate or prevent late pancreatic cancer-related pain.

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S- Editor Wang XL and Guo SY **L- Editor** Elsevier HK **E- Editor** Cao L

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World J Gastroenterol 2006 February 7; 12(5): 768-771
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Portal vein pulsatility index is a more important indicator than congestion index in the clinical evaluation of right heart function

Cheng-Yen Shih, Sien-Sing Yang, Jui-Ting Hu, Chin-Lin Lin, Yung-Chih Lai, Cheng-Wen Chang

Cheng-Yen Shih, Chin-Lin Lin, Jui-Ting Hu, Yung-Chih Lai, Liver Unit, Cathay General Hospital, Taipei 106, Taiwan，China
Sien-Sing Yang, Liver Unit, Cathay General Hospital, Taipei 106, Taiwan and Medical Faculty, China Medical University, Taichung 400, Taiwan, China
Cheng-Wen Chang, Department of Cardiology, Cathay General Hospital, Taipei 106, Taiwan, China
Supported by the grant from the Cathay Groups, Taipei, Taiwan, China
Correspondence to: Sien-Sing Yang, MD, Professor of Medicine, Director of Liver Unit, Cathay General Hospital, 280 Jen-Ai Road, Sec. 4, Taipei 106, Taiwan, China. yangss@cgh.org.tw
Telephone: +886-2-2708-2121-3123 Fax: +886-2-2707-4949
Received: 2005-08-08 Accepted: 2005-08-26

Abstract

AIM: To study the changes of portal blood flow in congestive heart failure.

METHODS: We studied the congestion index (CI) and portal vein pulsatility index (PI) in patients with varied degrees of congestive heart failure using ultrasonic Doppler. Ten patients with mean right atrial pressure (RA) < 10 mmHg were classified as group 1 and the remaining 10 patients with RA ≥ 10 mmHg as group 2.

RESULTS: There were no difference on cardiac index (HI, $P = 0.28$), aortic pressure (AO, $P = 0.78$), left ventricular end-diastolic pressure (LVED, $P = 0.06$), maximum portal blood velocity (V_{max}, $P = 0.17$), mean portal blood velocity (V_{mean}, $P = 0.15$) and portal blood flow volume (PBF, $P = 0.95$) between the two groups. Group 2 patients had higher pulmonary wedge pressure (PW, 29.9 ± 9.3 mmHg *vs* 14.6 ± 7.3 mmHg, $P = 0.002$), pulmonary arterial pressure (PA, 46.3 ± 13.2 mmHg *vs* 25.0±8.2 mmHg, $P = 0.004$), RA (17.5±5.7 mmHg *vs* 4.7 ± 2.4 mmHg, $P < 0.001$), right ventricular end-diastolic pressure (RVED, 18.3 ± 5.6 mmHg *vs* 6.4 ± 2.7 mmHg, $P < 0.001$), CI (8.7 ± 2.4 *vs* 5.8 ± 1.2, $P = 0.03$), and PI (87.8 ± 32.3% *vs* 27.0 ± 7.4%, $P < 0.001$) than Group 1. CI was correlated with PI ($P < 0.001$), PW ($P < 0.001$), PA ($P < 0.001$), RA ($P = 0.043$), RVED ($P = 0.005$), HI ($P < 0.001$), AO ($P < 0.001$), CO ($P < 0.001$), LVED ($P < 0.001$), V_{max} ($P < 0.001$), V_{mean} ($P < 0.001$), cross-sectional area of the main portal vein ($P < 0.001$) and PBF ($P < 0.001$). CI could be as high as 8.3 in patients with RA < 10 mmHg and as low as 5.9 in those with RA ≥ 10 mmHg.

CONCLUSION: Our data show that RI is a more significant indicator than CI in the clinical evaluation of high RA ≥ 10 mmHg, whereas CI is better than PI in the assessment of left heart function.

Key words: Portal blood flow; Heart failure; Ultrasonic Doppler; Congestion index; Portal vein pulsatility index

Shih CY, Yang SS, Hu JT, Lin CL, Lai YC, Chang CW. Portal vein pulsatility index is a more important indicator than congestion index in the clinical evaluation of right heart function. *World J Gastroenterol* 2006; 12(5): 768-771

http://www.wjgnet.com/1007-9327/12/768.asp

INTRODUCTION

Congestive heart failure increases the pressure in the inferior vena cava and hepatic veins[1-3]. Ultrasonic Doppler is a safe and non-invasive method in the clinical evaluation of portal blood flow and portal hypertension[4-9]. Portal vein pulsatility index (PI) is calculated by the percentage of peak-to-peak maximum portal vein velocities[10,11]. In our earlier study[10], patients with right heart failure developed transient reduced, stagnant, or hepatofugal portal blood flow with increased PI. However, the change of portal flow pattern and PI did not correlate with left heart function.

The congestion index (CI) has been used to assess the pathophysiological hemodynamics of portal venous system in different forms of liver diseases[12-14]. The correlation between CI and PI and the role of CI on right heart function remain uncertain. Therefore, we have studied the changes of portal blood flow in patients with different degrees of heart failure using non-invasive ultrasonic Doppler[15,16].

MATERIALS AND METHODS

We studied the portal hemodynamic profiles in 20 patients (9 males, 11 females, mean age: 49 ± 13 years) who received cardiac and Swan-Ganz catheterizations for cardiovascular disorders (16 rheumatic heart disease, 4 atherosclerotic heart disease) to compare with 20 healthy volunteers. All

Table 1 Clinical and biochemical data in patients with heart failure (mean ± SD)

	Controls	RA < 10 mmHg	RA ≥ 10 mmHg
Gender (M/F)	10/10	4/6	5/5
Age (yr)	46±12	50±13	47±19
Total protein (g/dL)	7.5±0.6	7.1±0.8	6.9±1.1
Albumin (g/dL)	4.3±0.2	3.9±0.7	3.7±0.5
Total serum bilirubin (mg/dL)	0.9±0.4	1.3±0.8	1.4±0.8
AST (U/L)	21±6	31±11	59±29[1,b]
ALT (U/L)	24±6	23±8	35±20
Prolonged prothrombin time (s)	-	1.1±0.9	1.4±0.8

RA, right atrial pressure ; [1]P=0.009 *vs* patients with RA < 10 mmHg and [b]P<0.001 *vs* controls.

Table 2 Cardiac profiles in patients with congestive heart failure(mean ± SD)

	RA < 10 mmHg	RA ≥ 10 mmHg	*P*
HI (L/min/m^2)	3.0±0.9	2.4±0.4	0.28
AO (mmHg)	89.0±9.6	87.3±12.8	0.78
LVED (mmHg)	12.2±6.7	22.1±10.9	0.06
PW (mmHg)	14.6±5.6	29.1±7.7	0.002
PA (mmHg)	25.0±6.8	42.4±12.0	0.004
RA (mmHg)	4.7±2.1	16.8±4.9	<0.001
RVED (mmHg)	6.4±2.1	17.8±4.4	<0.001

RA, right atrial pressure; HI, cardiac index; LVED, left ventricular end-diastolic pressure; AO, mean aortic pressure; PW, pulmonary wedge pressure; PA, mean pulmonary arterial pressure; RA, mean right atrial pressure; RVED, right ventricular end-diastolic pressure.

patients had medications affecting the hemodynamics such as isosorbide dinitrate and furosemide, and their systemic blood pressure and body weight were measured to be constant for more than 48 h prior to the study. Patients with fever, infection, and shock were excluded. All patients had no history of liver disease, alcoholism or other metabolic disorders. None of the patients received transfusion, inotropic agents or dopamine. All patients had an abdominal sonography to exclude chronic liver disease or splenomegaly. Patients with severe orthopnea were excluded if they were not able to remain in the supine position for the study of ultrasonic Doppler.

Cardiac profiles including cardiac index (HI), left ventricular end-diastolic pressure (LVED), mean aortic pressure (AO), pulmonary wedge pressure (PW), mean pulmonary arterial pressure (PA), mean right atrial pressure (RA), right ventricular end-diastolic pressure (RVED) were recorded during the cardiac and Swan-Ganz catheterizations. Ten patients with RA < 10 mmHg (range: 1-7 mmHg) and without right heart failure were classified as Group 1. The remaining 10 patients with right heart failure and RA ≥ 10 mmHg (range: 10-28 mmHg) were classified as Group 2.

The portal profiles were assessed using an ultrasonic Doppler composed of a real-time mechanical sector scanner and a 3.5 mHz pulsed Doppler flowmetry (Aloka Echo Camera, Model SSD-1700, Tokyo) within 12 h of cardiac catheterization. After more than 8 h of fasting, portal profiles were measured in the supine position for more than 30 min. Portal blood flow was measured from the main portal vein at a site just entering or immediately after entering the liver with the patient in expiratory apnea. The flow angle formed by the directions of ultrasonic beam and the portal blood flow below 55 degree was corrected to minimize the variation caused by the angle of insonation. The Doppler signal could be viewed on the screen and heard through a build-in speaker. Portal blood flow was measured by the same physician (SY) to avoid interobserver variation[17].

For each measurement, at least three reproducible spectral patterns were recorded for calculating the mean maximum portal blood velocity (V_{max}) over a period of 3-4 s to ensure accuracy. Mean portal blood velocity (V_{mean}) was calculated by the equation "$V_{mean}=0.57\times V_{max}$" as described by Moriyasu *et al*.[18]. Cross-sectional area (area, cm^2) was also recorded at the site of main portal vein where portal blood velocity was measured. The direction of portal blood velocity, antegrade or retrograde, was also measured. Positive velocity indicates the blood flow towards the transducer and vice versa. Portal blood flow volume (PBF, mL/min) was obtained by the equation "PBF=area$\times V_{mean}\times 60$"[17,18]. PI was calculated by the equation "PI=(maximum-minimum)/maximum frequency shift"[6,15,17]. The waveforms were classified as continuous (PI ≤ 40%), decreased (PI 41-99%), stagnant (PI = 100%), or retrograde (PI>100%)[10,19]. CI was calculated by the equation "CI = (area/V_{mean})×100"[12].

The study protocol was reviewed and approved by the Institutional Review Committee under the guidelines of the 1975 Declaration of Helsinki. Statistical analysis was performed using Student's *t*-test and simple linear regression as appropriate.

RESULTS

The biochemical data of the 20 patients (Table 1) showed total protein 7.0 ± 0.8 g/dL, albumin 3.8 ± 0.5 g/dL, total bilirubin 1.3 ± 0.6 mg/dL, AST 49.5 ± 23.4 IU/L, ALT 28.7 ± 10.4 IU/L, and prolonged prothrombin time 1.2 ± 0.9 s (normal < 3 s). All controls had normal blood chemistries. Gender (P = 0.11), age (P = 0.61), total protein (P = 0.85), albumin (P = 0.62), total bilirubin (P = 0.83), ALT (P = 0.15) and prolonged prothrombin time (P = 0.19) were not different between those with RA < 10 mmHg and ≥ 10 mmHg. Patients with RA ≥ 10 mmHg had higher serum AST activities (P = 0.009), which were related to ischemic hepatitis.

HI (3.0 ± 0.9 L/min/m^2; range: 1.6-5.3 L/min/m^2 *vs* 2.4 ± 0.4 L/min/m^2; range: 1.7-2.9 L/min/m^2; P = 0.28), AO (89.0 ± 9.6 mmHg; range: 85-100 mmHg *vs* 87.3 ± 12.8 mmHg; range: 65-115 mmHg; P = 0.78), and LVED (12.2 ± 6.7; range: 4-34 mmHg *vs* 22.1 ± 10.9 mmHg; range: 10-40 mmHg; P = 0.06) were not statistically different between Groups 1 and 2 (Table 2).

For all Group 1 patients, the values of PW (mean: 14.6 ± 5.6 mmHg; range: 5-28 mmHg), PA (mean: 25.0 ± 6.8 mmHg; range: 16-38 mmHg), RA (mean: 4.7 ± 2.1 mmHg; range: 1-7 mmHg), and RVED (mean: 6.4 ± 2.1 mmHg; range: 2-11 mmHg) were within the nor-

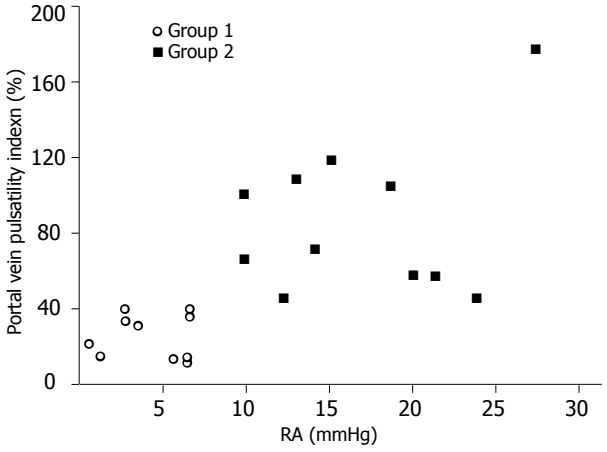

Figure 1 Portal vein pulsatility index of patients with right atrial pressure < 10 mmHg (Group 1) and ≥ 10 mmHg (Group 2).

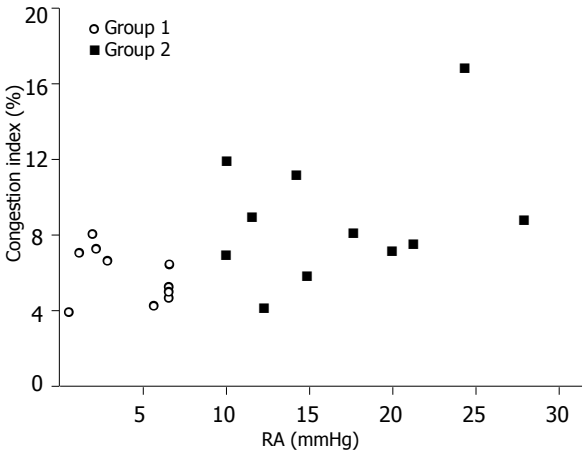

Figure 2 Congestion index of patients with right atrial pressure (RA) < 10 mmHg (Group 1) and ≥ 10 mmHg (Group 2).

Table 3 Portal profiles in patients with congestive heart failure(mean ± SD)

	Controls (n = 20)	RA ≤ 10 mmHg (n = 10)	RA > 10 mmHg (n = 10)
V_{max} (cm/s)	20.1±3.1	24.5±3.9	21.1±4.8
V_{mean} (cm/s)	11.2±1.9	14.0±2.3	12.0±2.7
Area (cm^2)	1.01±0.20	0.80±0.13	0.96±0.13
PBF (mL/min)	685±136	678±172	672±162
PI (%)	23.3±6.3	27.0±7.4	87.8±32.3[b]
Congestion index	5.3±1.2	5.8±1.2	8.7±2.4[1]

RA, right atrial pressure; V_{max}, maximum portal velocity; V_{mean}, mean portal velocity; PBF, portal blood flow; PI, portal vein pulsatility index. [b]P<0.001, [1]P=0.03 *vs* controls and RA≤10 mmHg.

mal limits.

All Group 2 patients had higher PW (mean: 29.1 ± 7.7 mmHg; range: 13-40 mmHg; $P = 0.002$), PA (mean: 42.4 ± 12.0 mmHg; range: 25-65 mmHg; $P = 0.004$), RA (mean: 16.8 ± 4.9 mmHg; range: 10-28 mmHg; $P < 0.001$), and RVED (mean: 17.8 ± 4.4 mmHg; range: 9-26 mmHg; $P < 0.001$) than Group 1 patients.

The healthy controls had V_{max} 20.1 ± 3.1 cm/s, V_{mean} 11.2 ± 1.9 cm/s, area 1.01 ± 0.20 cm^2, PBF 685 ± 136 mL/min, PI 23.3 ± 6.3%, and CI 5.3 ± 1.2. The mean values of V_{max} (24.5 ± 3.9 cm/s; range 17-33 cm/s *vs* 21.1 ± 4.8 cm/s; range 14-33 cm/s; $P = 0.17$), V_{mean} (14.0 ± 2.3 cm/s; range: 9.7-18.8 cm/s *vs* 12.0±2.7 cm/s; range: 8.6-18.8 mmHg; $P = 0.15$) and PBF (678 ± 172 mL/min; range: 373-1 120 mL/min *vs* 672 ± 162 mL/min; range: 432-922 mL/min; $P = 0.95$) between Groups 1 and 2 did not show any statistical difference (Table 3). Group 2 patients had a larger area of portal vein than that of Group 1 (0.80 ± 0.13 cm^2; range: 0.64-1.13 cm^2 *vs* 0.96 ± 0.13 cm^2; range: 0.79-1.33 cm^2; $P = 0.04$).

All the 10 patients in Group 1 had a continuous antegrade portal flow with a mean PI 27.0 ± 7.4% (range: 17-40%) (Figure 1). The mean PI of the 10 patients in Group 2 was 87.8 ± 32.2% (range: 43-194%). In Group 2, all the patients had a PI > 40%. Six of them had transient reduced portal blood flow, one had stagnant flow, and three had hepatofugal flow.

Group 2 patients (mean: 8.7 ± 2.4, range: 5.9-16.7) had a higher CI than that of Group 1 patients (mean: 5.8 ± 1.2, range: 3.9-8.3; $P = 0.03$). Although Group 2 had a higher mean CI than Group 1, the CI could be as low as 5.9 in Group 2 and as high as 8.3 in Group 1 (Figure 2).

Using linear regression, CI showed a good correlation with PI ($P < 0.001$), PW ($P < 0.001$), PA ($P < 0.001$), RA ($P = 0.043$), RVED ($P = 0.005$), HI ($P < 0.001$), AO ($P < 0.001$), CO ($P < 0.001$), LVED ($P < 0.001$), V_{max} ($P < 0.001$), V_{mean} ($P < 0.001$), area ($P < 0.001$) and PBF ($P < 0.001$).

DISCUSSION

It is well known that the passive "backward" congested liver develops into hepatomegaly, synchronous pulsation, engorged and dilated terminal hepatic veins, atrophy of hepatocytes and eventually cardiac cirrhosis. The high hepatic vein pressure can transmit through the liver to cause post-sinusoidal portal hypertension, cardiac ascites and change of portal vein flow patterns[12,13]. Therefore, the changes of portal flow may help the assessment of heart function.

Prolonged right heart failure may result in atrophy of hepatocytes and eventually cardiac cirrhosis[3]. In the present study, we have strived to exclude those patients with chronic liver disease. The abdominal sonographies showed no splenomegaly or coarse liver echogenicity and the peripheral blood showed no abnormal reduction of leukocyte, hemoglobin or platelet account, which were common in cirrhosis. Furthermore, the portal flow pattern did not show reduced fluctuation, which was common in cirrhosis with portal hypertension[4]. Our patients were not likely to develop obvious cardiac cirrhosis.

In the present study, all patients with RA ≥ 10 mmHg had a PI > 40% and all patients with RA < 10 had a PI < 40% or less. The findings were consistent with our prior study[10] that PI showed a good correlation with PW, PA, RA, and RVED. The waveform changes of portal blood flow correlate well with right heart function, and the measurement of PI change is a simple and non-

invasive method to identify right heart failure[10]. Our data also demonstrated that PI had no any correlation with HI, AO, CO, LVED, V_{max}, V_{mean} and PBF. Furthermore, the waveform changes of portal blood flow correlated well with right heat function; and the PI is helpful for the diagnosis of stagnant or hepatofugal portal blood flow but not by the CI[10]. Therefore, CI is better than PI in the assessment of left heart function.

In addition to the assessment of left heart function, the CI correlated with all PBF, V_{max}, V_{mean}, area, PI, HI, PW, PA, RA, AO, CO, LVED, and RVED. These results suggest that CI also correlates well with right heart profiles. Our findings were consistent with earlier studies[12,20,21]. However, the CI values could be as high as 8.3 in patients with RA < 10 mmHg and as low as 5.9 in those with RA ≥ 10 mmHg. If the CI value is between 5.9 and 8.3, it is difficult to predict whether or not the RA values ≥ 10 mmHg. Therefore, RI is a more significant indicator than CI in the clinical evaluation of high RA ≥ 10 mmHg.

The occurrence of congestive liver is not uncommon in patients with congestive heart failure. In addition to the occurrence of congestive hepatomegaly and dilatation of inferior vena cava and hepatic veins during abdominal sonography, the measurement of both CI and PI is helpful for the indirect non-invasive evaluation of cardiac function.

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S- Editor Guo SY **L- Editor** Elsevier HK **E- Editor** Cao L

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World J Gastroenterol 2006 February 7; 12(5): 772-775
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Successful laparoscopic management for cholecystoenteric fistula

Wen-Ke Wang, Chun-Nan Yeh, Yi-Yin Jan

Wen-Ke Wang, Chun-Nan Yeh, Yi-Yin Jan, Department of General Surgery, Chang Gung Memorial Hospital, Chang Gung University, Taiwan, China
Correspondence to: Chun-Nun Yeh, MD, Department of General Surgery, Chang Gung Memorial Hospital, Chang Gung University, 5, Fu-Hsing Street, Kwei-Shan, Taoyuan 333, Taiwan, China. ycn@adm.cgmh.org.tw
Telephone: +886-3-3281200-3219 Fax: +886-3-3285818
Received: 2005-06-07 Accepted: 2005-07-08

Abstract

AIM: Since 1987, laparoscopic cholecystectomy (LC) has been widely used as the favored treatment for gallbladder lesions. Cholecystoenteric fistula (CF) is an uncommon complication of the gallbladder disease, which has been one of the reasons for the conversion from LC to open cholecystectomy. Here, we have reported four cases of CF managed successfully by laparoscopic approach without conversion to open cholecystectomy.

METHODS: During the 4-year period from 2000 to 2004, the medical records of the four patients with CF treated successfully with laparoscopic management at the Chang Gung Memorial Hospital-Taipei were retrospectively reviewed.

RESULTS: The study comprised two male and two female patients with ages ranging from 36 to 74 years (median: 53.5 years). All the four patients had right upper quadrant pain. Two of the four patients were detected with pneumobilia by abdominal ultrasonography. One patient was diagnosed with cholecystocolic fistula preoperatively correctly by endoscopic retrograde cholangiopancreatography and the other one was diagnosed as cholecystoduodenal fistula by magnetic resonance cholangiopancreatography. Correct preoperative diagnosis of CF was made in two of the four patients with 50% preoperative diagnostic rate. All the four patients underwent LC and closure of the fistula was carried out by using Endo-GIA successfully with uneventful postoperative courses. The hospital stay of the four patients ranged from 7 to 10 d (median, 8 d).

CONCLUSION: CF is a known complication of chronic gallbladder disease that is traditionally considered as a contraindication to LC. Correct preoperative diagnosis of CF demands high index of suspicion and determines the success of laparoscopic management for the subset of patients. The difficult laparoscopic repair is safe and effective in the experienced hands of laparoscopic surgeons.

Key words: Laparoscopic management; Cholecystoenteric fistula

Wang WK, Yeh CN, Jan YY. Successful laparoscopic management for cholecystoenteric fistula. *World J Gastroenterol* 2006; 12(5): 772-775

http://www.wjgnet.com/1007-9327/12/772.asp

INTRODUCTION

Since 1987, laparoscopic cholecystectomy (LC) has been the favored treatment for gallbladder lesions[1]. With experience gained from laparoscopic surgery, LC has successfully been attempted in every kind of gallbladder disease.

Cholecystoenteric fistula (CF) is an uncommon disease affecting both biliary and the gastrointestinal tracts, with reported incidences ranging from 0.15% to 5% of biliary disease[2]. Chronic cholecystitis with gallstones is the primary etiology in as many as 75% of CF patients. Cholecystoduodenal type accounts for as many as 80% of CF[3]. CF is generally considered to be a relative contraindication to LC because of the difficulties involved in its management intraoperatively[4]. Laparoscopic stapling techniques have been reported as feasible and safe methods for treating such fistula[2,3,5-7]; however, these procedures are not always performed successfully. We have reported four cases of CF managed successfully by laparoscopic approach due to the correct preoperative diagnosis and experienced laparoscopic techniques.

MATERIALS AND METHODS

From January 2000 to December 2003, 4131 patients underwent LC for gallbladder lesions at the Department of General Surgery, Chang Gung Memorial Hospital (Taipei, Taiwan), 4 (0.1%) of them were identified as having CF. CF was defined as an abnormal communication between gallbladder and the neighboring organ. This study retrospectively reviewed the medical records of these four patients. All the patients' history was recorded and under-

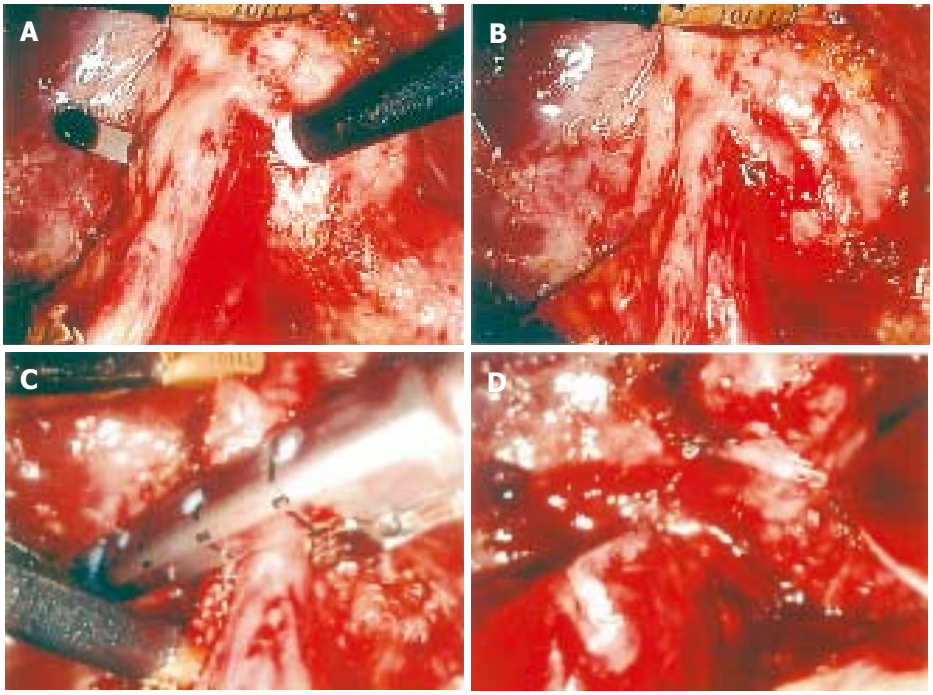

Figure 1 Procedures showing management of cholecystoenteric fistula. **A**: Identification of cholecystocolic fistula; **B**: fistula is clearly exposed; **C**: fistula tract communicating with the transverse colon is resected using an Endo-GIA device; **D**: fistula is disconnected.

went physical examination, ultrasonography (USG), or endoscopic retrograde cholangiopancreatography (ERCP), or computed tomography (CT), or magnetic resonance cholangiopancreatogram (MRCP) to establish a preoperative diagnosis. Preoperative ERCP was done routinely in patients with a history of jaundice, cholangitis or pancreatitis, patients with dilated common bile duct (CBD) displayed on abdominal USG, and patients with abnormal liver function tests. Patients with CBD stones had the stones removed endoscopically before LC. Moreover, endoscopic papillotomy (EPT) with stone retrieval and/or endonasobiliary drainage (ENBD) was performed to relieve fever, jaundice, and/or CBD stone retrieval. Data were collected on patients' age, sex, preoperative diagnoses, operative findings, operative methods, morbidity, and management.

Surgery was performed under general anesthesia using the standard four-cannula technique. The cystic artery generally was first controlled using metallic clips and divided. Moreover, the fistula was cleaned until the anatomy was clear and adequate space existed to apply an Endo-GIA (US Surgical Corp., Norwalk, CT, USA). Endo-GIA was applied via the epigastric port and then was applied to the adjacent organ end of the fistula using an applicator (Ethicon Endo-surgery). One of the blades of the Endo-GIA with the locking mechanism at its end must be seen clearly behind the fistula tract and the locking mechanism should be free of the intervening tissue to facilitate and ensure Endo-GIA locking. The gallbladder end of the fistula was ligated and simultaneously the fistula was divided (Figures 1A-1D). The gallbladder was dissected from the liver using electrocautery and removed via the umbilical or epigastric wound with an endo-bag. The postoperative course and complications were recorded. Patients were followed up in the outpatient clinic one month after the surgery and at three-monthly intervals for one year. Patients who operated more than one year before were interviewed by telephone to identify any unrecorded complications before the conclusion of the study (2004, June).

Table 1 Demographic and laboratory data of patients with cholecystoenteric fistula

Patients	1	2	3	4
Age (yr)	63	74	36	44
Gender	F	M	M	F
Symptoms and signs	RUQ pain	RUQ pain, fever, and jaundice	RUQ pain	RUQ pain
Hemoglobin (g/dL)	7.9	9.7	8.2	9.5
WBC (1 000/dL)	11.4	14.3	14.1	13.5
Bilirubin (T) (mg/dL)	0.8	8.3	1.3	0.4
AST (IU/L)	20	339	17	51
ALT (IU/L)	15	319	30	62
ALP (IU/L)	347	182	74	82

M: male; F: female; WBC: white blood cell; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; RUQ: right upper quadrant.

RESULTS

Age and sex

The study comprised two male and two female patients with ages ranging from 36 to 74 years (median: 53.5 years, Table 1). All the four patients exhibited right upper quadrant pain. Table 1 summarizes the laboratory data of these four patients. All the four patients revealed anemia and leukocytosis. Only one patient revealed elevated bilirubin level due to common bile duct stone and cholangitis.

Preoperative investigations, diagnoses, managements, and operative outcomes

Table 2 displays the preoperative investigations, diagnoses, managements, and operative outcomes. Two of the four patients were detected as gallbladder stone with pneumobilia by abdominal USG. One patient had gallbladder stone and a cholecystocolic fistula displayed by ERCP (patient 1,

Table 2 Preoperative investigations, diagnoses, managements, and operative outcomes of patients with cholecystoenteric fistula

Patients	1	2	3	4
USG	GB stone, pneumobilia, and CBD stone	GB stone and biliary tree dilatation	Contracted GB with stone and biliary tree dilatation	GB stone and pneumobilia
ERCP/EPT/stone retrieval	+ (fistula)/+/+	+ (pneumobilia)/+/+	+/-/-	-/-/-
CT/MRCP	-/-	-/-	-/-	-/+ (fistula)
Preoperative diagnosis	Cholecystocolic fistula	GB stone and CBD stone	Cystic duct stone and normal bile duct	Cholecystoduodenal fistula
Associate biliary lesion	GB stone and CBD stone	GB stone and CBD stone	GB stone	GB stone
Operative findings	Cholecystocolic fistula	Cholecystoduodenal fistula	Cholecystocholedochal fistula (Mirizzi syndrome type II)	Cholecystoduodenal fistula
Operative method	LC and closure of fistula using Endo-GIA	LC and closure of fistula using Endo-GIA	LC and closure of fistula using Endo-GIA	LC and closure of fistula using Endo-GIA
Cause of conversion	Nil	Nil	Nil	Nil
Morbidity	Nil	Nil	Nil	Nil
Hospital stay (d)	8	8	7	10

GB: gallbladder; CBD: common bile duct; ERCP: endoscopic retrograde cholangiopancreatography; EPT: endoscopic papillotomy; MRCP: magnetic resonance cholangiopancreatography; CT: computed tomography; USG: ultrasonography.

Figure 2A). ERCP revealed opacification of the gallbladder and abnormal fistula communication with colon demonstrated by haustration. The other one had pneumobilia revealed by ERCP (patient 2, Figure 2B). MRCP diagnosed cholecystoduodenal fistula in one patient as demonstrated by air-fluid level and abnormal communication between gallbladder and duodenum in T2-weighed image (patient 4, Figures 2C and 2D). Correct preoperative diagnosis of CF was made in two of the four patients with 50% preoperative diagnostic rate. Operative findings revealed two cholecystoduodenal fistula (patients 2 and 4), one cholecystocolic fistula (patient 1), and one cholecystocholedochal fistula (Mirizzi syndrome type II, patient 3). All the four patients underwent LC and closure of the fistula by Endo-GIA (after successful identification of the fistula). None of the patients needed to be converted to open cholecystectomy and all the four patients had uneventful postoperative course. None of the CF was caused by malignancy. The hospital stay of the four patients ranged from 7 to 10 d (median, 8 d). Length of follow-up ranged from 8 to 48 mo (median, 36.5 mo).

DISCUSSION

As we know, the development of a fistulous tract from the gallbladder is associated with gallstones in 90% of cases. Our study demonstrated CF was associated with gallstones in 100% cases. As seen in this study, patients with a biliary-enteric fistula are commonly presented with signs and symptoms similar to those of chronic cholecystitis. These lack specific symptoms that suggest the presence or development of a biliary-intestinal fistula. Right upper quadrant pain was present in all the CF patients, irrespective of the level of the fistula. Yamashita *et al* [8] reviewed 33 (1.3%) of 1 929 consecutive patients who had been treated for biliary tract diseases during a 12-year period had biliary fistulas. In this study, we found that the incidence of CF was only 0.1%, showing a decreased incidence these years in Asia corresponding to Western observations. CF is generally considered to be a relative contraindication to LC because of the difficulties involved in its management intraoperatively, and CF is therefore usually managed by laparotomy. This may partly explain the reason of lower incidence in this series.

Preoperative findings can be suggestive of internal biliary fistula. Pneumobilia with an atrophic gallbladder adherent to neighboring organs seen on CT scanning or USG is highly suggestive of the presence of cholecystoenteric fistula, as seen in patients 1 and 4[4]. However, the reliability of a diagnosis based on the presence or absence of pneumobilia has been questioned. Similar to the report by Yamashita *et al* [8], ERCP was the most valuable diagnostic method for revealing the presence of biliary-enteric fistula. As seen in the patient 1, ERCP could be used to diagnose the cholecystocolic fistula by demonstration of the haustration of the colon. In this study, we were able to diagnose biliary-enteric fistula preoperatively by USG, ERCP, or MRCP, and could therefore decide on a treatment strategy preoperatively. Besides, MRCP provides another reliable diagnostic tool to clearly demonstrate the abnormal communication, especially in T2-weighed image. However, only 50% of CF was diagnosed preoperatively in our series.

Treatment advocated for CF is cholecystectomy and closure of the fistula communication. Making a knot either intracorporeally or extracorporeally is time-consuming and cannot secure this difficult fistula closure. An alternative is applying laparoscopic intracorporeal suturing interruptedly or continuously with absorbable or non-absorbable material to close the inflammatory fistula, as performed in the open procedure. However, this method is technically demanding and time-consuming[5]. Ligation of the fistula with an endoloop offers a solution to the problem. The technique for the application of an endoloop consists

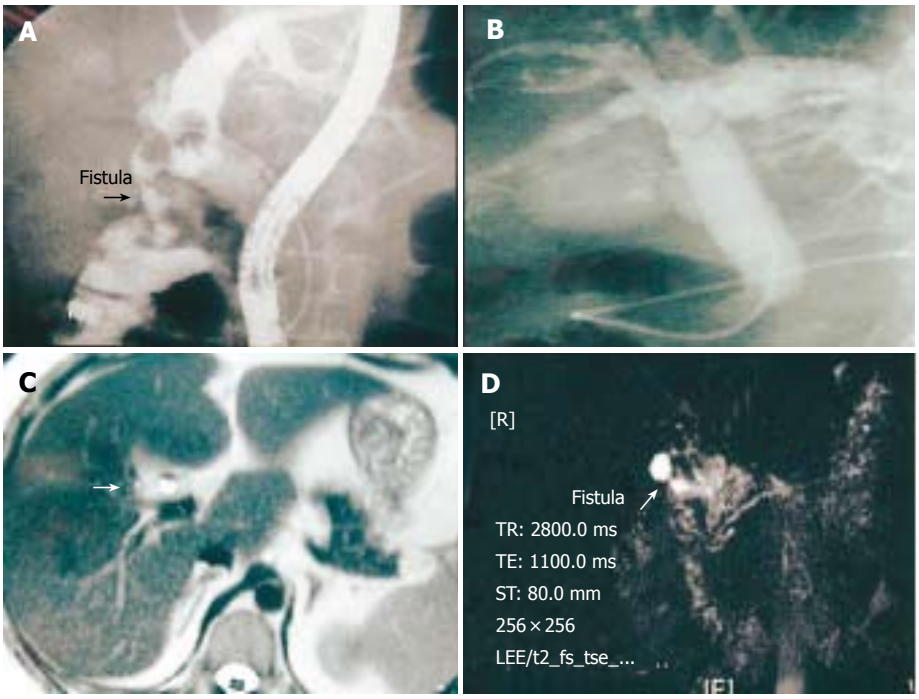

Figure 2 Detection of cholecystoenteric fistula radiologically. **A**: ERCP showing appearance of pneumobilia with common bile duct stone and a fistula between collapsed gallbladder and transverse colon; **B**: ERCP showing common bile duct stone and pneumobilia; **C**: MRCP T2-weighed image showing pneumobilia and air-fluid level within the gallbladder; **D**: MRCP T2-weighed image revealing fistula between collapsed gallbladder and duodenum.

of dividing the fistula and then applying the endoloop. This becomes more difficult if, after the fistula is divided, loss of traction on the communicating organ results in retraction of the divided fistula stump outside of the laparoscopic field of view. Although endoloop is cheaper than Endo-GIA, some new devices are needed to apply to avoid this difficulty[9]. Endo-GIA is easy to use and effective. However, the following important safety points must be noted. First, the fistula must be cleaned until the anatomy of the abnormal communication is clear and adequate space exists to apply an Endo-GIA. Second, one of the blades of the Endo-GIA with the locking mechanism at its end must be seen clearly behind the communicating organ and the locking mechanism should be free of the intervening tissue to ensure successful Endo-GIA locking. The above ensures safe closure of the fistula tract with the communicating organ. Especially, when resecting the fistula laparoscopically, it is important to ensure that part of the colonic wall is included in the excised specimen. Two reasons explain the maneuver, including that histological examination is essential to rule out colonic carcinoma at the site of fistula and unhealthy fistula or gallbladder tissue, if left on the wall of the colon, can become ischemic at a later date, thereby subsequently leading to the perforation of the wall[6]. However, no malignancy was seen in the colonic wall of the cholecystocolonic fistula in this study.

In conclusion, this report shows that with increasing experience and confidence, contraindications to laparoscopic cholecystectomy are diminishing. Laparoscopic management of CF with Endo-GIA is feasible and safe, if CF is diagnosed correctly preoperatively and managed by experienced laparoscopic surgeons.

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S- Editor Kumar M and Guo SY **L- Editor** Elsevier HK **E- Editor** Cao L

PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

World J Gastroenterol 2006 February 7; 12(5): 776-779
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Interleukin-1β gene polymorphism associated with hepatocellular carcinoma in hepatitis B virus infection

Nattiya Hirankarn, Ingorn Kimkong, Pittaya Kummee, Pisit Tangkijvanich, Yong Poovorawan

Nattiya Hirankarn, Immunology Unit, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand
Ingorn Kimkong, Pittaya Kummee, Inter-Department of Medical Microbiology, Graduate School, Chulalongkorn University, Bangkok 10330, Thailand
Pisit Tangkijvanich, Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand
Yong Poovorawan, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand
Supported by the Thailand Research Fund, RSA4680021
Correspondence to: Dr Nattiya Hirankarn, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Rama IV Road, Bangkok 10330, Thailand. fmednpt@md.chula.ac.th
Telephone: +66-2-256-4000-3667 Fax: +66-2-252-5952
Received: 2005-05-10 Accepted: 2005-08-26

Abstract

AIM: To examine the effect of interleukin-1-beta (IL-1β) promoter region C-511T and IL-1 receptor antagonist (IL-1RN) polymorphism among the patients with chronic hepatitis B virus (HBV) infection (HCC and non-HCC).

METHODS: Genomic DNA from 136 Thai patients with chronic HBV infection (HCC = 46 and non-HCC = 90) and 152 healthy individuals was genotyped for IL-1β gene polymorphism (-511) using polymerase chain reaction with sequence specific primers (PCR-SSP). The variable number of tandem repeats (VNTR) of IL-1RN gene was assessed by a PCR-based assay. The association between these genes and status of the disease was evaluated by χ^2 test.

RESULTS: IL-1B-511 genotype C/C was found to be significantly different in patients with HCC when compared with healthy individuals ($P = 0.036$, OR = 2.29, 95%CI = 1.05-4.97) and patients without HCC (P=0.036, OR = 2.52, 95%CI=1.05-6.04). Analysis of allele frequencies of IL-1B-511 showed that IL-1B-511 C allele was also significantly increased in patients with HCC, compared to that in healthy control ($P = 0.033$, OR = 1.72, 95%CI=1.04-2.84). However, no significant association in IL-1RN gene was found between the two groups.

CONCLUSION: IL-1B-511C allele, which may be associated with high IL-1B production in the liver, is a genetic marker for the development of HCC in chronic hepatitis B patients in Thai population.

Key words: Interleukin-1 beta gene; Polymorphism; Hepatocellular carcinoma; Hepatitis B

Hirankarn N, Kimkong I, Kummee P, Tangkijvanich P, Poovorawan Y. Interleukin-1β gene polymorphism associated with hepatocellular carcinoma in hepatitis B virus infection. *World J Gastroenterol* 2006; 12(5): 776-779

http://www.wjgnet.com/1007-9327/12/776.asp

INTRODUCTION

Hepatitis B virus (HBV) is the most common cause of acute and chronic liver disease worldwide, especially in Asia and Africa. It has been estimated that more than 350 million people worldwide, representing more than 5% of world population are carriers of HBV infection[1,2]. Approximately 250 000 deaths occur each year resulting from fulminant hepatic failure, cirrhosis, and hepatocellular carcinoma (HCC)[3]. Family studies in China provide some evidence that the host genetic factors influence viral persistence, as a higher concordance rate has been found for HBeAg persistence in identical twins compared with non-identical twins[4]. Thus, it is conceivable that genetic difference may affect the different outcomes of patients with HBV infection. Several genetic studies on the hosts have reported that human leukocyte antigen (HLA) genes[5-7] and various cytokine genes (TNF-α, IFN-γ, IL-12, IL-18, TGF-β, IL-10, IL-4) are associated with HBV susceptibility and/or HBV persistence or disease severity[8-11].

Interleukin-1 (IL-1) is a proinflammatory cytokine with multiple biological effects[12]. The IL-1 gene family (including IL-1A, IL-1B, and IL-1RN) on chromosome 2q13-21 encodes three proteins, which comprise the agonists IL-1α, IL-1β, and their naturally occurring inhibitor, IL-1 receptor antagonist (IL-1RN)[12,13]. Recently, allele 2 of IL-1RN intron 2 has been reported as a resistant marker of HBV infection, suggesting the role of IL-1 polymorphisms in the pathogenesis of developing chronic hepatitis B[14]. This allele is associated with enhanced IL-1β production *in vitro*[15] and *in vivo*[16]. Therefore, Zhang *et al*[14] hypothesized that high production of IL-1β may help

increase the production of other cytokines such as IL-2, IL-6, and TNF-α and trigger the complex immunological processes to eliminate the virus. Interestingly, besides its major role as a proinflammatory cytokine, IL-1β has been implicated as an important factor for tumor growth[17-19]. Several independent lines of evidence have also suggested that genetic polymorphisms within IL-1β gene are associated with gastric cancer and HCC in HCV infection[20-22].

The aim of the present study was to determine the genotype and allele frequencies of IL-1B-511 and IL-1RN VNTR polymorphisms among the Thai patients with chronic HBV infection (HCC and non-HCC) and healthy individuals to assess whether these genes are involved in chronic HBV susceptibility and/or HCC development.

MATERIALS AND METHODS

Subjects

One hundred and thirty-six Thai patients with chronic HBV infection were recruited into this study from Chulalongkorn Memorial Hospital. The diagnosis of chronic hepatitis B was established by seropositivity for HBsAg over a 6-month period and did not have any other type of liver diseases such as chronic hepatitis C or alcoholic liver disease. In addition, all the patients had elevated serum ALT and AST levels. Patients with chronic HBV infection were further divided into two groups: without ($n=90$) and with HCC ($n=46$) according to the absence or presence of concurrent HCC. Diagnosis of HCC was based on histopathology and/or a combination of mass lesion in the liver from hepatic imaging and serum alpha fetoprotein level > 400 ng/mL. Moreover, 152 ethnically and geographically matched controls from healthy blood donors of the Thai Red Cross Society were recruited.

Genotyping for SNPs of IL-1B and IL-1RN genes

Molecular genetic analysis was performed on genomic DNA obtained from peripheral blood leukocytes using standard salting-out method as previously described[23]. SNP at position -511 of IL-1B was genotyped by polymerase chain reaction (PCR) with sequence specific primer (PCR-SSP) (F-5' CTCATCTGGCATTGATCTGG-3' and R-5' GGTGCTGTTCTCTGCCTCGA-3')[24]. The PCR conditions were established as previously described[25].

The VNTR of IL-1RN gene was assessed by a PCR-based assay. Oligonucleotides F-5' CTCAGCAACACTCCTAT-3' and R-5' TCCTGGTCTGCAGGTAA-3' flanking this region were used as primers[24]. The PCR conditions were an initial denaturation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 20, at 59 °C for 50 s, at 72 °C for 20 s, and a final extension at 72 °C for 7 min. Each allele was identified according to its size[24].

Statistical analysis

The association between these genes and disease status was evaluated by the statcalc from Epi info version 6 program[26] to calculate the odds ratio (OR) and 95% confidence interval (CI), Yates' corrected χ^2 and associated P values. $P<0.05$ was considered statistically significant. The groups were tested for conformity to the Hardy-Weinberg equilibrium by 2×2 χ^2 test comparing observed and expected numbers.

Table 1 IL-1β and IL-1RN polymorphisms in patients with HBV and healthy controls

Polymorphisms		Patients with HBV: Without HCC $n=90$ (%)	Patients with HBV: With HCC $n=46$ (%)	Healthy controls $n=152$ (%)
IL-1B-511				
	Genotype frequencies			
	C/C	17 (18.89)	17 (36.96)[1,2]	31 (20.39)
	C/T	51 (56.67)	21 (45.65)	79 (51.97)
	T/T	22 (24.44)	8 (17.39)	42 (27.63)
	Allele frequencies			
	C	85 (47.22)	55 (59.78)[3]	141 (46.38)
	T	95 (52.78)	37 (40.22)	163 (53.62)
IL-1RN				
	Genotype frequencies			
	1/1	74 (82.22)	38 (82.61)	121 (79.61)
	1/2	15 (16.67)	8 (17.39)	29 (19.08)
	2/2	1 (1.11)	0	1 (0.66)
	1/4	0	0	1 (0.66)
	Allele frequencies			
	1	163 (90.56)	84 (91.30)	272 (89.47)
	2	17 (9.44)	8 (8.70)	31 (10.20)
	4	0	0	1 (0.33)

[1]$P=0.036$ *vs* healthy controls, OR (95%CI) = 2.29 (1.05–4.97); [2]$P=0.036$ *vs* patients without HCC, OR (95%CI) = 2.52 (1.05–6.04); [3]$P=0.033$ *vs* healthy controls, OR (95%CI) = 1.72 (1.04–2.84).

RESULTS

The genotype and allele frequencies of IL-1B and IL-1RN in healthy control subjects and patients with chronic hepatitis B, including patients with and without HCC are shown in Table 1. All the three groups were in Hardy-Weinberg equilibrium with no significant χ^2 values compared to the observed and expected genotype frequencies of each of the tested polymorphisms. The heterozygous C/T of IL-1B was the most common genotype in all the three groups (51.97% in healthy controls, 45.65% in patients with HCC and 56.67% in patients without HCC). The homozygous T/T was the second most common genotype in healthy controls (27.63%) and patients without HCC (24.44%), followed by the C/C genotype that was found in 20.39% of healthy controls and in 18.89% of patients without HCC, respectively. In contrast to these two groups, the C/C was the second most common genotype in patients with HCC (36.96%), followed by the T/T genotype (17.39%). Comparison of IL-1B-511 genotype revealed that IL-1B-511 C/C genotype was significantly increased in patients with HCC compared to that in healthy controls ($P=0.036$, OR = 2.29, 95%CI = 1.05-4.97) and patients without HCC ($P=0.036$, OR = 2.52, 95%CI = 1.05-6.04). Analysis of allele frequencies of IL-1B-511 showed

that IL-1B-511 C allele was also significantly increased in patients with HCC, compared to that in the healthy controls ($P = 0.033$, OR = 1.72, 95%CI = 1.04-2.84). The effect of IL-1B-511 C allele was similar to autosomal recessive mode of inheritance. The presence of two C alleles (CC) was required to increase the likelihood of HCC development.

Four genotypes of IL-1RN (1/1, 1/2, 2/2, 1/4) were found in this study. The IL-1RN 1/1 genotype was the most common genotype in all the three groups (79.61% in healthy controls, 82.61% in patients with HCC and 82.22% in patients without HCC), followed by 1/2 genotype that was 19.08% in healthy controls, 17.39% in patients with HCC and 16.67% in patients without HCC, respectively. The 2/2 genotype was 0.66% in healthy control and 1.11% in patients without HCC, whereas the 1/4 genotype was 0.66% in healthy controls. There were no significant differences in genotype or allele frequencies of IL-1RN between patients with chronic hepatitis B and healthy controls.

DISCUSSION

The association between the development of chronic hepatitis B and the polymorphisms of IL-1B gene (-511C/T) and IL-1RN gene (VNTR at intron 2) was not observed in this study. However, the -511C allele of IL-1B gene was identified as a genetic marker for the development of HCC in patients with chronic HBV infection. The hypothesis regarding IL-1 genetic polymorphism and hepatocarcinogenesis is based on the assumption that carriers of these genotypes are associated with increased levels of IL-1B in the liver in response to HBV infection and hepatocyte damage that may finally lead to the development of HCC. This hypothesis is supported by the observation that IL-1B level is increased in the liver tissue surrounding the tumor tissue[27]. IL-1B is a proinflammatory cytokine as well as a tumor growth factor. There are several lines of evidence that support its role in tumor growth development. First, IL-1B can increase the production of prostaglandin E2 and hepatocyte growth factor[17]. Second, IL-1B can induce angiogenesis which is an important step in promoting tumor growth by either upregulating COX-2 or inducing nitric oxide[19] and vascular endothelial growth factor (VEGF)[28]. Third, IL-1B can also attenuate interferon-induced antiviral activity and STAT1 activation in the liver[29].

However, the IL-1B polymorphism in the promoter area in association with cancer remains controversial. Briefly, a number of studies mostly in the Caucasian population support that -511T in linkage disequilibrium with -31C is a risk haplotype for the development of gastric cancer[30-34]. There are more conflicting data regarding the effect of IL-1B-511/-31 haplotype on the risk of gastric cancer and HCV-related HCC in Asian population, while some studies support the result from the Caucasian group[16,21,35] and a number of studies reported that -511C/-31T is a risk haplotype for the development of cancer[22,36-40]. Interestingly, functional studies of IL-1B genotype seem to support that -511C/-31T haplotype is associated with high-production of IL-1B. First, the IL-1B -31 polymorphism involves a TATA sequence in the promoter and the -31T allele is associated with a five-fold elevated binding activity with the transcription initiation factor[30,31]. Second, mucosal IL-1B level is higher than IL-1B-31T level in *H pylori*-infected gastric cancer patients[40]. Although IL-1B -511T/-31C is associated with high level of IL-1B in the plasma[41], it is likely that gene expression in each organ is differently regulated and the assessment of IL-1B level in targeted organ is more reliable. The functional study in liver tissue is also required for the better understanding of the role of IL-1B genotype in chronic hepatitis and HCC development.

In conclusion, IL-1B-511C allele which may be associated with the high IL-1B production in the liver is a genetic marker for the development of HCC in chronic hepatitis B patients in Thai population.

ACKNOWLEDGMENTS

The authors greatly appreciate the participants and all the staff members who participated in chronic HBV study at King Chulalongkorn Memorial Hospital. Also, we would like to thank the National Blood Center for the recruitment of healthy controls and collection of research materials.

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S- Editor Wang XL and Guo SY **L- Editor** Elsevier HK **E- Editor** Cao L

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World J Gastroenterol 2006 February 7; 12(5): 780-783
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Increased heat shock protein 70 expression in the pancreas of rats with endotoxic shock

Xue-Lian Wang, Ying Li, Jin-Song Kuang, Yue Zhao, Pei Liu

Xue-Lian Wang, Ying Li, Pei Liu, Department of Infectious Diseases, The Second Affiliated Hospital, China Medical University, Shenyang 110004, Liaoning Province, China
Jin-Song Kuang, Department of Internal Medicine, the Fourth People's Hospital of Shenyang, Shenyang 110007, Liaoning Province, China
Yue Zhao, Department of Infectious diseases, the First Affiliated Hospital of Jinzhou Medical College, Jinzhou 121004, Liaoning Province, China
Supported by the National Natural Science Foundation of China, No. 30170849
Correspondence to: Dr. Pei Liu, Department of Infectious Diseases, The Second Affiliated Hospital, China Medical University, Shenyang 110004, Liaoning Province, China. syliupei2003@yahoo.com.cn
Telephone: +86-24-83956451 Fax: +86-24-83956451
Received: 2005-02-24 Accepted: 2005-07-12

Abstract

AIM: To investigate the ultra-structural changes and heat shock protein 70 (HSP70) expression in the pancreas of rats with endotoxic shock and to detect their possible relationship.

METHODS: A total of 33 Wistar rats were randomly divided into three groups: control group (given normal saline), small dose lipopolysaccharide (LPS) group (given LPS 5 mg/kg) and large dose LPS group (given LPS 10 mg/kg). Pancreas was explanted to detect the ultra-structural changes by TEM and the HSP70 expression by immunohistochemistry and Western blot.

RESULTS: Rats given small doses of LPS showed swelling and loss of mitochondrial cristae of acinar cells and increased number of autophagic vacuoles in the cytoplasm of acinar cells. Rats given large doses of LPS showed swelling, vacuolization, and obvious myeloid changes of mitochondrial cristae of acinar cells, increased number of autophagic vacuoles in the cytoplasm of acinar cells. HSP70 expression was increased compared to the control group ($P<0.05$).

CONCLUSION: Small doses of LPS may induce stronger expression of HSP70, promote autophagocytosis and ameliorate ultra-structural injuries.

Key words: HSP70; Pancreas; Lipopolysaccharide; Ultra-structural changes

Wang XL, Li Y, Kuang JS, Zhao Y, Liu P. Increased heat shock protein 70 expression in the pancreas of rats with endotoxic shock. *World J Gastroenterol* 2006; 12(5):780-783

http://www.wjgnet.com/1007-9327/12/780.asp

INTRODUCTION

Septic shock is a clinical syndrome resulting from the systemic response of the body to infection. Septic shock is characterized by hypoperfusion of major organs, leading to multiple organ failure and finally to death. Though advances have been achieved in supportive and anti-microbial treatment, septic shock continues to be a common cause of intensive care with the mortality rate being approximately 40%[1]. Sepsis is associated with secretary pancreatic dysfunction[2].

Heat shock proteins (HSPs) are highly conserved molecules present in both prokaryotic and eukaryotic cells and play an important role in cellular function and stress conditions. HSPs function as molecular chaperones and are categorized into HSP90, HSP70, HSP60, HSP20, and HSP8.5 on the basis of their molecular weight. HSP70 proteins constitute the central part of the chaperone system. In mammals, two isoforms of HSP70 exist in cytoplasm: a 73-ku constitutively expressed form (HSC70) and a 72-ku stress-inducible form (HSP70). HSPs are usually constitutively present at low concentrations in cells, but are actively synthesized to reach much higher concentrations as cells react to aggressive situations[3].

This study was to investigate the effect of different doses of LPS on the expression of HSP70 in a model of LPS-induced septic shock.

MATERIALS AND METHODS

Experimental animals

A total of 33 Wistar rats weighing 250±10 g were supplied by China Medical University Animal Department. All animals received humane care.

Induction of sepsis

A total of 33 Wistar rats were randomly divided into three groups. Small dose LPS group ($n=12$) was given a single dose of 5 mg/kg LPS, large dose LPS group ($n=12$) was

Table 1 Expression of HSP70 in the pancreas of the three groups

Groups	*n*	−	+	++	+++
LPS5 mg/kg	12	2	0	3	7
LPS10 mg/kg	12	4	1	3	4
Control group	9	2	6	1	0

given 10 mg/kg LPS, control group ($n=9$) was given the same volume of 0.9% normal saline. All rats were anesthetized with sodium pentobarbital (36 mg/kg). LPS (SIGMA, $O_{127}B_8$) was given via the femoral vein. A catheter was connected to the femoral artery to measure the mean arterial pressure. Death occurred after 3.5 h. The rats that survived were killed at 6 h. Pancreas were removed immediately and stored at -70 ℃ for later use.

Immunohistochemistry

Pancreatic samples were fixed in neutral buffered formalin, embedded in paraffin, sectioned, and mounted on slides. After being deparaffinized, endogenous peroxidase activity was quenched with 10% H_2O_2 for 10 min. After being blocked with goat serum, the sections were incubated with anti-mouse IgG polyclonal HSP70 antibody at 37 ℃ for 90 min, followed by incubation with S-A/HRP at 37 ℃ for 20 min. The Ag-Ab complex was visualized using diaminobenzidine method. Slides were then counterstained with hematoxylin for 1 min. After dehydration, the slides were coverslipped. To ensure specificity of the immunoreactions, control sections were subjected to the same immunohistochemical method with the exception that the primary Ab antibody was replaced with PBS.

Electromicroscopic study

Pancreatic tissue was cut into 1 mm × 1 mm × 1 mm pieces and placed in a freshly prepared fixative buffer (2.5% gluteraldehyde) at 4 ℃ for 2 h. After being fixed in 1% osmium tetroxide at 4 ℃ for 1 h, the tissue was exposed twice to an increased concentration of ethanol (30%, 50%, 70%, and 99.5%) at 5-min intervals for partial dehydration. Tissue was embedded with resin in gelatin capsules and incubated at 55-60 ℃ for 24 h. Ultra-thin (70 nm) sections were cut and transferred to 300-mesh nickel griads. After being stained with tannic acid, saturated uranyl acetate in 50% alcohol and 0.01% lead citrate, the samples were examined under transmission electron microscope (JEM-1200EX, Tokyo, Japan).

Western blot

Tissues were sonicated on ice for approximately 10 min until the tissues were completely homogenized in a solution containing 2 mmol/L EDTA, 2 mmol/L EGTA, 0.2 mmol/L NaCl, 20 mmol/L Tris-HCl, 1% Triton, 1 mmol/L DTT, and 1 mmol/L aprotinin. The homogenate was then centrifuged at 12000 *g* for 60 min at 4 ℃ and the resulting supernatant fraction was transferred to a fresh tube.

An equal amount (30 μg) of protein extracts was loaded and separated by 8% SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the proteins on the gel were transferred to polyvinylidene difluoride (PVDF) membrane. After being blocked with 5% nonfat dry milk at 4 ℃ overnight, the PVDF membranes were incubated with anti-rabbit polyclonal antibody of HSP70 diluted 1:200 with TPBS at 37 ℃ for 2 h. Then the blots were reacted with goat anti-rabbit antibody at room temperature for 2 h. Pre-stained molecular mass was used to estimate the positions of various proteins on the gel. Blots were stripped and incubated with a monoclonal Ab against β-actin (Sigma) to confirm that an equal protein was loaded. The target was then detected by enhanced chemiluminescence and exposed to X-ray film for appropriate time. The pictures were analyzed by GIS TANON.

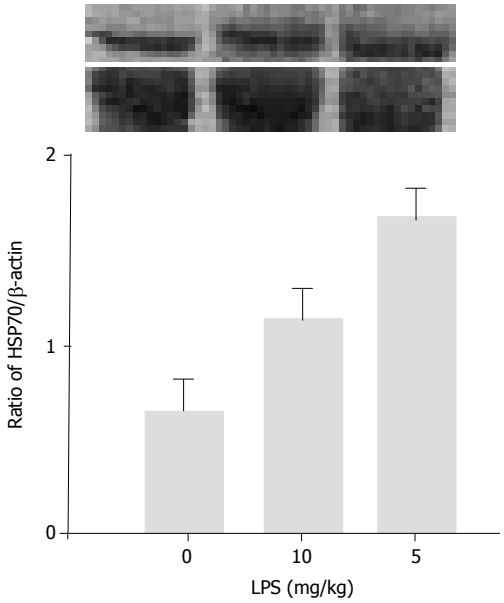

Figure 1 HSP70 expression in three groups.

Statistical analysis

All data were expressed as mean ± SD. $P<0.05$ was considered statistically significant.

RESULTS

TEM changes

No obvious ultra-structural damage was found in the control group. Swelling and loss of mitochondrial cristae of acinar cells and increased number of autophagic vacuoles in the cytoplasm of acinar cells were found in small dose LPS group. Swelling, vacuolization, and obvious myeloid changes of mitochondrial cristae of acinar cells and increased number of autophagic vacuoles in the cytoplasm of acinar cells were found in large dose LPS group (figure 2).

Immunohistochemistry changes

HSP70 was expressed at a low level in pancreatic acinar cells of the control group. After injection of LPS, HSP70 was highly expressed in the cytoplasm and nuclei of acinar cells and endothelia of blood vessels. Immunohistochemical examination showed overexpression

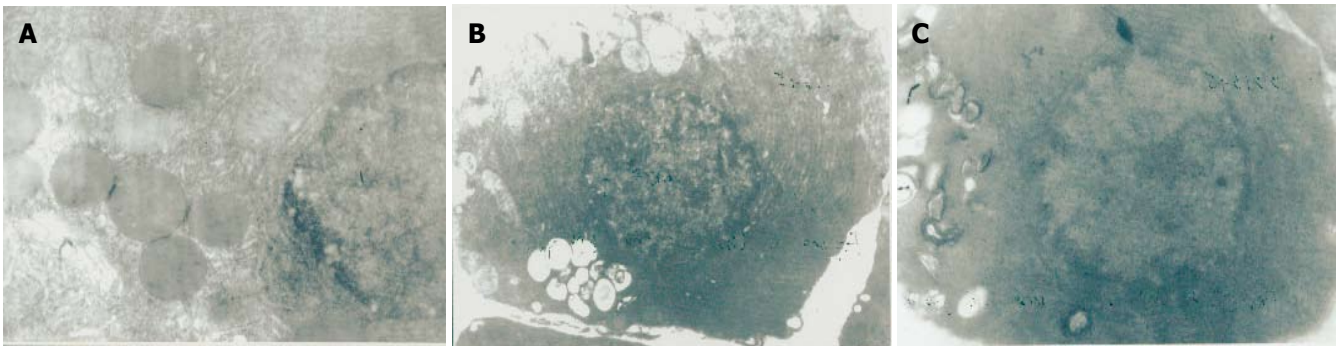

Figure 2 Ultra-structural changes of the pancreas in control (**A**),small dose LPS (**B**) and large dose LPS (**C**) groups.

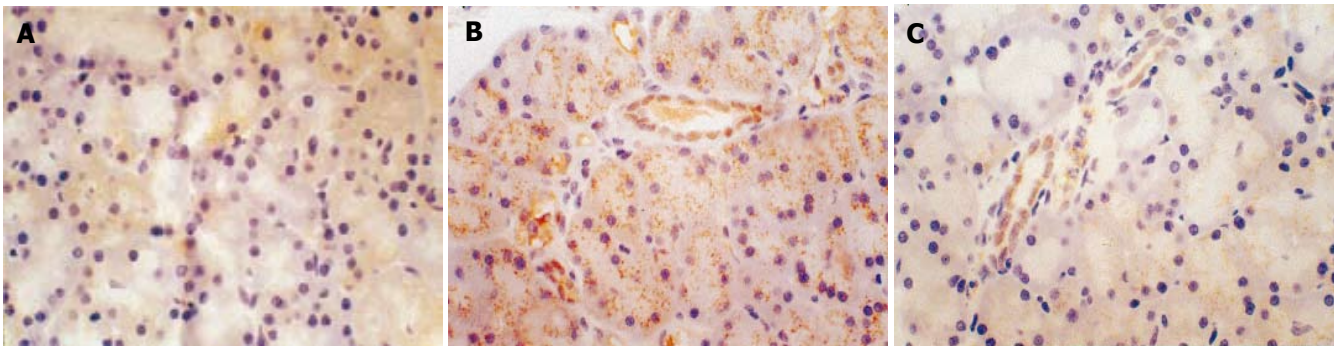

Figure 3 HSP70 expression in the pancreatic tissue of control (**A**), small dose LPS (**B**) and large dose LPS (**C**) groups.

of HSP70 in the vascular endothelial cells and cytoplasm of pancreatic tissue of the small dose LPS group. There were no significant differences between the two groups (Table 1).

Western blots

HSP70 expression in the control group was 112 ± 18.9 (0.62 ± 0.04). HSP70 expression in the large dose LPS group was 169 ± 13.4 (0.974 ± 0.04). HSP70 expression in the small dose LPS group was 211 ± 11.5 (1.34 ± 0.17) (Figure 1 and 3). β-Actin expression was constant, indicating the equal protein loading on the blots. HSP70 expression increased compared to the control group ($P < 0.05$).

DISCUSSION

This study showed that LPS could induce HSP70 expression in the pancreas of rats with endotoxic shock and the upregulation of HSP70 was associated with the doses of LPS and the extent of pancreatic ultra-structural injury. Endotoxin caused mitochondrial changes in the acinar cells of the pancreas and large doses of endotoxin led to myeloid changes of mitochondria. Small doses of LPS induced obvious overexpression of HSP70 and autophagocytosis, suggesting that high level of HSP70 promotes degradation of damaged proteins and ameliorates ultra-structural damages.

HSPs can protect cells from injury induced by environmental challenges, such as hypoxia, ischemia, high temperature, endotoxin, infection, and fever[4]. The protective effects of HSPs have been confirmed in various animal models of sepsis[5]. Several studies have demonstrated that a preceding HSP reaction reduces both mortality and organ dysfunction in experimentally induced severe sepsis[6]. Induction of HSPs in response to stress correlates with increased resistance to subsequent cellular damage. HSP70 is believed to act in a chaperone-like manner aiding in the passage of proteins across membrane barriers, preventing misfolding of newly synthesized proteins, facilitating elimination of improperly assembled, misfolded or aggregated proteins[7]. HSP70 contributes to the delivery of dysfunctional proteins to lysosomes for proteolytic degradation[8]. HSP70 is also known to facilitate antigen presentation in cells such as macrophages and dendrites[9]. Elevations in intracellular HSP levels have been shown to improve cell tolerance to inflammatory cytokines, such as TNF-α and IL-1β[10]. TNF-α and IL-1β are thought to be the main mediators in the pathogenesis of septic shock[11]. HSP70 expression protects the lung against ventilator-induced lung injury by decreasing cytokine transcription[12].

Mitochondrial oxidative phosphorylation is responsible for over 90% of total body oxygen consumption and ATP generation. HSP70 upregulation protects mitochondrial function after ischemia-reperfusion injury[13]. Increased levels of HSP70 through gene transfection lead to a greater protection of mitochondrial function after ischemia-reperfusion in a donor heart preservation protocol[13]. This may be one mechanism by which HSP70 overexpression leads to a better protection. Overexpression of HSP70 in the whole pancreas can protect mitochondrial integrity[14]. It was reported that structural deformity and decrease of respiratory chain enzyme activity in mitochondria and decline of ATP content are highly correlated with the deterioration of cardiac function during sepsis[15]. Heat shock

pretreatment prevents cardiac mitochondrial dysfunction during sepsis[16], thus achieving a protective goal.

HSP70 protection against LPS is most probably mediated through the modulation of iNOS activation and the subsequent decreased synthesis of nitric oxide (NO)[17]. NO plays an important role in the pathogenesis of septic shock[18]. A key role of HSP70 in the natural resistance of human beta cells against NO-induced injury is by preserving mitochondrial function[19]. Constitutive expression of HSP70 in human beta cells is essential for the natural resistance against NO-induced injury[19]. HSP70 prevents secretogogue-induced cell injury in the pancreas[20]. Hyperthermia protects against arginine-induced pancreatitis and induces HSP70 in the pancreas[21].

After entering blood, endotoxin combines with CD14[22]. Both CD14 and TLR2 are receptors of LPS[22]. TLR-4 and CD14 are involved in HSP70-mediated proinflammatory responses[23] and in HSP70-mediated activation of innate immunity[24]. Further investigation is necessary to elucidate the mechanism underlying the induction of endotoxin by HSP70.

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S- Editor Wang XL and Guo SY **L- Editor** Elsevier HK **E- Editor** Cao L

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World J Gastroenterol 2006 February 7; 12(5):784-790
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Interferon alpha plus ribavirin combination treatment of Japanese chronic hepatitis C patients with HCV genotype 2: A project of the Kyushu University Liver Disease Study Group

Norihiro Furusyo, Masaki Katoh, Yuichi Tanabe, Eiji Kajiwara, Toshihiro Maruyama, Junya Shimono, Hironori Sakai, Makoto Nakamuta, Hideyuki Nomura, Akihide Masumoto, Shinji Shimoda, Kazuhiro Takahashi, Koichi Azuma, Jun Hayashi, Kyushu University Liver Disease Study Group

Norihiro Furusyo, Jun Hayashi, Department of General Medicine, Kyushu University Hospital, Fukuoka, Japan
Masaki Katoh, Yuichi Tanabe, Department of Medicine, Fukuoka City Hospital, Fukuoka, Japan
Eiji Kajiwara, Department of Internal Medicine, Nippon Steel Yawata Memorial Hospital, Kitakyushu, Japan
Toshihiro Maruyama, Department of Medicine, Kitakyushu Municipal Medical Center, Kitakyushu, Japan
Junya Shimono, Department of Medicine, Yahata Saiseikai Hospital, Kitakyushu, Japan
Hironori Sakai, Department of Gastroenterology, National Hospital Organization Kyushu Medical Center, Fukuoka, Japan
Makoto Nakamuta, Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan
Hideyuki Nomura, Department of Internal Medicine, Shin-Kokura Hospital, Kitakyushu, Japan
Akihide Masumoto, Department of Clinical Research, National Hospital Organization, Kokura Hospital, Kitakyushu, Japan
Shinji Shimoda, Department of Medicine and Biosystemic Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan
Kazuhiro Takahashi, Department of Medicine, Hamanomachi Hospital, Fukuoka, Japan
Koichi Azuma, Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan
Correspondence to: Norihiro Furusyo, MD, PhD, Assistant Professor, Department of General Medicine, Kyushu University Hospital, Higashi-Ku, Fukuoka 812-8582, Japan. furusyo@genmedpr.med.kyushu-u.ac.jp
Telephone: +81-92-642-5909 Fax: +81-92-642-5916
Received: 2005-07-06 Accepted: 2005-08-03

Abstract

AIM: To determine the efficacy of an interferon alpha and ribavirin combination treatment for Japanese patients infected with hepatitis C virus (HCV) of genotype 2, a multi-center study was retrospectively analyzed.

METHODS: In total, 173 patients with HCV genotype 2 started to receive interferon-alpha subcutaneously thrice a week and 600–800 mg of ribavirin daily for 24 wk.

RESULTS: The overall sustained virological response (SVR), defined as undetectable HCV RNA in serum, 24 wk after the end of treatment, was remarkably high by 84.4%, (146/173) by an intention-to-treat analysis. A significant difference in SVR was found between patients with and without the discontinuation of ribavirin (46.9% *vs* 92.9%), but no difference was found between those with and without a dose reduction of ribavirin. A significant difference in SVR was also found between patients with less than 16 wk and patients with 16 or more weeks of ribavirin treatment (34.8% *vs* 92.0%).

CONCLUSION: The 24-wk interferon and ribavirin treatment is highly effective for Japanese patients with HCV genotype 2. The significant predictor of SVR is continuation of the ribavirin treatment for up to 16 weeks.

Key words: Hepatitis C virus; Interferon; Ribavirin; Genotype 2

Furusyo N, Katoh M, Tanabe Y, Kajiwara E, Maruyama T, Shimono J, Sakai H, Nakamuta M, Nomura H, Masumoto A, Shimoda S, Takahashi K, Azuma K, Hayashi J, Kyushu University Liver Disease Study Group. Interferon alpha plus ribavirin combination treatment of Japanese chronic hepatitis C patients with HCV genotype 2: A project of the Kyushu University Liver Disease Study Group. *World J Gastroenterol* 2006; 12(5): 784-790

http://www.wjgnet.com/1007-9327/12/784.asp

INTRODUCTION

The heterogeneity of the hepatitis C virus (HCV) genome has warranted the classification of the virus into different genotypes, with six major genotypes and more than 50 subtypes of HCV having been described till date[1-3]. The different genotypes may be important to the pathogenesis of the disease[4], response to antiviral therapy[5], and the diagnosis[6], as shown by molecular epidemiological studies and research on vaccine development.

A currently popular treatment regimen for the treat-

ment of chronic HCV infection in the world is pegylated interferon (IFN) alpha in combination with ribavirin. However, there was no data of response to such combination treatment for Japanese patients, because the treatment was just approved by the Japanese Minister of Health, Labour and Welfare in December 2004. Treatment with these drugs has resulted in a high rate of sustained virological response (SVR), over 50%[7,8]; however, the treatment duration is long, 48 wk and it causes various side effects, which are sometimes serious. Such a combination treatment is also expensive; a 24-wk treatment course costs approximately $20 000[9]. The efficacy and economic aspects need to be analyzed. Quite recently, a very short duration treatment for acute hepatitis C was shown to be highly effective[10].

The HCV genotype has been reported to be the most important predictor of IFN treatment response[7-13]. Patients infected with genotypes 2 and 3 have achieved about 65% SVR in a trial of 24-wk IFN alpha in combination with ribavirin, in contrast to patients with genotype 1 who had under 30% SVR[14,15]. Recently, multicenter studies in Europe and North America showed that patients with genotypes 2 and 3 were able to achieve a high SVR in a trial of 14-16 wk of pegylated IFN alpha in combination with ribavirin[16,17]. However, their analysis included very few genotype 2 patients: one included 23 genotype 2 patients and the other had 43 patients.

The distribution of HCV genotypes in Japan includes about 70% genotype 1b, with the remaining 30% genotypes 2a and 2b[18]. The SVRs to treatment of even shorter duration have not yet been reported for Japanese patients. Data are needed to define whether or not the duration of treatment with IFN alpha in combination with ribavirin can be reduced from 24 wk without compromising antiviral efficacy in patients chronically infected with HCV of genotype 2. This investigation has assessed the efficacy of a 24-wk combination treatment of IFN alpha and ribavirin for Japanese patients with HCV genotype 2 infection and focussed on the issue of the relationship between the duration of treatment and the efficacy.

MATERIALS AND METHODS

Patients

A retrospective study was done on Japanese patients treated between December 2000 and March 2004 that included 173 patients, 20 years or older, who satisfied the following criteria: (1) chronically infected with HCV genotype 2a or 2b; and (2) a history of an increased alanine aminotransferase (ALT) level for over 6 months. Criteria for exclusion were: (1) clinical or biochemical evidence of hepatic decompensation; (2) hemoglobin level less than 115 g/L, white blood cell count less than 3×10^9/L, and platelet count less than 50×10^9/L; (3) concomitant liver disease other than hepatitis C (hepatitis B surface antigen- or human immunodeficiency virus-positive); (4) alcohol or drug abuse; (5) suspected hepatocellular carcinoma; (6) severe psychiatric disease; and (7) treatment with antiviral or immunosuppressive agents prior to enrolment. Patients who fulfilled the above criteria were recruited at Kyushu University Hospital and 32 affiliated hospitals in the northern Kyushu area of Japan.

Informed consent was obtained from all the patients before enrollment in this study. The study was approved by the institutional Ethics Committees of the hospitals involved and conducted in accordance with the ethical guidelines of the Declaration of Helsinki and the International Conference on Harmonization of guidelines for good clinical practice.

Study design

All patients were treated with 6-10 MU of IFN alpha-2b (Intron A; Schering-Plough, Osaka, Japan) subcutaneously daily for the first 2 wk, then thrice a week for 22 wk. Ribavirin (Rebetol; Schering-Plough) was administrated orally for 24 wk at a daily dose of 600-800 mg based on the body weight (600 mg for patients weighing less than 60 kg and 800 mg for those weighing 60 kg or more). The above duration and dose were approved by the Japanese Minister of Health, Labour and Welfare. The 48-wk combination treatment and the ribavirin dosage of 1 000-1 200 mg recommended by the international guidelines were not permitted under the rules of the Japanese national health insurance system during the period of this study. The dose of ribavirin was reduced by 200 mg if the hemoglobin level fell to 100 g/L. Patients were considered to have ribavirin-induced anemia if the hemoglobin level decreased to less than 100 g/L. In such cases, a reduction in the dose of ribavirin was required. Both IFN alpha-2b and ribavirin were discontinued if the hemoglobin level, white blood cell count, or platelet count fell below 85 g/L, 1×10^9/L, and 2.5×10^9/L, respectively. The treatment was also discontinued if severe malaise developed, the continuation of treatment was judged not to be possible by the attending physician, or the patient desired to discontinue treatment.

Grouping by continuation or discontinuation of treatment

Patients were divided into the following four categories: Group A, patients who well tolerated the 24-wk combination treatment with IFN and ribavirin without a reduction in the dose of either drug; Group B, patients who received the full 24-wk combination treatment but who needed a reduction of the dose of IFN or ribavirin, or both; Group C, patients who discontinued the ribavirin treatment but continued the 24-wk IFN treatment; and Group D, patients who did not complete the 24 wk of treatment, because of adverse effects or who dropped out.

Determination of HCV RNA and HCV genotype and serotype

The serum HCV RNA level was examined with an Amplicor HCV monitor assay (version 2.0) (Roche, Tokyo, Japan), with a lower limit of quantitation of 500 IU (135 copies/mL) and an outer limit of quantitation of 850 000 IU/mL. Samples with HCV RNA over the limit of 850 000 IU/L were not diluted to determine the levels between 850 000-5 000 000 IU/mL. HCV RNA was also examined with the qualitative Amplicor HCV assay (Roche). HCV genotype was determined by type-specific primer from the core region of the HCV genome. The protocol for genotyping was carried out as described earlier[11,12].

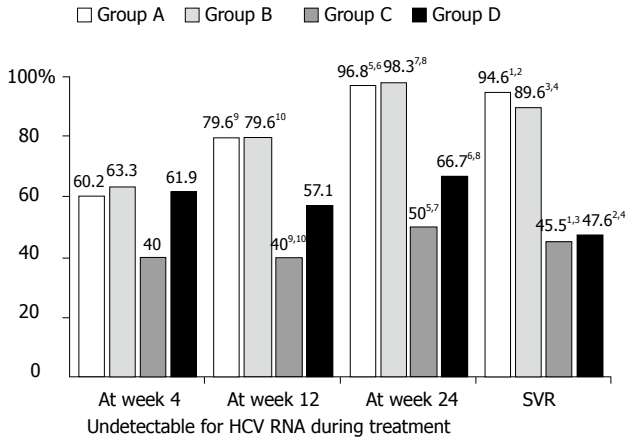

Figure 1 The sustained virological response (SVR) rate and undetectable hepatitis C virus (HCV) RNA rates during the treatment of 173 patients, classified by continuation and discontinuation of interferon and ribavirin combination treatment. Group A patients ($n=93$) who well tolerated the 24-week treatment with IFN and ribavirin in combination without any reduction in the dose of either drug; Group B patients ($n=48$) received the 24-week combination treatment, but needed a dose reduction of IFN or ribavirin, or both; Group C patients ($n=11$) discontinued the ribavirin treatment, but continued the full 24 weeks of IFN treatment; Group D patients ($n=21$) did not complete the 24 weeks of treatment because of adverse effects ($n=17$) or dropped out ($n=4$). [1]$P=0.0001$; [2]$P<0.0001$; [3]$P=0.0031$; [4]$P=0.0003$; [5]$P=0.0001$; [6]$P=0.0002$; [7]$P=0.0003$; [8]$P=0.0002$; [9]$P=0.124$; [10]$P=0.0182$.

Histological examination

Liver biopsy was done for 117 patients infected with genotype 2 within the 6 months before the start of the treatment. For each specimen, a stage of fibrosis and a grade of activity were established according to the following criteria. Fibrosis was staged on a scale of 0-4: F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = few septa, F3 = numerous septa without cirrhosis, F4 = cirrhosis. The grading of activity, including the intensity of the necroinflammation, was scored as follows: A0 = no histological activity, A1 = mild activity, A2 = moderate activity, A3 = severe activity. Liver biopsy was not available from 56 patients who declined to have a biopsy.

Efficacy of treatment

The SVR was defined as undetectable HCV RNA by the qualitative Amplicor HCV assay (Roche) and a normal ALT level (under 40 IU/L) at 6 months after the end or stoppage of the treatment. Patients not achieving a SVR were considered as non-SVR. Patients who had undetectable HCV RNA within 4 wk of the start of treatment were considered to have had an early virological response (EVR).

Statistical analysis

The analysis of SVR was done on an intention-to-treatment basis, including dropouts, who were counted as non-sustained virological responders, and patients who stopped treatment. The χ^2 test or Fisher's exact test was used to examine the association between baseline characteristics and SVR. The Mann-Whitney *U* test was also used to compare responders and non-responders with regard to various characteristics, when appropriate. Independent factors associated with SVR were studied using forward stepwise logistic regression analysis of the variables. Forward stepwise logistic regression analysis was done using a commercially available software package (BMDP Statistical Software Inc., Los Angeles, CA, USA) for the IBM 3090 system computer. A *P*-value of less than 0.05 was considered significant. All *P*-values were two tailed.

RESULTS

Patient characteristics, dose reduction and discontinuation of treatment regimen

The distribution of Groups A, B, C, and D patients was 93 (53.8%), 48 (27.7%), 11 (6.4%), and 21 (12.1%), respectively. Completing the 24-week ribavirin treatment were 141 patients in Groups A and B. Thirty-two patients of Groups C and D discontinued the ribavirin treatment.

The pretreatment characteristics of these four groups of patients are summarized in Table 1 and 2. The median age was significantly younger in Group A (51 years) than in Groups B (56 years) and C (59 years). Significantly more men were in Group A (71.0%) than in Group B (33.3%). The median creatinine clearance was significantly higher in Group A (110 mL/min) than Groups B (92 mL/min) and C (85 mL/min). The median hemoglobin level was significantly higher in Group A (150 g/L) than Groups B (136 g/L), C (134 g/L), and D (134 g/L). The median platelet count was significantly higher in Group A (168 $\times 10^9$/L) than in Group C (127×10^9/L). No notable differences between the groups were found in body weight, ribavirin dose, HCV RNA level, genotype, or histology.

Virological response

SVR was achieved by 146 (84.4%) of 173 patients. The SVR did not differ between patients with genotypes 2a and 2b (83.1% *vs* 84.6%). The SVRs were 82.4% (14 of 17) (under 100 kIU/mL), 84.2% (16 of 19) (100-199 kIU/mL), 85.7% (24 of 28) (200-299 kIU/mL), 83.3% (15 of 18) (300-399 kIU/mL), 100% (12 of 12) (400-499 kIU/mL), 76.9% (10 of 13) (500-599 kIU/mL), 77.8% (7 of 9) (600-699 kIU/mL), 90.9% (10 of 11) (700-799 kIU/mL), and 82.6% (38 of 46) (800 and over kIU/mL). The SVRs were 76.9-100%. The SVRs of the HCV genotype 2 patients with any level of viremia level did not significantly differ.

Figure 1 shows the SVR and undetectable HCV viremia rate during the treatment of 173 patients, classified by continuation and discontinuation of combination treatment. The SVRs were significantly higher in Groups A (94.6%) and B (89.6%) than in Groups C (45.5%) and D (47.6%). A significant difference of SVR was found between patients with and without discontinuation of ribavirin (46.9%, 15 of 32 of Groups C and D patients *vs* 92.9%, 131 of 141 of Groups A and B patients, $P<0.0001$). During the treatment period, except for at week 4, the rates of undetectable HCV RNA were also significantly higher in Groups A and B than in Groups C and D.

Figure 2 shows the relationship between SVR and the ribavirin treatment period in all the patients. A significant difference was found between patients with less than 16 wk of treatment period and patients with longer periods

Table 1 Baseline characteristics

Characteristic	Complete Ribavirin treatment (*n* = 141)		Discontinued Ribavirin treatment (*n* = 32)		All patients (*n* = 173)
	Group A (*n* = 93)	Group B (*n* = 48)	Group C (*n* = 11)	Group D (*n* = 21)	
Median age (yr)	51[1,2]	56[1]	59[2]	50	53
(range)	(20-73)	(25-70)	(53-73)	(29-73)	(20-73)
Male (%)	66 (71.0)[3]	16 (33.3)[3]	5(50.0)	13 (61.9)	100 (57.8)
Body weight					
60 kg or more (%)	60 (64.5)	23 (47.9)	6 (54.5)	12 (57.1)	101 (58.3)
Ribavirin dose by weight					
12 mg/kg or more (%)	24 (25.8)	20 (41.7)	4 (36.4)	9 (42.8)	57 (32.9)
Creatinine clearance (mL/min)	110[4,5]	92[4]	85[5]	101	102
(range)	(53-261)	(46-167)	(60-111)	(41-203)	(41-261)
HCV RNA level					
500 kIU/mL or more (%)	44 (47.3)	22 (45.8)	4 (36.4)	9 (42.8)	79 (45.7)
Genotype 2a (%)	67 (72.0)	28 (58.3)	6 (54.5)	13 (61.9)	114 (65.9)

[1]$P = 0.0401$; [2]$P = 0.0044$; [3]$P < 0.0001$; [4]$P = 0.0002$; [5]$P = 0.0248$

Table 2 Baseline characteristics (continued)

Characteristic	Complete Ribavirin treatment (*n* = 141)		Discontinued Ribavirin treatment (*n* = 32)		All patients (*n* = 173)
	Group A (*n* = 93)	Group B (*n* = 48)	Group C (*n* = 11)	Group D (*n* = 21)	
Histology					
Stage of fibrosis					
F0 – F1 (%)	27 (43.5)	17 (50.0)	4 (50.0)	9 (42.9)	56 (47.9)
F2 – F3 (%)	35 (56.5)	15 (44.1)	4 (50.0)	6 (28.6)	59 (50.4)
F4 (%)	0 -	2 (5.9)	0 -	0 -	2 (1.7)
Not determined	31	14	3	6	54
Grade of activity					
A0 – A1 (%)	27 (43.5)	17 (50.0)	4 (50.0)	9 (42.9)	43 (47.9)
A2 (%)	35 (56.5)	15 (44.1)	4 (50.0)	6 (28.6)	58 (50.4)
A3 (%)	0 -	2 (5.9)	0 -	0 -	16 (1.7)
Not determined	31	14	3	6	54
Median hemoglobin (g/L)	150[6,7,8]	136[6]	134[7]	134[8]	144
(range)	(117-171)	(116-163)	(121-152)	(12.1-153)	(116-171)
Median platelet count ($X 10^9$ /L)	168[9]	167	127[9]	157	162
(range)	(79-385)	(58-363)	(55-181)	(57-240)	(55-385)

[6]$P = 0.0003$; [7]$P = 0.0063$; [8]$P = 0.0225$; [9]$P = 0.0120$

(34.8%, 8 of 23 *vs* 92.0%, 138 of 150, $P < 0.0001$), showing that 16 wk of ribavirin treatment significantly contributed to a SVR. Of the 173 studied patients, 104 (60.1%) had an EVR, defined as undetectable HCV RNA within 4 wk of the start of treatment. The SVR was 94 (90.4%) of these 104 patients with EVR, which was significantly higher than the non-EVR patients (52 of 69, 75.4%) ($P = 0.0142$). No significant differences were found between patients with and without undetectable HCV RNA at 8 or 12 wk of the start of treatment. Moreover, we analyzed the relationship between SVR and the length of ribavirin treatment in the 104 patients with EVR. A significant difference was found between patients with less than 16 wk of ribavirin treatment and those with a longer treatment period (46.2%, 6 of 13 *vs* 96.7%, 88 of 91, $P < 0.0001$). These findings showed that 16 wk of ribavirin treatment significantly contributed to a SVR, even in patients with EVR.

Factors contributing to SVR

To assess the independent role of the IFN and ribavirin combination treatment on SVR, an adjustment by forward stepwise logistic regression analysis for all other independent risk factors identified was done. The continuation of ribavirin treatment ($P < 0.0001$) was significantly associated with SVR in analysis of all the patients. A higher SVR (odds ratio = 13.15) was found for patients who continued to receive ribavirin treatment than for those who discontinued it. Other factors such as sex, age, HCV genotype, pretreatment-HCV RNA level, histological findings, pretreatment platelet count and creatinine clearance, history

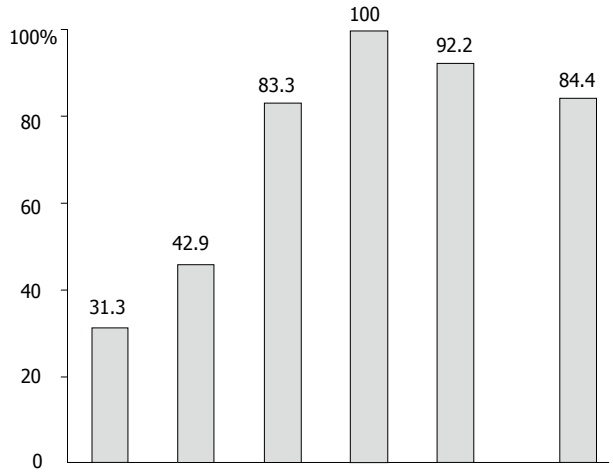

Figure 2 Relationship between the sustained virological response rates and the length of ribavirin treatment period of the 173 studied patients.

of prior IFN, and dose reduction of IFN or ribavirin were not significantly, independently associated with a SVR.

DISCUSSION

The large number of Japanese HCV genotype 2 patients enrolled in this study was sufficient to provide for meaningful statistical analysis, even though it was retrospective. This study demonstrated that a 24-wk IFN and ribavirin combination treatment was highly effective and resulted in a remarkably high SVR (84.4%) in genotype 2 patients, as expected. Importantly, we also showed that dose reductions of ribavirin were not associated with a poor outcome in these patients, only ribavirin discontinuation, and that the addition of ribavirin for up to 16 wk contributed to the high SVR.

In December 2004, pegylated IFN plus ribavirin combination treatment received the official approval in Japan. The combination treatment was not yet approved for clinical use for patients with chronic HCV viremia by the Japanese Ministry of Health, Labour and Welfare at the time of the present study. So far, our most effective and available treatment is the 6-month IFN-alpha plus ribavirin combination.

Remarkably high SVRs were observed for our patients with genotype 2 who took the IFN and ribavirin combination treatment. IFN monotherapy does not result in a satisfactory outcome for patients with chronic hepatitis C, particularly those with genotype 1, which is known to be IFN-resistant, whereas genotype 2 is IFN-sensitive[11-13]. The addition of ribavirin, a synthetic purine nucleoside analog, to IFN enhances the virological response[8,9,13-17]. Our research group, KULDS, also analyzed the data of patients with genotype 1 who were treated with this 24-wk combination treatment: SVR was achieved by 21% of 528 patients with genotype 1 by intention-to-treat analysis (data not published). Differences between genotype 1 and 2 patients still existed following the ribavirin combination treatment. Moreover, a striking finding in our study was that there were no differences among the patients with genotype 2 of any HCV RNA level (76.9-100%). The precise mechanism is unclear, although it possibly originates in different nucleotide sequence of their genome. Further study is needed to clarify the reasons for the differences in antiviral effect, by the use of novel and new tools for the quantification of the HCV replication system[19,20].

How long the ribavirin needs to be administrated to achieve the best efficacy with IFN alpha-treated patients of genotype 2 is unclear. In the present study, SVR after 16 or more weeks of treatment ranged from 83.3% to 100% and was not dependent on the dose reduction of ribavirin treatment but on the discontinuation of IFN or ribavirin treatment. A pilot study from Norway showed that patients with genotype 2 and an EVR obtained a high SVR after 14 weeks of pegylated IFN and ribavirin combination treatment[21]. The Zeuzem group also demonstrated a very high SVR in a 24-week pegylated IFN and ribavirin treatment for genotype 2 patients, and 16-week treatment duration was observed to be a significant independent predictor[17]. In view of the adverse effects, high cost of ribavirin, and the above mentioned findings along with our results, a 16-week ribavirin addition to IFN treatment would seem to produce a high rate of SVR for patients with genotype 2, especially for those with EVR, defined as undetectable HCV RNA within 4 wk of the start of the treatment.

The Davis group attempted to confirm that an EVR in patients with chronic hepatitis C undergoing initial treatment with a combination therapy of pegylated IFN alpha and ribavirin was predictive of SVR[22]. Retrospective analysis of data from other trials[23] has also suggested that patients who do not attain EVR have a nominal chance of SVR with additional weeks of treatment. While the primary goal, or "holy grail", of treatment of chronic hepatitis C is SVR, it must be acknowledged there are other secondary goals that compel physicians to continue treatment without EVR. In fact, patients who do not achieve EVR or SVR may have histological benefit[24], leading to a decreased risk of hepatocellular carcinoma[19]. Thus, it remains to be determined whether or not early discontinuation of treatment would reduce economic costs if a long-term perspective is taken.

Several adverse reactions are associated with ribavirin. One of the most significant reactions is hemolytic problems, especially anemia[15]. Most of our patients who had to have a dose reduction or who discontinued ribavirin were observed to have anemia. It is important to reduce the dose of ribavirin at as early a stage as possible to allow the safe continuation of the combination treatment. The Nomura group pointed out that careful administration is necessary in patients over 60 years, in female patients, and in patients receiving a ribavirin dose by body weight of 12 mg/kg or more[21]. Our forward stepwise logistic regression analysis showed that the continuation of ribavirin treatment was significantly associated with SVR. This combination treatment, which could depend on hemolytic adverse reaction, has a high efficacy, if physicians are able to continue the ribavirin treatment for as short a period as

16 wk, even when taking into account of the dose reductions necessary for patients with a dangerous decrease of hemoglobin caused by ribavirin, as often seen in genotype 2 patients with a low hemoglobin level at pretreatment.

In conclusion, the 24-week IFN and ribavirin combination treatment was highly effective and resulted in a remarkably high SVR in Japanese HCV patients with genotype 2 from the retrospective study of ours. The most significant predictor was continuation of the ribavirin treatment for up to 16 wk. These findings are not pertinent to the other different genotypes.

ACKNOWLEDGMENTS

In addition to the authors, the following investigators of the KULDS Group were involved in the present study: H Nakashima and M Murata, Haradoi Hospital, Fukuoka, K Toyoda, Yokota Hospital, Hirokawa, Fukuoka, H Takeoka, T Kuga and A Mitsutake, Mitsutake Hospital, Iki, Nagasaki, R Sugimoto, Harasanshin Hospital, Fukuoka: H Amagase and S Tominaga, Mihagino Hospital, Kitakyushu: K Yanagita, Saiseikai Karatsu Hospital, Karatsu: K Ogiwara, Kyusyu Rosai Hospital, Kitakyushu: M Tokumatsu, Saiseikai Fukuoka Hospital, Fukuoka: S Tabata, Hayashi Hospital, Fukuoka: M Yokota, National Kyushu Cancer Center, Fukuoka: H Tanaka, Chihaya Hospital, Fukuoka: S Nagase, Fukuoka Teishin Hospital, Fukuoka: S Tsuruta, Nakabaru Hospital, Fukuoka: S Tada, Moji Rosai Hospital, Kitakyushu: M Nagano, Kyushu Koseinenkin Hospital, Kitakyushu: M Honda, Nishi-Fukuoka Hospital, Fukuoka: T Umeno, Sawara Hospital, Fukuoka: T Sugimura, National Hospital Organization Fukuoka Higashi Hospital, Fukuoka: S Ueno, Kitakyushu Municipal Wakamatsu Hospital, Kitakyushu: K Miki, Kitakyushu Municipal Moji Hospital, Kitakyushu: H Okubo, Shineikai Hospital, Kitakyushu: H Fujimoto, Mitsubishikagaku Hospital, Kitakyushu: N Higuchi, Shin-Nakama Hospital, Kitakyushu: S Shigematsu, Kouseikan Hospital, Saga: N Higashi, National Hospital Organization Beppu Hospital, Beppu, Japan.

We greatly thank Hironori Ebihara, Kazukuni Kawasaki and Toshihiro Ueda for their advice for this study.

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S- Editor Guo SY **L- Editor** Elsevier HK **E- Editor** Wu M

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World J Gastroenterol 2006 February 7; 12(5):791-795
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Omeprazole maintenance therapy prevents recurrent ulcer bleeding after surgery for duodenal ulcer

Konstantinos Demertzis, Dimitrios Polymeros, Theodoros Emmanuel, Konstantinos Triantafyllou, Pericles Tassios, Spiros D Ladas

Konstantinos Demertzis, Dimitrios Polymeros, Theodoros Emmanuel, Konstantinos Triantafyllou, Pericles Tassios, Spiros D Ladas, Hepatogastroenterology Unit, 2nd Department of Internal Medicine, "Attikon" University General Hospital, 11528 Athens, Greece
Correspondence to: Professor Spiros D Ladas, MD, PhD, Hepatogastroenterology Unit, 2nd Department of Internal Medicine, "Attikon" University General Hospital, 23 Sisini Street, 11528 Athens, Greece. sdladas@hol.gr
Telephone: +30-210-5326442 Fax: +30-210-5326422
Received: 2005-05-24 Accepted: 2005-07-20

Abstract

AIM: To evaluate the omeprazole maintenance therapy in patients with recurrent ulcer bleeding after surgery for duodenal ulcer.

METHODS: We studied 15 consecutive patients with recurrent ulcer bleeding after surgery for duodenal ulcer. Omeprazole (20 mg/d) maintenance therapy was given after ulcer healing. In addition to clinical follow-up, ambulatory 24-h gastric pH assay was performed before and during omeprazole therapy in those patients and controls with previous duodenal ulcer surgery but no ulcer recurrence.

RESULTS: All the 15 ulcers were healed after being treated with omeprazole (40 mg/d) for 2 mo. Eleven patients with two (1-9) episodes of recurrent ulcer bleeding completed the follow-up (43, 12-72 mo). None of them had a bleeding episode while on omeprazole. One patient discontinued the therapy and had recurrent bleeding. The median 24-h fraction time of gastric pH <4 in patients was 80, 46-95%, and was reduced to 32, 13-70% by omeprazole ($P = 0.002$).

CONCLUSION: Long-term maintenance therapy with omeprazole (20 mg/day) is effective in preventing recurrent ulcer bleeding.

Key words: Duodenal ulcer; Gastrectomy; Vagotomy; Omeprazole

Demertzis K, Polymeros D, Emmanuel T, Triantafyllou K, Tassios P, Ladas SD. Omeprazole maintenance therapy prevents recurrent ulcer bleeding after surgery for duodenal ulcer. *World J Gastroenterol* 2006; 12(5):791-795

http://www.wjgnet.com/1007-9327/12/791.asp

INTRODUCTION

H pylori eradication therapy and the use of potent proton pump inhibitors (PPIs) have dramatically reduced the need for surgical therapy of peptic ulcer disease. Still, about 10% of duodenal ulcer patients undergo emergency surgical therapy for acute ulcer bleeding[1]. However, recurrent ulcer is not uncommon as it occurs in 10-15% of patients after vagotomy and drainage and in 2-5% of patients after gastric resection[2]. This may be complicated by life threatening acute recurrent ulcer bleeding in certain patients, requiring hospitalization.

Several studies have investigated the rate of ulcer recurrence after duodenal ulcer surgery[2,3] and the completeness of vagotomy[4,5], but only a few studies have evaluated the anastomotic ulcer healing rates after being treated with H_2 receptor antagonists (H_2RA)[6,7] or PPI[8] therapy. Studies have shown that *H pylori* infection of the gastric mucosa is not related to ulcer recurrence after gastric surgery[4,9,10]. Furthermore, it has been shown that 28% of anastomotic ulcers recur within 6 wk after discontinuing cimetidine therapy[7], and 33% relapse within a year while on cimetidine maintenance therapy[6].

These patients are often treated with a second operation[1]. However, to the best of our knowledge, there are no studies investigating the long-term outcome of patients with recurrent post-surgical ulcer and whether maintenance acid suppression therapy with PPIs may prevent recurrent ulceration and/or re-bleeding. Therefore, the present prospective open label study was conducted to investigate gastric pH profile and the effect of omeprazole maintenance therapy in patients presented with recurrent ulcer bleeding after duodenal ulcer surgical therapy.

MATERIALS AND METHODS

Over a 7-year period, this prospective open label study included 15 consecutive male patients admitted to our department due to recurrent acute ulcer bleeding. All patients underwent gastric surgery for duodenal ulcer disease at least 2 years ago.

Clinical study

In each case, emergency endoscopy was performed to confirm recurrent ulcer bleeding. The finding of an ulcer was considered as the bleeding cause if active bleeding or stigmata of recent hemorrhage were noted in the absence of other lesions. The recurrent ulcers were peristomal or duodenal in location. At the same time, detailed history was obtained about the indication and time of past gastric operation and the number of hospital admissions with hematemesis or melena after gastric surgery. History specifically included questions about the use of H_2RA, PPIs or non-steroidal anti-inflammatory drugs (NSAIDs)[11], smoking and alcohol abuse. In all the patients fasting serum gastrin and salicylate concentrations were determined to exclude Zollinger–Ellison syndrome and recent consumption of non-steroidal antiinflammatory drugs. Patients who were on non-steroidal anti-inflammatory drugs were excluded. During endoscopy, multiple gastric mucosal biopsies were obtained to investigate *H pylori* infection.

All patients were initially treated with intravenous omeprazole (20 mg every 12 h) and then orally after discharge from the hospital. *H pylori* eradication therapy was not used to prevent ulcer recurrence[10,12], but *H pylori* was eradicated in two patients because of severe *H pylori* gastritis. Follow-up endoscopy was scheduled at 2 mo, while on oral omeprazole (40 mg/d) to confirm ulcer healing. Thereafter, the patients were instructed to receive oral omeprazole (20 mg/d) maintenance therapy, to avoid the use of any non-steroidal anti-inflammatory drugs and to have follow-up every 6 mo as outpatients.

Twenty-four-hour gastric pH studies

Twenty-four-hour gastric pH studies were performed in the following groups on omeprazole therapy (20 mg/d) but not on antisecretory therapy: patients with first or second degree reflux esophagitis (Los Angeles classification) ("normal" controls); patients with duodenal ulcer; controls who underwent vagotomy and pyloroplasty or gastrojejunostomy for duodenal ulcer but had no ulcer recurrence; controls who had Billroth II partial gastrectomy and patients who had recurrent anastomotic ulcer bleeding after gastric surgery for duodenal ulcer. In the latter group of patients (test group), 24-h gastric pH studies were performed while on omeprazole (40 mg/d, 20 mg/d). Omeprazole was then discontinued and the patients were treated with ranitidine 150 mg twice daily for 2 d, followed by a 2-d washout period before the pH study in patients not on antisecretory therapy. The duodenal ulcer group included patients admitted to our department for acute ulcer bleeding and volunteered to have 24-h pH studies. The vagotomy and gastrectomy control groups included patients who attended the outpatient clinic for various epigastric symptoms and volunteered to participate in the study after having a negative gastroscopy.

Gastric ambulatory pH monitoring was performed using a monocrystalline antimony pH catheter. The electrode was passed transnasally into the stomach, 10-15 cm below the detectable esophagogastric junction by endoscopy. The catheter was connected to a portable pH-recording device (Digitrapper Mk III, Synectics Medical AB, Stockholm, Sweden). Recorded data were uploaded into the "EsopHogram Analysis Software" for analysis and review. During the 24-h pH studies, patients were encouraged to keep up their usual activities and diet in order to maximize the diagnostic yield of the test. The 24-h fraction (%) time when the gastric pH was below 4 was calculated.

All patients and volunteers gave oral consent after being informed of the purpose of the study by the investigator. The study protocol was approved by the Ethics Committee on Human Studies, Department of Internal Medicine, Medical School of the Athens University. The trial was conducted according to the declarations of Helsinki.

Statistical analysis

We used non-parametric statistics ["Statgraphics-Plus" version 4 for Windows (Manugistics Inc., Rockville, USA)], because of the small number of observations included in each group. Results were presented as median with ranges. We used the non-parametric Mann-Whitney (Wilcoxon) two-sided *U*-test and the Kruskal-Wallis *t*-test for two and multiple-sample comparison analysis, respectively[13]. $P < 0.05$ was considered statistically significant.

RESULTS

Clinical data and follow-up

Over a 7-year period, 15 consecutive male patients with recurrent ulcer bleeding after surgery for duodenal ulcer were admitted to our department because of acute bleeding episode. Serum gastrin was normal and salicylates were not detected in the serum in any of the 15 patients during their hospitalization. In addition, all patients denied the use of non-aspirin, non-steroidal anti-inflammatory drugs after thorough interrogation.

Ulcer in all the 15 patients was healed after treatment with omeprazole (40 mg/d) for 2 mo. However, four of them did not consent to gastric pH studies and were lost to clinical follow-up within the first year from entering the study. Therefore, data were presented for 11 patients (Table 1).

Two out of the eleven patients had a history of two gastric operations because of recurrent ulcer bleeding. The time of recurrent ulcer bleeding after gastric surgery was 6 years, averaged 2-18 years. The number of hospital admissions because of post surgical ulcer bleeding was 2, averaged 1-9. At study entry, 3/11 patients were smokers and 5/11 were *H pylori*-positive. *H pylori* was successfully eradicated in two of these five patients upon admission to the study. One of the *H pylori*-negative patients had successful triple eradication therapy at another hospital a year ago.

No recurrent ulcer or re-bleeding occurred during the maintenance therapy (omeprazole 20 mg/d) over the follow-up period (43 mo, averaged 12-72 mo). One patient discontinued treatment and had recurrent bleeding 8 mo after stopping omeprazole. Currently, he was symptom-free on omeprazole maintenance therapy. There were no significant adverse events related to treatment.

Table 1 Background information of patients who had recurrent ulcer bleeding after surgery for peptic ulcer disease

Case	Age (yr)	Smoking status	*H. pylori* status	Type of operation	Years since operation	Bleeding episodes after surgery	Follow -up (mo)
1	62	Never	Positive1	TV+D[2]	5, 2[3]	3	72
2	60	Ex-smoker	Positive	TV+D	6	8	62
3	61	Smoker	Positive1	BII	2	1	61
4	66	Never	Negative	BII+V[2]	4, 3[3]	2	56
5	49	Smoker	Positive	TV+D	7	1	52
6	70	Ex-smoker	Negative	BII	18	2	43
7	64	Never	Negative	BII	3	2	35
8	59	Ex-smoker	Negative	BII	16	4	32
9	66	Smoker	Negative	TV+D	7	5	28
10	52	Ex-smoker	Positive	TV+D	5	2	18
11	48	Ex-smoker	Negative	TV+P	13	9	12

[1]Successful *H. pylori* eradication therapy upon entering the study. [2]Final operation. [3]Years elapsed since the first and second gastric operation, upon entering the study. TV+D: truncal vagotomy and gastrojejunostomy. TV+P: truncal vagotomy and pyloroplasty. BII: Billroth II partial gastrectomy.

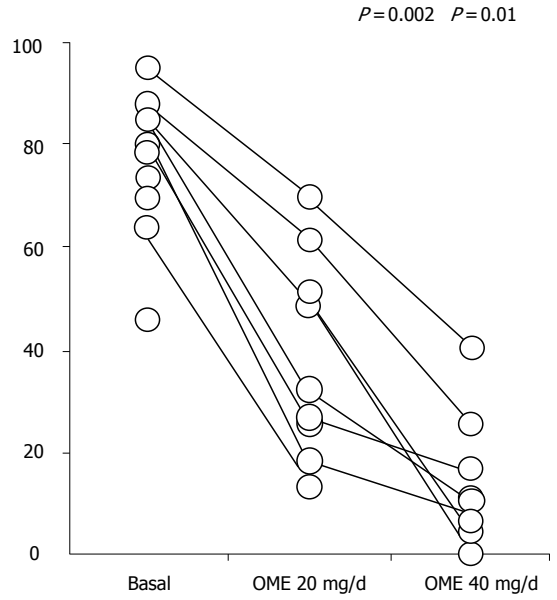

Figure 1 Individual data points of the 24-h intra gastric pH monitoring studies while on no treatment (baseline), and during therapy with 20 or 40 mg/d omeprazole (OME) in patients with recurrent post-surgical ulcer bleeding.

Twenty-four-hour gastric pH studies

Results of the 24-h intragastric pH monitoring studies are shown in Table 2. The "normal" control group included 10 patients with first or second degree reflux esophagitis, who were matched for age, sex and *H pylori* status with the recurrent post-surgical ulcer patients. They were selected from a pool of patients with reflux esophagitis on 24-h esophageal-gastric pH monitoring. The duodenal ulcer control group included seven patients hospitalized because of duodenal ulcer bleeding. The vagotomy control group included seven patients who had truncal vagotomy and drainage for duodenal ulcer. The gastrectomy control group included seven patients who had partial gastrectomy (Billroth II) for duodenal ulcer. Volunteers of the vagotomy and gastrectomy control groups studied at least 2 years postoperatively had no postoperative ulcer recurrence. The test group included ten of the eleven patients who had truncal vagotomy ($n = 5$) or Billroth II gastrectomy ($n = 5$) and recurrent anastomotic ulcer bleeding. Intolerance to nasogastric pH probe was not studied in another patient who had vagotomy and drainage. There were three failures out of 30 24-h gastric pH recordings in the test group, as shown by missing values in Figure 1. One of the vagotomy controls refused to have the 24-h pH study on omeprazole.

Treatment-free (baseline) 24-hour gastric pH studies

The 24-h gastric pH measurements of the groups studied during treatment-free period are shown in Table 2. There was a statistically significant difference in the 24-h fraction time of gastric pH < 4 among the six groups ($T = 29.10$, $P < 0.001$), with the lowest values recorded in the Billroth II gastrectomy control group. Patients who had vagotomy and recurrent ulcer bleeding had a significantly different (higher) 24-h fraction time of gastric pH < 4 as compared to vagotomy controls ($U = 32$, $P = 0.023$). This difference was also significant when bleeding patients after gastrectomy were compared to those of the gastrectomy control group ($U = 35$, $P = 0.006$).

Twenty-four hour gastric pH studies (omeprazole 20 mg/day)

Gastric pH studies were performed in the control and patient (test) groups on omeprazole (20 mg/d). Billroth II gastrectomy control group did not undergo this study because of the low fraction time of gastric pH < 4 recorded during treatment-free gastric pH monitoring. Omeprazole therapy (20 mg/d) significantly reduced the 24-h fraction time of gastric pH < 4 in both control and patient groups (Table 2).

Twenty-four hour gastric pH studies in patient group

Eight out of the ten patients underwent all (baseline 20 mg/day and 40 mg/day omeprazole) 24-h gastric pH studies. One additional patient underwent only baseline, and another patient both baseline and omeprazole (20 mg/day) 24-h gastric pH studies. Individual data are shown in Figure 1. The 24-h fraction time of gastric pH < 4 (80, 46-95%) was significantly reduced by omeprazole (20 mg/d) to 32, 13-70% ($U = 6$, $P = 0.002$) and by omeprazole (40 mg/d) to 11, 1-40% (omeprazole 20 mg/day *vs* 40 mg/day, $U = 9$, $P = 0.01$).

DISCUSSION

The data of the present study showed that recurrent ulcers after gastric surgery for duodenal ulcer can heal after 2 months of 40 mg/day omeprazole therapy. Even more important, none of our patients had a re-bleeding episode while on omeprazole maintenance therapy over a 3.5-year follow-up period.

A large review is available on the complications associated with ulcer recurrence following gastric surgery including 130 studies published on this topic over a

Table 2 Demographic data of patients and control groups and results (median, range) of the 24-h fraction (%) time of gastric pH < 4 before (basal) and while on 20 mg/d omeprazole.

Groups studied	Number of patients	Age (yr)	*H pylori* (+)	Treatment-free (baseline)	Omeprazole 20 mg/d	*P* value
Normal controls	10	60, 47–73	2/9	87.7, 71.3–98.5	34.6, 4.3–72.7	<0.001
Duodenal ulcer controls	7	61, 38–71	7/7	92.3, 86.6–98.8	45.2, 25.8–72.0	0.002
Vagotomy controls[2]	7	54, 41–72	2/6	72.8, 34.0–87.3	17.8, 6.5–35.1	0.005
Gastrectomy controls	7	66, 46–72	1/7	6.2, 4.6–39.6	ND[1]	
Vagotomy patients	5	57, 49–72	3/5	84.9, 79.1–95.2	49.4, 18.3–69.9	0.012
Gastrectomy patients	5	68, 61–72	0/5	69.8, 45.8–85.2	28.8, 13.3–51.3	0.037

[1]ND, not done. [2]Truncal vagotomy and gastrojejunostomy (n=5), truncal vagotomy and pyloroplasty (n=2).

30-year period[14]. The authors estimated that vagotomy plus drainage is associated with a 9% recurrence rate and a risk of hemorrhage of 1.7%. Partial gastrectomy has a lower recurrence rate (<1%) but a similar risk of hemorrhage (1.3%). Such patients with recurrent ulcer bleeding are often submitted to a second gastric operation to cure recurrent ulcer. Similarly, most of our patients had a history of two or more hospital admissions for recurrent ulcer bleeding after surgery and two of them had a second gastric operation that failed to cure recurrent ulcer.

Cimetidine has been used in a few clinical trials including a small number of patients with recurrent ulcer bleeding following surgery for peptic ulcer disease[6,7,15-17]. Cimetidine heals about 85% of ulcers within 8 weeks[17], but about 30% of ulcers relapse within 6 months after discontinuing therapy[7,17]. Furthermore, 33% of ulcers recur 1 year after surgery while on cimetidine maintenance therapy[6], probably because of tolerance to H_2RA, which is more significant after 4 wk of therapy[18]. Finally, there is only one clinical trial using omeprazole therapy for 12 patients with recurrent ulcer after vagotomy or gastrectomy and all ulcers are healed after 8 weeks of omeprazole therapy[8], suggesting that maintenance therapy with PPIs is the only alternative for surgical intervention. Since as many as one-third of patients with anastomotic ulcer have recurrent ulcer within a year after discontinuing acid reduction therapy[6,15], an open trial of PPI maintenance treatment should be considered ethically justifiable.

The role of *H pylori* in recurrent peptic ulcer disease after gastric surgery has been investigated in several clinical studies[9,10,12,19], showing that *H pylori* does not play an important role in post surgical ulcer recurrence. Less than half of our patients with recurrent ulcer bleeding were *H pylori*-positive upon admission to the study. Furthermore, one of our patients who had successful *H pylori* eradication therapy during a previous hospitalization was admitted to our department for a new episode of anastomotic ulcer bleeding. Another patient had two gastric operations for recurrent ulcer bleeding. Upon study entry, he had successful *H pylori* eradication therapy, followed by omeprazole (20 mg/d) maintenance therapy. He was healthy for 2 years and then lost his follow-up. He was readmitted to another hospital for recurrent ulcer bleeding 8 mo after discontinuing PPI maintenance therapy, though he was *H pylori*-negative.

With regard to the underlying mechanism of ulcer recurrence, our study showed that patients with recurrent ulcer bleeding after truncal vagotomy or partial gastrectomy had a higher treatment-free 24-h intragastric acidity than controls with no ulcer recurrence after the same operation, suggesting that vagotomy may be incomplete due to a retained antrum[20,21]. Our data showed that ulcer healing therapy with omeprazole (40 mg/d) could strongly inhibit gastric acid secretion. The intragastric acidity could also be significantly reduced with oral omeprazole maintenance treatment (20 mg/d).

Other factors that may be implicated in ulcer recurrence after surgical treatment for duodenal ulcer include a decreased resistance to acid in the jejunal mucosa, the site of pyloroplasty and anastomosis, possibly due to local ischemia and scarring. Hirschowitz and Lanas[11] also showed that aspirin abusers develop intractable recurrent ulceration following gastric surgery for peptic ulcer disease. However, our patients refused to use non-steroidal anti-inflammatory drugs and their serum salicylates were negative. Zollinger-Ellison syndrome was also excluded by normal fasting serum gastrin.

In conclusion, omeprazole (20 mg/d) maintenance therapy should be the treatment of choice for patients with recurrent ulcer after gastric surgery for duodenal ulcer disease.

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S- Editor Wang XL and Guo SY **L- Editor** Elsevier HK **E- Editor** Wu M

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World J Gastroenterol 2006 February 7; 12(5):796-799
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Involvement of mitochondrial Na+–Ca2+ exchange in intestinal pacemaking activity

Byung Joo Kim, Jae Yeoul Jun, Insuk So, Ki Whan Kim

Byung Joo Kim, Insuk So, Ki Whan Kim, Department of Physiology and Biophysics, College of Medicine, Seoul National University, Republic of Korea
Jae Yeoul Jun, Department of Physiology, College of Medicine, Chosun University, Republic of Korea
Supported by the Seoul National University Hospital Research Fund (03-2004-008), Korea Research Foundation Grant funded by Korea Government (MOEHRD, Basic Research Promotion Fund, KRF-2004-041-E00022) and BK21 project for medicine, dentistry, and pharmacy
Correspondence to: Insuk So, MD, PhD, Department of Physiology and Biophysics, College of Medicine, Seoul National University, 28 Yongon-Dong, Chongno-Gu, Seoul 110-799, Republic of Korea. insuk@plaza.snu.ac.kr
Telephone: +82-2-740-8228 Fax: +82-2-763-9667
Received: 2005-07-13 Accepted: 2005-08-31

Abstract

AIM: Interstitial cells of Cajal (ICCs) are the pacemaker cells that generate slow waves in the gastrointestinal (GI) tract. We have aimed to investigate the involvement of mitochondrial Na^+-Ca^{2+} exchange in intestinal pacemaking activity in cultured interstitial cells of Cajal.

METHODS: Enzymatic digestions were used to dissociate ICCs from the small intestine of a mouse. The whole-cell patch-clamp configuration was used to record membrane currents (voltage clamp) and potentials (current clamp) from cultured ICCs.

RESULTS: Clonazepam and CGP37157 inhibited the pacemaking activity of ICCs in a dose-dependent manner. Clonazepam from 20 to 60 μmol/L and CGP37157 from 10 to 30 μmol/L effectively inhibited Ca^{2+} efflux from mitochondria in pacemaking activity of ICCs. The IC_{50}s of clonazepam and CGP37157 were 37.1 and 18.2 μmol/L, respectively. The addition of 20 μmol/L $NiCl_2$ to the internal solution caused a "wax and wane" phenomenon of pacemaking activity of ICCs.

CONCLUSION: These results suggest that mitochondrial Na^+-Ca^{2+} exchange has an important role in intestinal pacemaking activity.

Key words: Mitochondrial Na^+-Ca^{2+} exchange; Interstitial cells of Cajal

Kim BJ, Jun JY, So I, Kim KW. Involvement of mitochondrial Na^+-Ca^{2+} exchange in intestinal pacemaking activity. *World J Gastroenterol* 2006; 12(5): 796-799

http://www.wjgnet.com/1007-9327/12/796.asp

INTRODUCTION

The interstitial cells of Cajal (ICCs) produce spontaneous rhythmic inward currents that are critical for the generation of slow waves in intestinal smooth muscle[1-3]. Pacemaker currents in ICCs result from the activation of a voltage-independent, non-selective cation conductance[4,5]. Pacemaking activity in ICCs is dependent upon metabolic activity[6] and Ca^{2+} release from intracellular stores[7]. Recent findings suggested that the pacemaker conductance in ICC is regulated by intracellular Ca^{2+} modulation[8]. The close association between IP_3 receptor-dependent Ca^{2+} stores, mitochondria, and ion channels in the plasma membrane creates a basic cellular structure[9,10]. Release of Ca^{2+} from IP_3 receptors does not directly initiate pacemaker currents in ICC, but rather, initiates Ca^{2+} uptake into the mitochondria. It was found that mitochondria in ICCs experience Ca^{2+} oscillations at the same frequency as pacemaker currents and that a rise in mitochondrial Ca^{2+} slightly precedes the activation of pacemaker currents[8]. This implies that pacemaker channels in the plasma membrane are activated by the falling phase of localized Ca^{2+} transients.

In isolated mitochondria, Ca^{2+} influx occurs via a Ca^{2+} uniporter driven by the membrane potential[11]. Ca^{2+} efflux occurs via Na^+-Ca^{2+} exchange and can be inhibited by diltiazem, clonazepam, CGP37157, and by high external Ca^{2+}[11-14]. However, the effect of inhibiting mitochondrial efflux, by using inhibitors of the Na^+-Ca^{2+} exchange, on pacemaking activity of ICCs has not yet been investigated. Therefore, we undertook to investigate the involvement of mitochondrial Na^+-Ca^{2+} exchange in pacemaking activity of ICCs.

MATERIALS AND METHODS

Preparation of cells and cell cultures

Balb/c mice (8-13 days old) of either sex were anesthetized with ether and killed by cervical dislocation. The small intestines from 1 cm below the pyloric ring to the cecum were removed and opened along the mesenteric border. Luminal contents were removed by washing with Krebs-Ringer bicarbonate solution. The tissues were pinned to

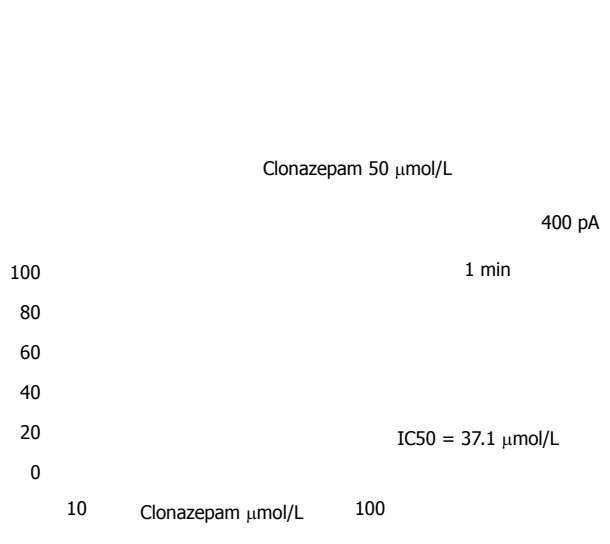

Figure 1 The effect of clonazepam on the pacemaking activity of ICCs. Clonazepam was applied to examine its effect on the pacemaking activity of ICCs. A: Under a voltage clamp at a holding potential of –60 mV, 50 μmol/L clonazepam inhibited the pacemaking currents of ICCs (n = 4). B: Clonazepam from 20 to 60 μmol/L effectively inhibited Ca^{2+} efflux from mitochondria in the pacemaking activity of ICCs. The IC_{50} of clonazepam was 37.1 μmol/L.

the base of a Sylgard dish and the mucosa removed by sharp dissection. Small tissue strips of the intestine muscle (consisting of both circular and longitudinal muscles) were equilibrated in Ca^{2+}-free Hanks solution (containing in mmol/L: KCl 5.36, NaCl 125, NaOH 0.34, Na_2HCO_3 0.44, glucose 10, sucrose 2.9, and HEPES 11) for 30 min. Then, the cells were dispersed using an enzyme solution containing collagenase (Worthington Biochemical Co., Lakewood, NJ, USA) 1.3 mg/mL, bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA) 2 mg/mL, trypsin inhibitor (Sigma) 2 mg/mL and ATP 0.27 mg/mL. Cells were plated onto sterile glass coverslips coated with murine collagen (2.5 μg/mL, Falcon/BD, Franklin Lakes, NJ, USA) in a 35-mm culture dish and then cultured at 37 °C in a 95% O_2, 50 mL/L CO_2 incubator in a smooth muscle growth medium (Clonetics Corp., San Diego, CA, USA) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, USA) and murine stem cell factor (SCF, 5 ng/mL, Sigma). ICCs were identified immunologically with anti-c-kit antibody (phycoerythrin-conjugated rat anti-mouse c-kit monoclonal antibody; eBioscience, San Diego, CA, USA) at a dilution of 1:50 for 20 min[15]. ICCs were morphologically distinct from other cell types in the culture and thus it was possible to identify the cells by phase contrast microscopy once they had been verified with anti-c-kit antibody.

Patch-clamp experiments

The whole-cell patch-clamp configuration was used to record membrane currents (voltage clamp) and potentials (current clamp) from cultured ICCs. An axopatch ID (Axon Instruments, Foster, CA, USA) was used to amplify membrane currents and potentials. The command pulse was applied using an IBM-compatible personal computer and pClamp software (version 6.1; Axon Instruments). Data obtained were filtered at 5 kHz and displayed on an oscilloscope, a computer monitor, and using a pen recorder (Gould 2200, Gould, Valley View, OH, USA).

Results were analyzed using pClamp and Origin (version 6.0) software. All experiments were performed at 30-32 °C.

Solutions and drugs

The physiological salt solution used to bathe cells (Na^+-Tyrode) contained (mmol/L): KCl 5, NaCl 135, $CaCl_2$ 2, glucose 10, $MgCl_2$ 1.2 and HEPES 10, adjusted to pH 7.4 with NaOH. The pipette solution contained (mmol/L): KCl 140, $MgCl_2$ 5, K_2ATP 2.7, NaGTP 0.1, creatine phosphate disodium 2.5, HEPES 5 and EGTA 0.1, adjusted to pH 7.2 with KOH.

Before the development of CGP37157, several benzodiazepines (except clonazepam) were used as mitochondrial Na^+-Ca^{2+} exchange inhibitors[12]. Clonazepam and CGP37157 were dissolved in dimethyl sulfoxide (DMSO) for 100 and 50 mmol/L stock solution, respectively and added (1 000 times dilution) to the bathing solution at the day of the experiment. The final concentration of DMSO in the bath solution was always <0.1%, and we confirmed that this concentration of DMSO did not affect the results that were recorded. Nickel chloride was directly added to the pipette solutions at the day of the experiment. CGP37157 was purchased from Tocris Cookson (Ellisville, MO, USA). The rest of the drugs were obtained from Sigma (Sigma Chemical Co., USA), unless otherwise stated. Diltiazem was not used because of its known effects on the cell membrane Ca^{2+} channels.

RESULTS

Effect of clonazepam on the pacemaking activity of ICCs

Under a voltage clamp at a holding potential of -60 mV, clonazepam 50 μmol/L inhibited the pacemaking currents of ICCs (n = 4, Figure 1A). Clonazepam from 20 to 60 μmol/L effectively inhibited Ca^{2+} efflux from mitochondria on the pacemaking activity of ICCs. Concentrations of clonazepam of >100 μmol/L produced no further inhibition. The IC_{50} of clonazepam was 37.1 μmol/L (Figure 1B).

Effect of CGP37157 on the pacemaking activity of ICCs

The benzodiazepine CGP37157 has been shown to be a more potent inhibitor than either clonazepam or diltiazem in terms of Ca^{2+} efflux measured in isolated mitochondria[12]. Thus, CGP37157 was applied to examine its effect on the pacemaking activity of ICCs. Under a current clamp (I = 0), CGP37157 was found to inhibit pacemaking potentials in a dose-dependent manner (n = 15, Figures 2A-2C). CGP37157 from 10 to 30 μmol/L effectively inhibited Ca^{2+} efflux from mitochondria on the pacemaking activity of ICCs. Concentrations of CGP37157 of >50 μmol/L produced no further inhibition. The IC_{50} of CGP37157 was 18.2 μmol/L (Figure 2D).

Effect of internal Ni^{2+} on the pacemaking activity of ICCs

Ni^{2+} is a competitive inhibitor of the Ca^{2+} carrier, but it is not transported into the mitochondria[16]. Micromolar concentrations of nickel (Ni^{2+}) chloride have been reported

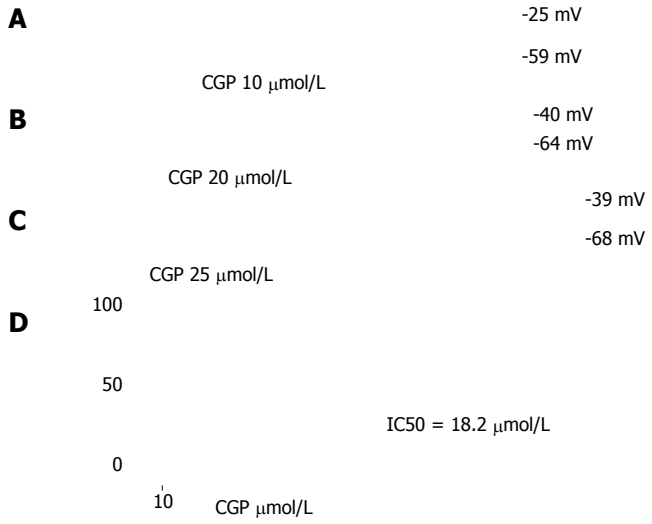

Figure 2 The effect of CGP37157 in the pacemaking activity of the ICCs. CGP37157 was applied to examine its effect on the pacemaking activity of ICCs. **A, B**, and **C:** Under a current clamp ($I=0$), CGP37157 inhibited the pacemaking potentials in a dose-dependent manner ($n=15$). **D:** CGP37157 from 10 to 30 μmol/L effectively inhibited Ca^{2+} efflux from mitochondria in the pacemaking activity of ICCs. The IC_{50} of CGP37157 was 18.2 μmol/L.

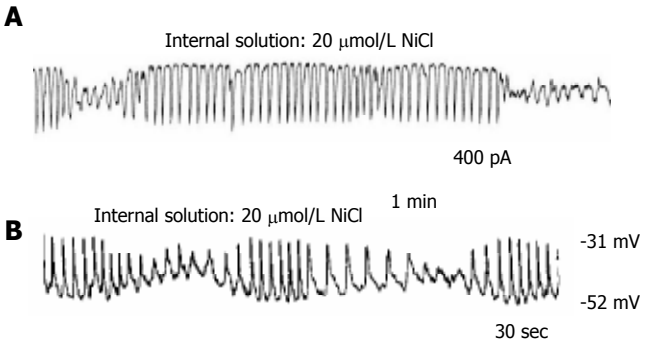

Figure 3 Effect of internal Ni^{2+} on the pacemaking activity of ICCs. In order to investigate the effect of $NiCl_2$ on the pacemaking activity of ICCs, we added 20 μmol/L of $NiCl_2$ to the internal solution. A: Under a voltage clamp at a holding potential of -60 mV, the pacemaking activity of ICCs showed a "wax and wane" phenomenon ($n=6$). B: Current clamp mode ($I=0$) showed the same phenomenon ($n=3$).

to inhibit Na^+-Ca^{2+} exchange in both vascular and non-vascular cells[17]. In order to investigate the effect of $NiCl_2$ on the pacemaking activity of ICCs, we added 20 μmol/L $NiCl_2$ to the internal solution. Under a voltage clamp mode at a holding potential of -60 mV, the pacemaking activity of ICCs showed a "wax and wane" phenomenon ($n=6$, Figure 3A). Also in current clamp mode ($I=0$), the same phenomenon was shown ($n=3$, Figure 3B). In case of 100 μmol/L $NiCl_2$, the pacemaking activity of ICCs stopped (data not shown).

DISCUSSION

Intracellular Ca^{2+} plays an important role in the regulation of various cellular functions including exocytosis, metabolic activity, contractile activity, and gene expression in excitable cells. In these cells, the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is lowered by various mechanisms such as Ca^{2+} extrusion due to the actions of Na^+-Ca^{2+} exchangers and Ca^{2+} pumps in the plasma membrane, Ca^{2+} uptake by Ca^{2+} pumps in the endoplasmic reticulum, and by Ca^{2+} uniporters in the mitochondria[17]. Sequestered Ca^{2+} in mitochondria is, in turn, released to the cytoplasma via various mechanisms[11]. Na^+-Ca^{2+} exchangers and/or permeability transition pores are proposed to be involved in Ca^{2+} efflux from the mitochondria[11].

In case of ICCs, pacemaking activity is associated with mitochondrial Ca^{2+} transients. Pacemaker currents and rhythmic mitochondrial Ca^{2+} uptake by ICCs are blocked by inhibitors of IP_3dependent Ca^{2+} release from the endoplasmic reticulum and by inhibitors of endoplasmic reticulum Ca^{2+} reuptake. Therefore, integrated Ca^{2+} management by endoplasmic reticulum and mitochondria is a prerequisite of electrical pacemaking in the gastrointestinal tract[8].

CGP37157 is a benzodiazepine derivative that inhibits electroneutral mitochondrial Na^+-Ca^{2+} exchanger with submicromolar potency. In the heart, for example, this transporter is inhibited by CGP37157 at 400 nmol/L[12,14]. Before the development of CGP37157, several related benzodiazepines (e.g., clonazepam and diltiazem) were used as mitochondrial Na^+-Ca^{2+} exchange inhibitors[14]. In general, few have reported that these compounds inhibit the cardiac plasmalemmal Na^+-Ca^{2+} exchanger[18,19]. Mitochondrial Na^+-Ca^{2+} exchanger protein participates in Ca^{2+} efflux and operates in opposition to a Ca^{2+} uniporter within the inner mitochondrial membrane. Thus, the inhibition of mitochondrial Na^+-Ca^{2+} exchanger leads to an increase in Ca^{2+} levels within the mitochondria[14]. Calcium within the mitochondria serves as an important regulator of several key enzymes involved in energy metabolism. For example, the upregulation of the steady-state level of mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_m$) result increases NADH production and stimulates oxidative phosphorylation[14].

The benzodiazepine CGP37157 has been shown to be a more potent inhibitor than either clonazepam or diltiazem on Ca^{2+} efflux, as measured in isolated mitochondria[12]. In ICCs, the IC_{50}s of clonazepam and CGP37157 were found to be 37.1 and 18.2 μmol/L, respectively. Thus, CGP37157 was about twofold more potent than clonazepam.

Ni^{2+} is a potent inhibitor of mitochondrial Ca^{2+} transport[20] and a competitive inhibitor of Ca^{2+} carrier[16]. Also micromolar concentrations of nickel (Ni^{2+}) chloride were found to inhibit Na^+-Ca^{2+} exchanger in both vascular and non-vascular cells[21]. Therefore, we have investigated the effect of $NiCl_2$ added to the internal solution. At 20 μmol/L, we observed a "wax and wane" phenomenon and at 100 μmol/L, the pacemaking activity of ICCs stopped.

Our data indicate that both clonazepam and CGP37157 inhibit the pacemaking activity of ICCs in a dose-dependent manner. The IC_{50}s of clonazepam and CGP37157 were 37.1 and 18.2 μmol/L, respectively. When 20 μmol/L $NiCl_2$ was added to the internal solution, the pacemaking activity of ICCs showed a "wax and wane" phenomenon.

We conclude that mitochondrial Na^+-Ca^{2+} exchange has an important role in intestinal pacemaking activity.

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S- Editor Guo SY **L- Editor** Elsevier HK **E- Editor** Li HY

PO Box 2345, Beijing 100023, China
www.wjgnet.com
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World J Gastroenterol 2006 February 7; 12(5):800-803
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Combined resection and multi-agent adjuvant chemotherapy for desmoplastic small round cell tumor arising in the abdominal cavity: Report of a case

Chang-Cheng Chang, Jun-Te Hsu, Jeng-Hwei Tseng, Tsann-Long Hwang, Han-Ming Chen, Yi-Yin Jan

Chang-Cheng Chang, Jun-Te Hsu, Tsann-Long Hwang, Han-Ming Chen, Yi-Yin Jan, Department of Surgery, Chang Gung Memorial Hospital, Taoyuan, Taiwan, China
Jeng-Hwei Tseng, Department of Radiology, Chang Gung Memorial Hospital, Taoyuan, Taiwan, China
Correspondence to: Dr Han-Ming Chen, Department of Surgery, En Chu Kong Hospital 399, Fuhsing Road, San-shia Town, Taipei Hsien 237, Taiwan, China. ming1838@yahoo.com
Telephone: +886-3-3281200- 3219 Fax: +886-3-3285818
Received: 2005-06-28 Accepted: 2005-07-15

Abstract

Desmoplastic small round cell tumor (DSRCT) is a rare, highly aggressive malignancy with distinctive histological features: a nesting pattern of cellular growth within dense desmoplastic stroma, occurring in young population with male predominance. The mean survival period is only about 1.5-2.5 years. The tumor has co-expressed epithelial, muscle, and neural markers in immunohistochemical studies. This work reports a 27-year-old man presenting with hematemesis and chronic constipation. Serial studies including endoscopy, upper gastrointestinal series, abdominal computed tomography and barium enema study showed disseminated involvement of visceral organs. The patient underwent aggressive surgery and received postoperative adjuvant chemotherapy consisting of 5-fluorouracil, cyclophosphamide, etoposide, doxorubicin, and cisplatin. He survived without any disease for 20 mo after the surgery. No standard treatment protocol has been established. Aggressive surgery combined with postoperative multi-agent adjuvant chemotherapy is justified not only to relieve symptoms but also to try to improve the outcome in this advanced DSRCT young patient.

Key words: Desmoplastic small round cell tumor; Surgery; Chemotherapy

Chang CC, Hsu JT, Tseng JH, Hwang TL, Chen HM, Jan YY. Combined resection and multi-agent adjuvant chemotherapy for desmoplastic small round cell tumor arising in the abdominal cavity: Report of a case. *World J Gastroenterol* 2006; 12(5): 800-803

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INTRODUCTION

Desmoplastic small round cell tumor (DSRCT) is a rare malignancy with highly aggressive behavior spreading widely along the serosal surface. Gerald and Rosai[1] first described the disease in 1989 in terms of distinctive pathologic findings: a nesting pattern of cellular growth within dense desmoplastic stroma, and immunohistochemical co-expression of epithelial, muscle and neural markers[1-3]. A specific chromosome abnormality, t (11;22) (p13;q11 or q12) was identified[4], and DSRCT is believed to be the result of involvement of both Wilms' tumor suppressor gene and Ewing's sarcoma gene located on Chromosome 11 and 22, respectively[5]. The tumors usually present in young male individual, as a single mass or multiple masses in the abdominal-pelvic cavity with metastases common to peritoneum, liver and lymphoid tissue[6]. Some cases had presentation sites in the scrotum[6,7], pleural space[6,8,9] and mediastenum[6,10]. Complete resection is generally difficult to obtain due to multiple foci and dissemination. The standard treatment protocol has not been well established. Chemotherapy currently seems to be the best potential treatment. This work reports on a case of DSRCT with no disease for 20 months following complete resection of tumors and postoperative multi-agent adjuvant chemotherapy consisting of 5-fluorouracil (5-FU) and PAVEP (cyclophosphamide, etoposide, doxorubicin and cisplatin).

CASE REPORT

The 27-year-old man had intermittent epigastralgia, abdominal fullness, and chronic constipation for 3 months. He presented sudden onset of hematemesis (500 mL) outside our hospital on September 15, 2003. Physical examination revealed only mild epigastric tenderness. The hemoglobin level was normal. Endoscopy showed esophageal and cardiac ulcers without complete study owing to poor compliance of the patient. Upper gastrointestinal (GI) series demonstrated a mass with external compression at the gastric antrum (Figure 1). Abdominal computed tomography (CT) indicated a 5 cm heterogeneous mass located among the gastric antrum, duodenum loop and pancreatic head (Figure 2). Barium enema study showed external compression of the middle transverse colon, ileocecal valve and ascending colon (Figure 3). Laparoscopic biopsy was performed because multiple intra-abdominal organs were involved. A histo-

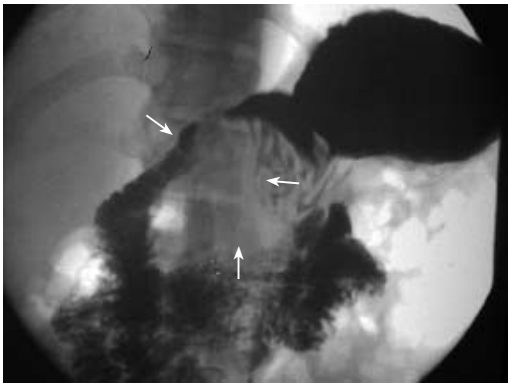

Figure 1 Upper gastrointestinal series shows that the gastric antrum (arrows) is deformed due to external compression.

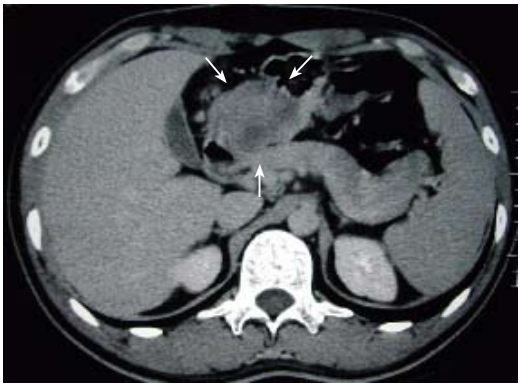

Figure 2 Abdominal computed tomography shows a 5 cm heterogeneous mass (arrows) located between the gastric antrum, duodenal loop, and pancreatic head.

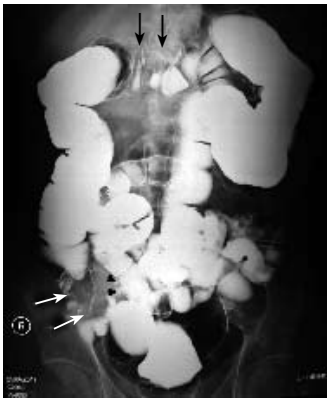

Figure 3 Barium enema study demonstrates a mass compressing the middle transverse colon with tethering appearance, suggesting gastrocolic ligament invasion (black arrows). Long segmental encasement of cecum, proximal ascending colon (white arrows), and ileocecal valve (arrows head) is also evident.

pathologic exam of the specimen revealed poorly differentiated carcinoma. The patient was then referred to our hospital for further treatment.

No significant medical or pertinent family history was found. Additionally, no history of exposure to carcinogens such as radiation, tobacco or asbestos was identified. Hematogram showed hemoglobin, 110 g/L and white blood cells (WBC), 11×10^9/L. Biochemistry studies yielded normal results. Tumor markers such as carcinoembryonic antigen and carbohydrate antigen 19-9 were within the normal rage. Laparotomy was performed on October 3, 2003 to relieve the patient's symptoms. During the operation, the gastric antrum, pancreas, terminal ileum and cecum, sigmoid colon, rectovesicular region and omentum were all involved by tumors. Right hydroureter was also identified due to external compression by the tumor. Subtotal gastrectomy, partial pancreatic resection, total omentectomy, proctocolectomy and J-pouch ileo-anal anastomosis were performed for complete resection of tumors. A double-J stenting was inserted through the ureterostomy by a urologist.

A histopathologic exam of the surgical specimens demonstrated invasive nests of poorly differentiated carcinoma, mainly involving omentum, pancreatic, and gastric, colonic and ileal muscular walls with focal mucosa extension. Large and small foci of tumor cells were observed in fibrous interstitium (Figure 4A). The tumor cells had a small spherical or spheroid nucleus, which was rich in chromatin (Figure 4B). One of 20 peri-colic lymph nodes was positive for metastasis. The section margins were free of tumor. The immunohistochemical tests were positive for desmin (Figure 4C), epithelium membrane antigen and neuron-specific enolase, and focal positive for vimentin, indicating DSRCT.

J-pouch ileo-anal anastomosis leakage with intra-abdominal abscess occurred on postoperative day 7. The patient recovered after treatment by antibiotics and CT-guidance drainage. Postoperative adjuvant chemotherapy with high dose 5-FU (2600 mg/m^2, day 1) was initiated on 24th October, 2003. The patient received altogether eight courses of chemotherapy of 5-FU thereafter. Another chemotherapeutic regimen consisting of PAVEP (cyclophosphamide 300 mg/m^2, d 1-3; etoposide 75 mg/m^2, day 1-3; doxorubicin 40 mg/m^2, day 1; cisplatin 100 mg/m^2, day 4) was administered on February 17, 2004. Neutropenic (WBC, 2×10^9/L) fever developed one week later even though prophylactic granulocyte colony-stimulating factor (G-CSF) was given the day following chemotherapy. The patient received broad-spectrum antibiotics treatment, and his fever was gradually controlled. He was discharged 12 d later when his WBC returned to normal. Another four chemotherapy courses with PAVEP regimens were completed on May 24, 2004. He survived without any disease 20 mo after the surgery.

DISCUSSION

DSRCT has a strong male predominance, and the majority arises below the diaphragm with serosal spreading and easily metastasizing to lung, liver, and lymphoid tissues[2,6,11]. The most common site was the pelvis (62%), followed by spreading widely on the peritoneal surface (42%)[12]. Symptoms such as abdominal pain (52.1%), increased abdominal girth (8.4%), and abdominal mass (5.6%) have been reported[14]. Patients also presented with GI or genitourinary obstruction. GI bleeding does not appear to have been reported in the literature. This patient was the first case with DSRCT presented with GI bleeding. The bleeding was associated with tumors directly invading to the gastric mucosa.

The prognosis of DSRCT is dismal with a mean survival of 1.5-2.5 years since the wide spread of the tumor makes radical resection difficult to achieve, and

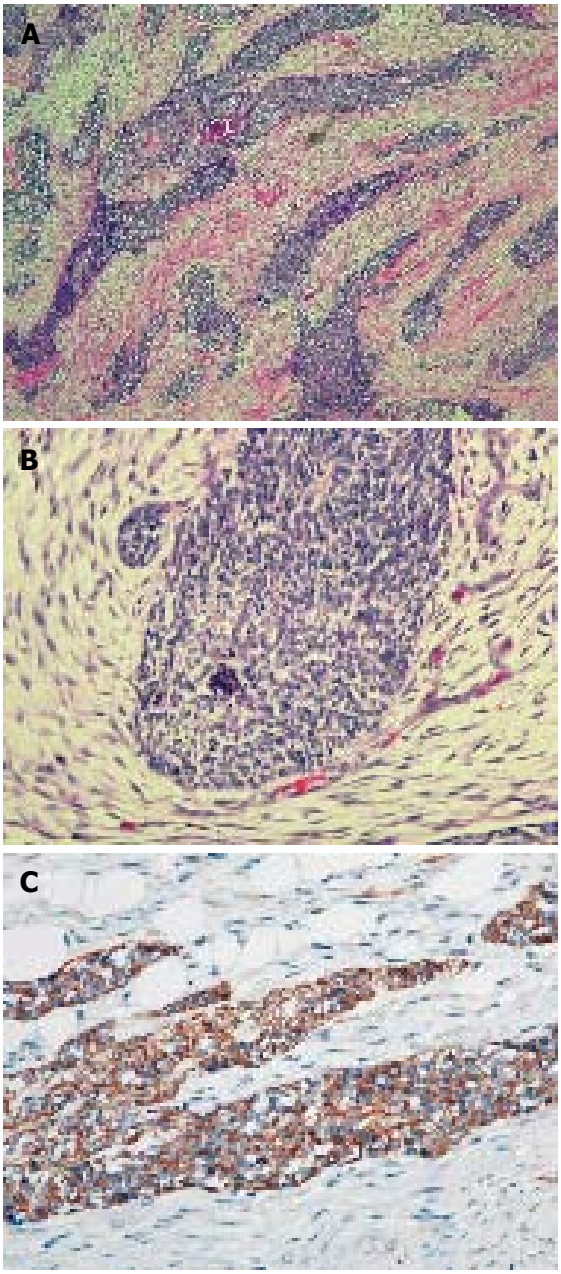

Figure 4A: Photomicrography depicts nests or clusters of small tumor cells outlined by characteristic desmoplastic stroma bands (hematoxylin and eosin staining, 100×). **B**: Photomicrography demonstrates the monomorphic, small round tumor cell population with a small spherical or spheroid nucleus, rich in chromatin (hematoxylin and eosin staining, 400×). **C:** The immunohistochemical staining of tumor cells is positive for desmin.

chemotherapeutic agents are only temporarily effective in treating this disease[6,12-15]. Farhat *et al* [13] recommended that PAVEP should be the first-line drug for treating DSRCT after reviewing 8 cases with complete remission from the treated 60 cases. However, grade four neutropenia occurred in 69% cycles while administering PAVEP regimens despite prophylactic use of G-CSF in 90% of cases in his report[15]. Considering the severe and life-threatening side effects and uncertainty of therapeutic results of PAVEP regimens, other safer and effective chemotherapeutic agents should be considered prior to PAVEP.

5-FU is well-known as a chemotherapeutic agent and a radioactive sensitizer broadly used for GI tract cancers and disseminated intra-abdominal metastatic adenocarcinoma. Gerald *et al* [2] reported a successful application of 5-FU in treating intra-abdominal DSRCT. Kretschmar *et al* [6] also reported 60% responsive rate to this tumor. Since DSRCT is a highly aggressive and progressive malignancy, post-operative adjuvant chemotherapy should be initiated as soon as possible. Post-operative anastomotic leakage and right urinary tract obstruction arose in our case. Therefore, 5-FU was applied instead of PAVEP regimen as the first-line drug to treat DSRCT considering the infection problems and the adverse effects of PAVEP regimens such as myelo-suppression and renal toxicity.

DSRCT is a disease so rare that no consistent response to chemotherapy was seen in the literature review[12]. Kurre *et al* [16] presented another alkylator-based chemotherapy protocol named P6 (cyclophosphamide, doxorubicin, vincristine, ifosfamide, and etoposide), which only improved the progression-free survival without significant survival benefit. Gil *et al* [12] recommended performing perioperative intraperitoneal chemotherapy when treating DSRCT patients, but no evidence of prolonged survival was observed.

The effect of complete resection of disseminated tumors on survival is still unknown because of the rarity of achieving complete resection at operation. Gil *et al* [12] reported that the median survival was 20 mo in 4 patients with complete resection of tumors compared with 11 mo in 3 cases without complete resection. Significantly, long-term survivors, including one patient who survived for 101 mo, have been reported when utilizing combined modality treatment comprising surgery and multi-agent chemotherapy. The influence of other salvage therapies, such as immunotherapy or bone marrow ablation, is still undetermined. In this work, the patient underwent complete resection of tumors and received postoperative adjuvant chemotherapy surviving without any disease and without gastrointestinal symptoms 20 mo after surgery, similar to that in Gil's report[12].

In conclusion, the optimal treatment for DSRCT remains to be determined, indicating the rarity and virulent behavior of the disease. In this advanced DSRCT patient, aggressive surgery combined with postoperative systemic multi-agent adjuvant chemotherapy was justified not only to relieve his symptoms but also to try to improve his outcome. The effectiveness and safety of chemotherapeutic regimens still need to be improved to treat this disease as early as possible postoperatively, especially when patients cannot tolerate the toxicity of alkylator agents after such an aggressive surgery.

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S- Editor Wang XL and Guo SY **L- Editor** Elsevier HK **E- Editor** Cao L

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World J Gastroenterol 2006 February 7; 12(5):804-808
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Ruptured angiosarcoma of the liver treated by emergency catheter-directed embolization

Christine Leowardi, Yura Hormann, Ulf Hinz, Moritz N Wente, Peter Hallscheidt, Christa Flechtenmacher, Markus W Büchler, Helmut Friess, Matthias HM Schwarzbach

Christine Leowardi, Yura Hormann, Moritz N Wente, Markus W Büchler, Helmut Friess, Matthias HM Schwarzbach, Department of Surgery, University of Heidelberg, Im Neuenheimer Feld 110, 69120 Heidelberg, Germany
Ulf Hinz, Unit for Documentation and Statistics, Department of Surgery, University of Heidelberg, Im Neuenheimer Feld 110, 69120 Heidelberg, Germany
Peter Hallscheidt, Department of Radiology, University of Heidelberg, Im Neuenheimer Feld 110, 69120 Heidelberg, Germany
Christa Flechtenmacher, Institute of Pathology, University of Heidelberg, Im Neuenheimer Feld 220, 69120 Heidelberg, Germany
Correspondence to: MHM Schwarzbach, C Leowardi, MD, Department of Surgery, University of Heidelberg, Im Neuenheimer Feld 110, 69120 Heidelberg,
Germany. matthias.schwarzbach@chir.ma.uni-heidelberg.de
Telephone: +49-621-3832227 Fax: +49-6221-805790
Received: 2005-07-16 Accepted: 2005-07-28

Abstract

Angiosarcoma is a rare primary malignant neoplasm of the liver with a poor prognosis. Here, we report a case of a patient with a ruptured hepatic angiosarcoma which was treated by emergency catheter-directed embolization, followed by left-sided hemihepatectomy.

Key words: Ruptured angiosarcoma; Liver; Embolization

Leowardi C, Hormann Y, Hinz U, Wente MN, Hallscheidt P, Flechtenmacher C, Büchler MW, Friess H, Schwarzbach MHM. Ruptured angiosarcoma of the liver treated by emergency catheter-directed embolization. *World J Gastroenterol* 2006; 12(5): 804-808

http://www.wjgnet.com/1007-9327/12/804.asp

INTRODUCTION

Angiosarcoma (AS) is a rare tumor entity which comprises less than 1% of all sarcomas[1]. Although rarely observed, it is the third most common primary malignant tumor of the liver. The occurrence of AS in the liver shows a predelection for elderly males[2]. The exposures to vinyl chloride, arsenic, thorotrast and irradiation are associated with an increased risk for the development of AS of the liver and are considered as etiologic cofactors[3-5]. Clinical diagnosis is usually hampered by the unspecific clinical signs and symptoms. Patients reported have been found with pain, loss of energy, and weight, hemorrhage and extrusion[6,7,8]. The therapeutical options described are oncological liver resection and chemotherapy[6,7,9]. Liver transplantation has not been reported to be significantly beneficial in this subgroup of sarcoma patients so far[10]. The clinical course of AS, even after curative surgery and chemotherapy is aggressive with a poor prognosis has been reported[7,11,12].

Here, we report about the rare case of a patient with acute bleeding to the abdominal cavity by a spontaneously ruptured AS of the liver.

CASE REPORT

A 76-year-old man was referred to the hospital with acute abdominal pain. With the onset of pain the patient collapsed. The subsequent consultation lead to emergency admission to the department of surgery. Apart from a slowly progressive local prostate cancer which was already resected transurethrally thrice (5 years ago, 1 year ago and palliatively 1 month before the admission), no significant disease was reported. The patient neither reported any kind of abdominal trauma nor prior abdominal surgery. Medication comprised antihypertensive drugs, iodine and androgene blockers since 1999. Besides that the patient had controlled his diabetes with an insulin pump.

At physical examination of the conscious patient, the abdomen was distended and showed a diffuse pain at deep palpation without clinical signs for peritonitis. Vital signs showed a slightly elevated blood pressure (140/70 mmHg) and a tachycardia (120 heart beats/min). Emergency blood test including hemoglobin, white blood count, electrolytes and hemostasis parameters revealed a reduced hemoglobin with 6.9 g/dL. Besides that, infection parameters were elevated with leukocytes of 16.0/nL and a C-reactive protein of 75.4 mg/L. Cholinesterase and albumin were also lowered significantly. Hemostasis parameters were normal.

The ultrasound examination of the abdomen, performed as the primary diagnostic modality, showed free abdominal fluid and a pathological finding in the left lobe of the liver, diagnosed as a ruptured liver lesion, at that time suspicious of a liver cell adenoma due to a 5-year

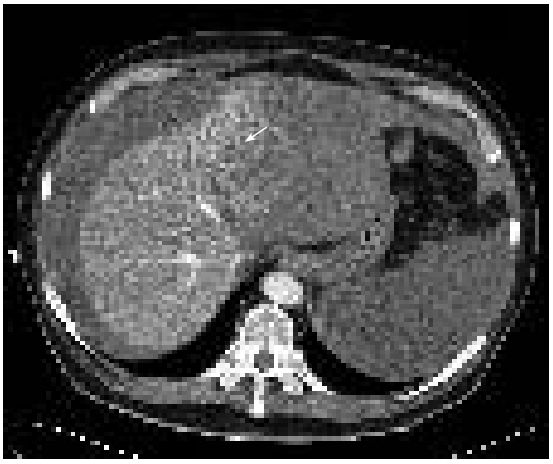


Figure 1 CT scan before embolization.

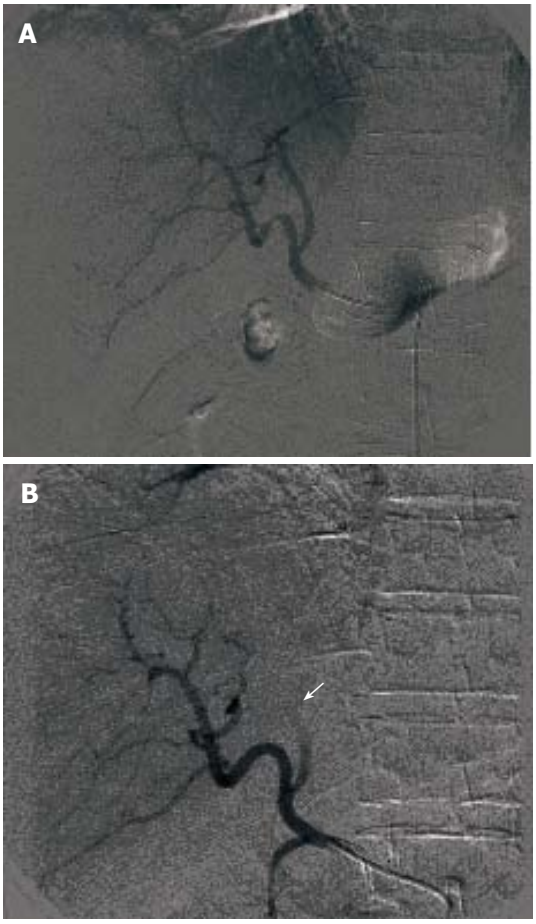


Figure 2 Angiography **A**: before embolization **B:** after embolization.

period of regular intake of androgene blockers. In order to identify the location of the active bleeding within the liver, an additive contrast enhanced computed tomography (CT) of the abdomen was carried out. The CT images confirmed a ruptured lesion of the left liver lobe with an ongoing active arterial bleeding located centrally within segments 2 and 3. In addition, a considerable amount of intra-abdominal blood (1-2 L) was diagnosed (Figure 1). The patient received blood transfusions (2 erythrocyte concentrates) and stayed hemodynamically stable. The interdisciplinary decision between radiologists and surgeons was made for therapeutic radiological intervention.

Subsequently, the patient was transferred to the radiological catheter room. The intervention was carried out by puncture of the right femoral artery and selective catheterization of the left hepatic artery. After cannulation of the left liver artery, the left lobe of the liver was completely and successfully embolized with 2 mL of embosphere 500-700 and 2 mL of embosphere 900-1 200. After intervention, there were no more signs for active bleeding, and blood pressure and heart rate stayed regular (Figure 2A, before embolization and Figure 2B after embolization). The patient was treated post-interventionally for 2 d at the intensive care unit before he was referred in a completely stable condition to the regular ward. Consecutively, elective surgery was scheduled to treat the abdominal hematoma and the ruptured lesion of the left lobe of the liver.

Preoperatively performed endoscopy of the upper GI tract and colonoscopy showed no substantial pathological findings (small axial hiatus hernia and a diverticulosis of the sigma without signs for inflammation).

Intraoperative findings

Surgery was performed 7 d after embolization. Explorative laparotomy revealed approximately 3 L of intra-abdominal hematoma without signs for active liver bleeding. Exploration of the left lobe of the liver revealed a ruptured hepatic tumor to the peritoneal cavity with a diameter of 12.5 cm which showed macroscopic characteristics of a hepatic adenoma. In order to treat the ruptured left lobe and to remove the liver tumor, a left sided hemihepatectomy was carried out. After resection, intraoperative sonography of the remaining liver parenchyma showed no pathological findings. Intraoperatively and during the early post-operative course, the patient received five erythrocyte-concentrates and two units of fresh frozen plasma. The post-operative course was uneventful and the patient could be discharged from hospital 19 d after the surgery.

Histopathologic findings

Histopathological examination of the specimen revealed a ruptured hematous hepatic malignancy of 12.5 cm diameter without sharp demarcation. The histopathological examination and the immunostaining diagnosed a tumor with endothelial proliferates along the liver sinusoids, large necroses and cell atypias consisting of solid and papillary tumor parts. Immunohistochemical staining for CD31, CD34, MIB-1 confirmed the diagnosis of a hemangiosarcoma. The final diagnosis was a ruptured AS of the left lobe of the liver with tumor-free resection margins at microscopic evaluation.

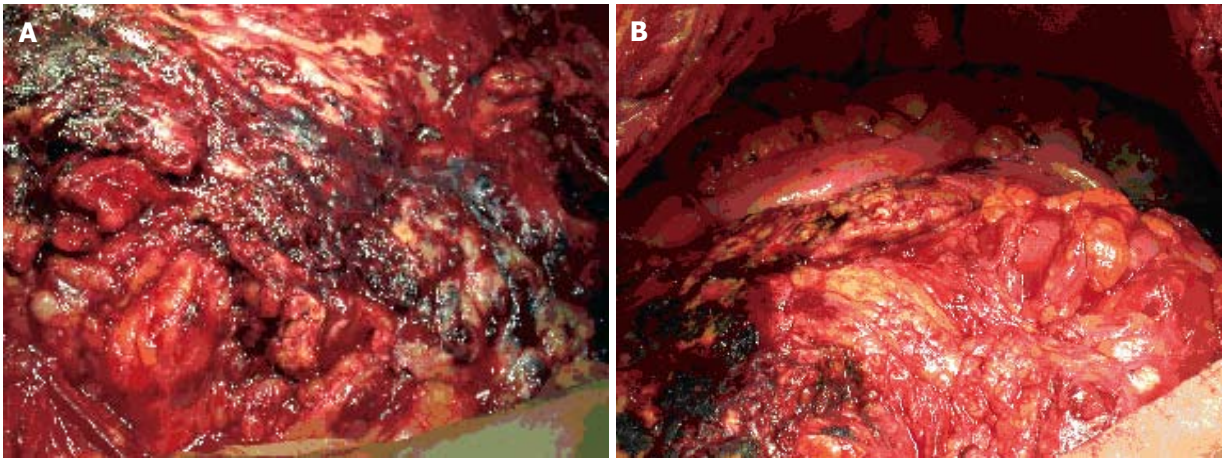
Postoperative course

Four weeks after primary surgery, the patient was readmitted to the hospital with the suspicion of recurrent acute intra-abdominal bleeding. A CT-scan showed free intra-abdominal fluid collection. An angiography showed small contracted vessels of the liver without proof of

Table 1 Case reports focusing on patients with hepatic AS undergoing surgery and/or embolization from 1995 to 2005

Reference	Year[1]	Number[2]	Age (yr)	Sex (M/F)	Clinical symptoms	Surgery	Resection margin	Additive Therapy	Metastasis at presentation	Emergency	Survival
Tordjman *et al*[27]	1995	1	34	M	?	No		Chemo-embolization	No	Yes	15 mo
Timaran *et al*[29]	2000	1	47	F	Right upper quadrant discomfort	Right hepatic lobectomy	Negative	No	No	No	Alive (120 mo postoperative)
Kirschstein *et al*[30]	2000	1	73	F	Abdominal pain	Right hepatectomy	Positive	No	Yes	No	6 wk
Hoppe *et al*[26]	2001	1	72	F	Pain, weight loss, hemodynamic instability	Liver segment resection	?	Preoperative embolization	No	Spontaneous rupture	2 wk
Ozden *et al*[10]	2003	1	54	F	?	Right hepatectomy + relaparotomy	Positive/ negative	Postoperative chemo-embolization	No	No	64 mo
Horger and Fendel[25]	2003	1	69	F	Right upper quadrant pain, bleeding	Right hepatectomy	?	Preoperative embolization	No	Spontaneous rupture	?
Almogy *et al*[8]	2004	2	33	F	Bleeding, right upper quadrant pain	Right hepatectomy	?	No	No	Yes	Died intra-operatively
			68	M	Bleeding	Right hepatectomy	Positive	Post-operative chemotherapy	No	Yes	7 mo

[1]Year of publication; [2]number of patients.



Figures 3 **A** and **B** Intraoperative pictures of peritoneal sarcomatosis.

active bleeding. The patient showed signs of hemorrhagic shock and received four erythrocyte concentrates and six units of fresh frozen plasma. Duplex-sonography showed reduced perfusion of the liver. Liver transaminases were elevated and the patient suffered simultaneously from a non-STEMI myocardial infarction without indication for acute catheter intervention.

In the following course the patient's abdomen became more and more distended with an aggravated pulmonary situation and progredient amounts of free intra-abdominal fluid. At this point, indication for explorative laparotomy was stated. Intraoperatively, multiple bleeding AS were found in the liver with massive peritoneal sarcomatosis (Figures 3A and 3B). Hemorrhage control of the diffuse bleeding could not be obtained surgically. The abdomen was closed and the patient was taken to the intensive care unit at basal infusion and kept on sedation and mechanical respiration. Few hours after the explorative laparotomy the patient died from persistent intra-abdominal bleeding from the angiosarcomatosis lesions.

DISCUSSION

Among malignant vascular tumors, AS is one of the most common subtypes diagnosed in patients undergoing surgery[13]. AS has a predilection for cutaneous sites, mostly in the head and neck region of elderly male patients[14]. AS also affects gastrointestinal organs[9,15-18] and hepatic AS is the second most common primary malignant neoplasm of the liver[19]. However, there have only been a few case reports available in the literature concerning primary hepatic AS over the past 10 years (Table 1).

The clinical course of AS is aggressive and prognosis is poor. In a study of 67 patients with AS, an overall 5-year survival of 35% has been reported[20]. Morgan *et al*[21] investigated 47 patients with cutaneous AS, outlining the

restricted prognosis and demonstrating a 5-year survival of 34%. Another analysis showed a median overall survival of 28.4 months in a cohort of 88 patients with AS of the scalp[14]. We observed a lethality of 65% in 43 patients who underwent surgery for AS with a median follow-up of 56 months[13].

AS presents with various unspecific clinical symptoms and a multilocular occurrence. Different sites of occurrence go along with a wide range of non-specific symptoms such as pain, swelling, extrusion, hemorrhage, or bowel obstruction[22]. Tumors of the skin and of superficial or deep soft tissue are usually biopsied preoperatively and thus, therapeutic decisions are made before surgery[13]. However, as also reported here, organotropic AS may be diagnosed consecutive to emergency treatment[23-26] or after the surgery for assumed benign disease. Thus, AS is often diagnosed unexpectedly after surgical procedures which do not meet oncological treatment standards.

To our knowledge, there are only two cases of hepatic AS reported to date, which have been treated by emergency catheter-directed embolization with consecutive liver resection (Table 1)[24,25]. Catheter-directed intervention is a useful method to cease acute arterial bleeding from the liver. The most common indication is traumatic laceration of the liver parenchyma[27]. Spontaneous bleeding and the formation of hematoma impede the clinical differentiation of the cause of the hemorrhage. In benign lesions (adenoma), a subsequent surgical resection of the affected parenchyma is curative. The catheter-directed embolization allows to perform a planned and well structured operation without the need of emergency liver surgery with high morbidity and lethality. In emergency cases with spontaneous bleeding, a differentiation of malignant or benign disease is impossible. Therefore, the final diagnosis is usually made by the histopathological evaluation of the resected specimen. At the time of the histopathological diagnosis, the prognosis of the disease becomes clear. In ruptured AS of the liver, the prognosis is devastating. Rupture in these cases leads to tumor cell spillage and consecutive diffuse angiosarcomatosis in the peritoneum. The angiosarcomatous lesions consecutively induce diffuse hemorrhage with subsequent loss of large amounts of blood. The acute bleeding from diffuse peritoneal angiosarcomatosis cannot be cured by surgical intervention and thus seems to be the leading cause for death in this rare subset of patients with AS. In an own observational series of 22 AS, 6 primary hepatic AS were found. Three of these patients died within 4 months and one patient 29 months after the diagnosis in spite of surgical intervention. The preliminary cause of death in the latter patients was peritoneal sarcomatosis with diffuse intra-abdominal bleeding[13].

The clinical course of the patient reported here shows that there is no effective treatment available till date either for cure or palliation of ruptured AS of the liver. The value of chemotherapy applied to the peritoneal cavity or systemically has not been clarified nor the value of experimental antitumoral strategies such as molecular therapies (e.g., with VEGF-antagonists) or hyperthermia. This clinical situation underlines the need for new therapy modalities and broader based clinical studies in patients with ruptured AS of the liver. An effort needs to be undertaken to include patients with ruptured AS of the liver after surgery into prospective trials. The opportunity to establish an international study group may, furthermore, allow a collection of tumor samples from AS in a reasonable number. Consecutive molecular analysis could help to establish targeted antitumoral therapies for these rare and extremely lethal malignancies.

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S- Editor Guo SY **L- Editor** Elsevier HK **E- Editor** Cao L

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World J Gastroenterol 2006 February 7; 12(5):809-811
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

First report of a *de novo* germline mutation in the *MLH1* gene

Rein P Stulp, Yvonne J Vos, Bart Mol, Arend Karrenbeld, Monique de Raad, Huub JC van der Mijle, Rolf H Sijmons

Rein P Stulp, Yvonne J Vos, Bart Mol, Monique de Raad, Rolf H Sijmons, Department of Clinical Genetics, University Medical Center Groningen, PO Box 30001, 9700 RB Groningen, The Netherlands
Arend Karrenbeld, Department of Pathology, University Medical Center Groningen, PO Box 30001, 9700 RB Groningen, The Netherlands
Huub JC van der Mijle, Department of Surgery, Nij Smellinghe Hospital, PO Box 20200, 9200 DA Drachten, The Netherlands
Correspondence to: Rein P Stulp, Department of Clinical Genetics, University Medical Center Groningen, PO Box 30001, 9700 RB Groningen, The Netherlands. r.p.stulp@medgen.umcg.nl
Telephone: +31-50-3632929 Fax: +31-50-3632950
Received: 2005-05-13 Accepted: 2005-07-15

Abstract

Hereditary non-polyposis colorectal carcinoma (HNPCC) is an autosomal dominant disorder associated with colorectal and endometrial cancer and a range of other tumor types. Germline mutations in the DNA mismatch repair (MMR) genes, particularly *MLH1, MSH2,* and *MSH6*, underlie this disorder. The vast majority of these HNPCC-associated mutations have been proven, or assumed, given the family history of cancer, to be transmitted through several generations. To the best of our knowledge, only a single case of a *de novo* germline MMR gene mutation (in *MSH2*) has been reported till now. Here, we report a patient with a *de novo* mutation in *MLH1*. We identified a *MLH1* Q701X truncating mutation in the blood lymphocytes of a male who had been diagnosed with rectal cancer at the age of 35. His family history of cancer was negative for the first- and second-degree relatives. The mutation could not be detected in the patient's parents and sibling and paternity was confirmed with a set of highly polymorphic markers. Non-penetrance and small family size is the common explanation of verified negative family histories of cancer in patients with a germline MMR gene mutation. However, in addition to some cases explained by non-paternity, *de novo* germline mutations should be considered as a possible explanation as well. As guidelines that stress not to restrict MMR gene mutation testing to patients with a positive family history are more widely introduced, more cases of *de novo* MMR gene germline mutations may be revealed.

Key words: *MLH1*; *de novo*; HNPCC; Germline mutation

Stulp RP, Vos YJ, Mol B, Karrenbeld A, de Raad M, van der Mijle HJC, Sijmons RH. First report of a *de novo* germline mutation in the *MLH1* gene. *World J Gastroenterol* 2006; 12(5): 809-811

http://www.wjgnet.com/1007-9327/12/809.asp

INTRODUCTION

Hereditary non-polyposis colorectal carcinoma (HNPCC) is a genetic disorder caused by germline mutations in DNA mismatch repair (MMR) genes, especially *MLH1*, *MSH2* and *MSH6*. HNPCC is characterized clinically by the early-onset colorectal and endometrial cancer as well as a range of other tumor types. In contrast to familial adenomatous polyposis (FAP), HNPCC lacks an easily recognizable clinical phenotype in individual patients. The original and revised Amsterdam criteria (AC and AC II) introduced to clinically define HNPCC therefore include specific characteristics of the family history that should be met[1,2]. However, it has become clear that HNPCC patients may have a negative family history. Small family sizes and less than 100% penetrance of the germline mutations involved are its most common explanation. Here, we report the rare phenomenon of a *de novo* germline MMR gene mutation as the cause of a negative family history in a patient with hereditary non-polyposis colorectal carcinoma.

CASE REPORT

A 35-year-old male had been referred to our department for genetic testing and counseling because he had very recently been diagnosed with a $T_4N_0M_0$ rectal adenocarcinoma. Family history of cancer was negative for the first- and second-degree relatives of the patient. The patient's mother, father, and sibling (aged 60, 61, and 38 years, respectively) were healthy.

As part of an ongoing study on the genetics of colorectal cancer, testing for tumor microsatellite instability (MSI), tumor immunohistochemistry for the MLH1, MSH2 and MSH6 proteins as well as mutation analysis of *MLH1, MSH2* and *MSH6* are offered to all the patients with colorectal cancer diagnosed before the age of 50 who are referred to our department for genetic testing and counseling.

DNA was extracted from formalin-fixed and paraffin-embedded tumor and non-tumor tissues as described previously[3]. Subsequent MSI analysis was performed using criteria and primers as described in the Bethesda

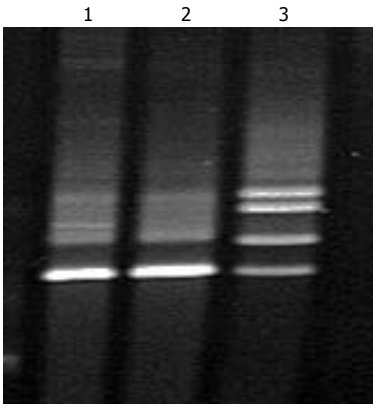

Figure 1 Denaturing gradient gel electrophoresis (DGGE) analysis of exon 18 of *MLH1* gene. Lanes 1 and 2: Father and mother, respectively; lane 3: index patient.

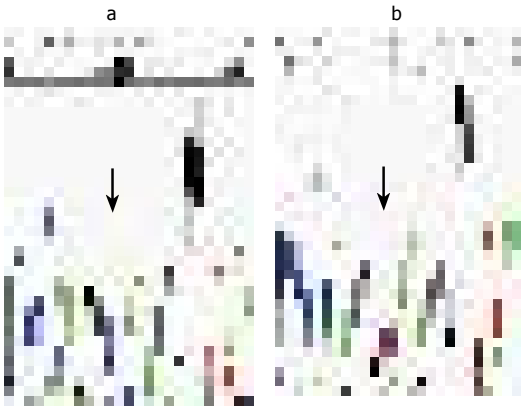

Figure 2 DNA sequence analysis of exon 18 of *MLH1* gene. (a) A control sample without mutation, (b) the index patient showing a C to T substitution of base 2 101 leading to Gln701Stop.

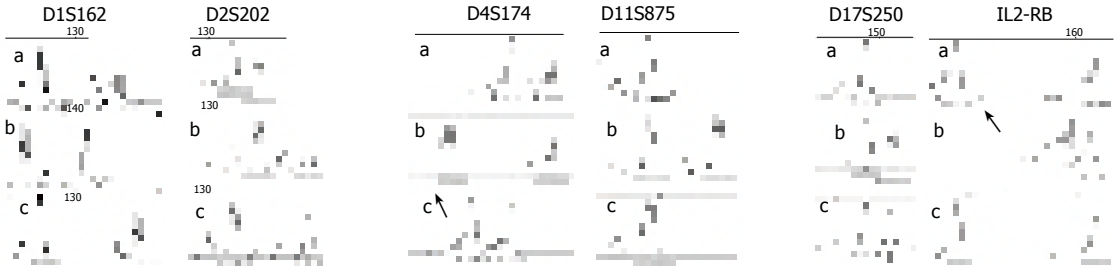

Figure 3 Paternity test using six highly informative microsatellites. a: The index patient; b: his father; c: his mother.

guidelines[4]. PCR products were analyzed on 60 g/L denaturing polyacrylamide gels with an LKB ALF DNA sequencer. For data analysis, the DNA fragment analyzer (Pharmacia) was used.

Immunohistochemistry for MLH1, MSH2, and MSH6 protein expression was carried out as described previously[5]. Protein expression in normal tissue surrounding the cancer served as internal control.

Mutation analysis of the *MLH1* and *MSH2* genes in DNA obtained from peripheral blood lymphocytes was carried out using denaturing gradient gel electrophoresis (DGGE), followed in case of aberrant patterns by direct sequencing of independently amplified PCR products as previously described[6,7]. To investigate paternity, a panel of six highly polymorphic markers was analyzed with PCR using markers D1S162, D2S202, D4S174, D11S875, D17S250, and IL2-RB in the patient and his parents. PCR products were analyzed on 60 g/L denaturing polyacrylamide gels with an LKB ALF DNA sequencer. For data analysis, the DNA fragment analyzer (Pharmacia) was used.

The tumor of the patient revealed microsatellite instability (MSI-H), as well as loss of immunohistochemical staining for the MLH1 protein with a positive internal control. Mutation analysis revealed a C to T substitution at nucleotide 2 101 in exon 18 of the *MLH1* gene, leading to an early stop codon (Gln701Stop). Neither the father nor the mother carried the mutation identified in their son (Figures 1 and 2). All six tested markers were in concordance with paternity (likelihood: >99.9%) (Figure 3)

DISCUSSION

Although the Q701X *MLH1* mutation has not been reported before in either the Human Gene Mutation Database (HGMD) and the ICG-HNPCC database, we have no reason to doubt its pathogenicity given its truncating nature and the findings of MSI-H and loss of staining for the MLH1 protein in the patient's tumor[8,9].

De novo germline mutations of the MMR genes in HNPCC are rare. To the best of our knowledge, only one single case, involving *MSH2*, has been reported so far[10]. We have identified a *de novo* germline mutation in the *MLH1* gene. The classification "germline" mutation is generally used in oncogenetics to distinguish mutations that can be transmitted to the offspring from the somatic mutations occurring locally in tissues and which may lead to local tumor development. For practical purposes, we therefore used the "germline" label. Strictly speaking, mutations classified as *de novo* germline mutations should refer to a single mutation occurring in a single germcell. Clinically, these often cannot be distinguished from an early postzygotic mutational event in the patient, or from germline mosaicism or somatic mosaicism extending to extra-gonadal tissues in the parents. We did not have the opportunity to test multiple tissues in our patient or his parents for the mutation. The patient's single sibling was offered mutation analysis and was shown to carry the wild-type *MLH1* alleles. Therefore, we lack proof of the germline nature of the *de novo* mutation reported by us and the same is true for the *MSH2* case published earlier by Kraus *et al* [10]. In clinical practice, this lack of proof does not lead to any obvious problems. One will simply discuss with the patient the fact that his or her siblings have a, probably small, risk to carry the mutation as well and one will offer to test them for it.

Non-penetrance and small family size is the common explanation of verified negative family histories of cancer

in patients with a germline MMR gene mutation. However, in addition to some cases due to non-paternity, *de novo* "germline" mutations should be considered as a possible explanation as well. The percentage of these mutations in HNPCC remains to be established. As guidelines (e.g., the Bethesda guidelines)[4] that stress not to limit MMR gene mutation testing to patients with a positive family history are more widely introduced, more cases of *de novo* "germline" MMR mutations are yet to be revealed.

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S- Editor Kumar M and Guo SY **L- Editor** Elsevier HK **E- Editor** Cao L

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World J Gastroenterol 2006 February 7; 12(5):812-814
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Successful percutaneous drainage of a giant hydatid cyst in the liver

István Battyány, Zsuzsanna Herbert, Tamás Rostás, Áron Vincze, Adrien Fülöp, Zoltán Harmat, Beáta Gasztonyi

István Battyány, Zsuzsanna Herbert, Tamás Rostás, Adrien Fülöp, Zoltán Harmat, Department of Radiology, University of Pécs, Hungary
Áron Vincze, Beáta Gasztonyi, 1st Department of Medicine, University of Pécs, Hungary
Correspondence to: István Battyány, MD, PhD, Chief Radiologist, Department of Radiology, Medical School, University of Pécs, Ifjúság u. 13., 7624 Pécs, Hungary. batyo@clinics.pote.hu
Telephone: +36-72-536-197 Fax: +36-72-536-199
Received: 2005-07-25 Accepted: 2005-08-26

Key words: Echinococcosis; Giant hydatid cyst; PAIR technique; Percutaneous drainage; Anaphylaxis

Battyány I, Herbert Z, Rostás T, Vincze Á, Fülöp A, Harmat Z, Gasztonyi B. Successful percutaneous drainage of a giant hydatid cyst in the liver. *World J Gastroenterol* 2006; 12(5): 812-814

http://www.wjgnet.com/1007-9327/12/812.asp

CASE REPORT

A 35-year-old female was hospitalized for abdominal pain, bloating, and vomiting. Plain X-ray pictures exhibited bowel obstruction for which she underwent emergency surgery. On the second postoperative day, she started to complain about dull right upper quadrant abdominal pain and bloating. Abdominal CT scans revealed very large cystic lesion with an inhomogeneous fluid content. The cyst occupied the whole right lobe of the liver compressing the surrounding organs and dislocating the left lobe of the liver towards the left hypochondrium (Figure 1). Additionally, several more cysts of various sizes were seen in the whole abdomen. This radio-morphology raised the possibility of a hydatid disease (caused by ruptured hydatid cyst) confirmed through positive echinococcus IgG serology (ELISA). Interestingly, no hypereosinophilia could be detected through repeated blood tests.

Mebendazole (200 mg/d for 3 d) was administered in order to prevent anaphylactic reactions and the dissemination of the disease. After obtaining written consent from the patient, the site of the puncture was defined to the X–XI intercostal space. The procedure was performed under ultrasound guidance (Diasonics Master Series VST) and the patient was closely monitored for any sign of anaphylaxis. The cyst that was punctured on its free surface with an 18 gauge needle contained a dense whitish fluid. Samples were taken for parasitological examinations. Because of the huge size of the cyst, an 8F pigtail catheter (polyurethane drainage catheter, COOK) with 32 side holes was introduced into the cyst. After aspiration of 6 200 mL of the cystic fluid (Figure 2), 150 mL of 15% hypertonic saline was injected into the cyst cavity. Twenty minutes later, the catheter was attached to a vacuum tube and left for suction drainage. This cycle (hypertonic 15% saline injection and 20 min later reaspiration) was repeated daily with 40 mL 15% sterile saline. The initial procedure lasted for 30 min. At the end of the flushing series, absolute alcohol of 30 mL was injected and removed after 20 min during the last 5 days. During the procedure the patient remained symptomless and no anaphylactic reaction could be observed. Ultrasonographically, the cyst decreased in size gradually with only a 7.6 mm subcapsular fluid collection remaining under the right diaphragm. The total time of percutaneous drainage took 43 d.

Under light microscopy, the aspirated cystic fluid showed clear evidence of hydatid scolices verifying the presumed diagnosis of echinococcosis. After 6 weeks of percutaneous drainage the catheter was removed. Abdominal CT scans exhibited total disappearance of the cyst. Subsequently, the patient was discharged and reported no symptom relapses on the follow-up period of 1 year.

DISCUSSION

Echinococcus granulosus is a small, 5-mm-long tapeworm being responsible for unilocular hydatid cyst disease. Human infection by *E. granulosus* occurs most commonly in sheep- and cattle-raising areas where dogs assist in herding; the infection is more frequent in Eastern Europe, the Mediterranean, Australia, New Zealand, Chile, Argentina, and Africa. Human beings are usually infected as intermediate hosts when they ingest egg-contaminated food or water. Over 50% of all human *E. granulosus* infections involve the liver[1]. Additional common sites for hydatid cysts are the lungs, spleen, kidneys, heart, bones, and brain[2].

Since hydatid cysts grow at a rate of about 1–5 cm/year, a long latent period is typical. The symptoms of hydatid disease are related primarily to the mass effect of the slowly enlarging cyst: right upper quadrant pain from the stretching hepatic capsule, jaundice from compression

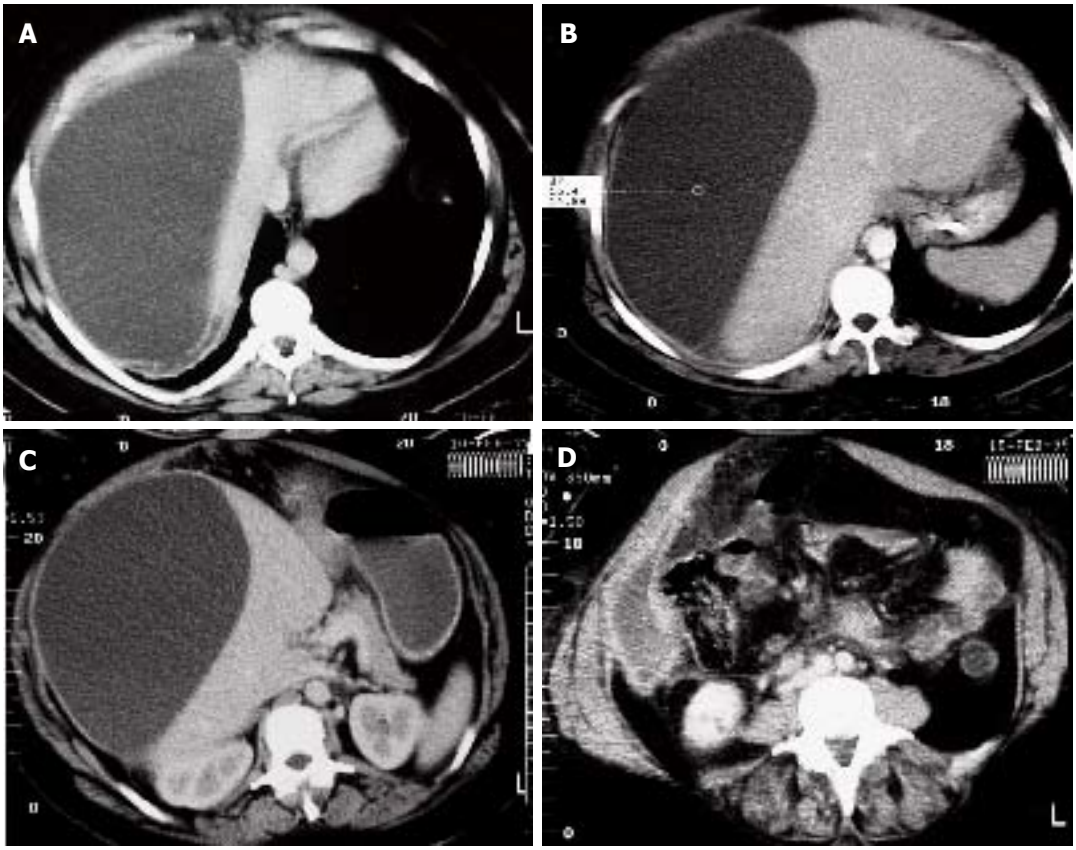

Figure 1 Abdominal CT scan. **A:** Axial CT scan shows the large inhomogeneous cystic fluid collection occupying the whole right lobe of the liver; **B:** CT scan of the cystic lesion on the level of spleen; **C:** the largest cystic lesion compresses the left lobe of the liver dislocating the nearby organs; **D:** cystic collection was seen also in the pararenal spaces and extended into the small pelvis.

Figure 2 **A**: The aspirated fluid (6 200 mL); **B**: drainage catheter with sucking bottle.

of the bile duct, or portal hypertension from portal vein obstruction. Following a presumably long latency, our patient presented with symptoms related to bowel obstruction caused by the space-occupying effect of the giant hydatid cyst. CT scans and ultrasonography are very useful for revealing well-defined cysts with thick or thin walls which correspond to our case as well[2]. The 6.2-L large cyst of our case is the largest hydatid cyst published in the literature. The visualization of daughter cysts within the larger cyst and the mural calcification helps to distinguish *E. granulosus* infection from carcinomas, bacterial or amebic liver abscesses, or hemangiomas[3]. Either spontaneously or as a result of a complication to surgery, free peritoneal rupture of the cyst can lead to fatal anaphylaxis. On the long term, leakage of the cyst may cause an intense antigenic response, resulting in eosinophilia, bronchial spasm, or anaphylactic shock.

Open surgical drainage of the hydatid cysts has been the preferred method of therapy. Several surgical techniques have been applied for the treatment of hydatid disease: simple-closure tube drainage, marsupialization, cystectomy, total pericystectomy, hepatic resection, and cavity management (omentoplasty, internal drainage)[4]. Among them, total pericystectomy was found to be the most effective method at reducing recurrence and improving resolution of the hepatic defect. The mortality rate after surgery ranges from 0% to 6.3%, while the rate of postoperative complications varies between 12.5% and 80%[4]. Jabbour *et al*[5] suggested that surgery should be the treatment of choice for large, multiple, complicated, and recurrent hydatid cysts of the liver.

The percutaneous treatment has been used in many hospitals as the alternative method to surgery[6]. A prospective randomized trial of 50 patients found that

percutaneous drainage and hypertonic saline irrigation of the cysts combined with albendazole is as effective as surgical drainage, with fewer complications and shorter durations of hospitalization[7]. A long-term follow-up study has also confirmed the effectiveness of percutaneous drainage with mortality and morbidity rates more favorably than those of conventional surgical approaches[8]. The generally accepted and safe technique for percutaneous drainage is called PAIR that stands for puncture, aspiration of cyst, injection of hypertonic saline and absolute alcohol, and reaspiration[9]. Hypertonic saline exerts a scolicidal effect and prevents the formation of daughter cysts through the irrigation of the germinative layer[10]. Absolute alcohol is the agent of sclerotherapy against the wall of the hydatid cyst. Formerly, the percutaneous drainage of the hydatid cyst was deemed to be a hazardous procedure due to the potential risk of anaphylaxis and dissemination[11]. Filice and Brunetti[12], however, observed no anaphylactic reaction or peritoneal dissemination having applied the PAIR technique for 231 cysts in 163 patients. In agreement with this report, Haddad *et al*[13] experienced only minor complications including pain, mild fever, pleural effusion, and transient hypernatremia in their series. In a 6-9 year follow-up involving ultrasonography, CT scans, and serology tests, no local recurrence or spread of the disease were found[13]. Pelaez *et al*[14] successfully treated 55 hydatid cysts in the liver in 34 patients with the combination of PAIR technique and albendazole prophylaxis. They reported only urticaria and subcapsular hematoma as minor complications and the mean hospitalization duration was 1.82 d. Akhan *et al*[4,8]. reported good accuracy rate and low complication rate in the percutaneous treatment of hydatid disease. Using the percutaneous drainage treatment combined with mebendazole prophylaxis in our patient, we could successfully manage the largest hydatid cyst ever reported. The applied drainage method seems to be useful for the treatment of huge hydatid cysts and has also been recommended as an effective and safe therapeutic choice.

In summary, this patient has demonstrated that percutaneous drainage is the appropriate and feasible alternative to surgery in treating hydatid cysts even of this giant size with a low risk of severe complications.

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World J Gastroenterol 2006 February 7; 12(5):815-817
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Small gastrointestinal stromal tumor concomitant with early gastric cancer: A case report

Ying-Lung Lin, Jeh-En Tzeng, Chang-Kou Wei, Chih-Wen Lin

Ying-Lung Lin, Jeh-En Tzeng, Chang-Kou Wei, Chih-Wen Lin, Department of Family Medicine, Pathology, General Surgery, Radiology, Buddhist Dalin Tzu Chi General Hospital, Chia Yi, Taiwan.
Correspondence to: Dr Jeh-En Tzeng, Department of Pathology, Buddhist Dalin Tzu Chi General Hospital, 2, Min Sheng Road, Dalin, Chia Yi, Taiwan. p121521@tzuchi.com.tw
Telephone: +886-5-2648000-5725
Received: 2005-06-07 Accepted: 2005-08-03

Abstract

The term gastrointestinal stromal tumors (GISTs) is defined diagnostically as the main group of mesenchymal tumors with spindle or epithelioid cells arising from the wall of the gastrointestinal tract with immunohistochemical reactivity for CD117 antibody. Previous studies revealed that cells in GISTs express a growth factor receptor with tyrosine kinase activity (termed c-kit), which is the product of the c-kit proto-oncogene. The most specific and practical diagnostic criteria for GISTs are: immunohistochemically determined c-kit (CD117) expression; mitotic score; and tumor size. A small GIST concomitant with early gastric cancer is rarely encountered clinically. Herein we have reported a case of a 1.1-cm GIST detected by esophagogastroduo denoscopy concomitant with a IIc type of early gastric cancer (signet ring cell type). It was detected during a routine physical health examination. To our knowledge, this is the first report of a small GIST concomitant with a signet ring cell type of early gastric cancer.

Key words: Gastrointestinal stromal tumor; Early gastric cancer; *H pylori* infection; Biopsy urease test; CD117

Lin YL, Tzeng JE, Wei CK, Lin CW. Small gastrointestinal stromal tumor concomitant with early gastric cancer: A case report. *World J Gastroenterol* 2006; 12(5): 815-817

http://www.wjgnet.com/1007-9327/12/815.asp

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are malignant or potentially malignant tumors and are considered to have a specific molecular pathogenesis. They are distinguished from other mesenchymal tumors by optimal immunostaining for CD117 and a prognostic classification is based on tumor size, mitotic score, and MIB-1 grade[1]. Gain-of-function mutations of the *c-kit* gene and immunoreactivity of the c-kit protein (CD117) in many GISTs support the idea that GISTs are a biologically distinct entity. We have reported a case of a small GIST concomitant with a IIc type of early gastric cancer (signet ring cell type) and provided a literature review.

CASE REPORT

A 70-year-old yellow race female came to our hospital for a physical health examination in November 2003. She had no past history of epigastralgia or peptic ulcer. Esophagogastroduodenoscopy (EGD) showed one approximately 0.4-cm sessile polyp with a smooth surface at the right upper posterior wall of the gastric fundus (Figure 1A). Biopsy was taken and three specimens were acquired. Histologically, one of the three specimens showed whorling bundles of spindle cells with mitosis. Only chronic gastritis was found in the other two specimens. A GIST was suspected. EGD simultaneously detected one approximately 1.7 cm × 1.4 cm, depressed, flat white-red based lesion with oozing hemorrhages at the gastric angle. The lesion looked like a IIc type of early gastric cancer (Figure 1B). Biopsy was carried out and 13 specimens were obtained. Pathological sections demonstrated an ill-defined tumor with signet ring cells within the gastric mucosa. A poorly differentiated adenocarcinoma was diagnosed and *Helicobacter* microorganisms were found. An imaging study with abdominal computed tomography demonstrated no remarkable mass lesion at the posterior wall of the gastric fundus and gastric angle. There was no fluid collection, and no mass lesion or lymph node was found in the intraperitoneal cavity, liver, kidneys, and other organs. Owing to its malignant nature, surgical intervention was performed one week later. Grossly, we observed a 1.1 cm × 0.8 cm × 0.7 cm fundal mass protruding from the inner muscularis propria to the mucosa (Figures 2A and 2B). Histologically, the gastric fundal tumor demonstrated whorling sheets of spindle cells which stained positively for CD117, CD34, NSE, S-100 protein, and actin-851 antibodies after immunohistochemical (IHC) staining (Figures 2C and 2D). GIST, with combined smooth muscle-neural differentiation, was diagnosed. The gastric angular tumor showed a residual adenocarcinoma of the signet ring cell type within the mucosa (Figures 2E and 2H). The postoperative period of the patient was uneventful, and

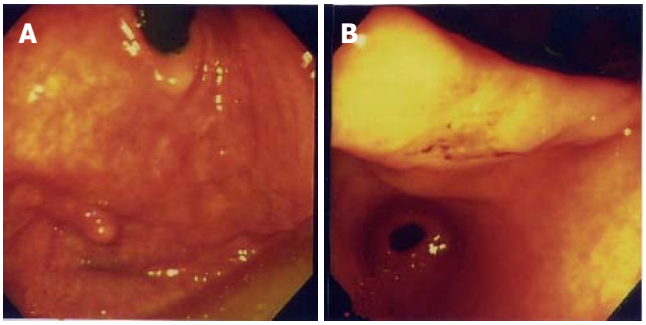

Figure 1 Esophagogastroduodenoscopic examination. **A:** A 0.4-cm sessile polyp with a smooth surface is seen at the right upper posterior wall of the gastric fundus. **B:** An approximately 1.7 cm × 1.4 cm, depressed, flat white-red based lesion with oozing hemorrhages is present at the gastric angle.

she was discharged one week later. No evidence of tumor recurrence was found after 14-months of follow-up.

DISCUSSION

GIST concomitant with early gastric cancer has rarely been reported. To the best of our knowledge, a small GIST concomitant with a signet ring cell type of early gastric cancer has never been reported. Although Japanese investigators reported some cases of gastric leiomyoblastoma associated with gastric cancer between 1971 and 2000, CD-117 immunohistochemical staining was not confirmed in those cases. Our 1.1-cm GIST detected by EGD was a small one. The origin of a GIST concomitant with an early gastric adenocarcinoma is unclear. This small GIST showed a positive microscopic finding of *H pylori* microorganisms and a positive CLO test (biopsy urease test) for *H pylori* infections. *H pylori* was implicated as a carcinogen of the stomach by the World Health Organization in 1994. We had previously detected two gastric GIST cases in 2003; both of those had a positive finding of *H pylori* microorganisms and a positive CLO test for *H pylori* infections. However, this finding is more likely an incidental finding rather than a causal associ ation.

GISTs are considered to be a group of mesenchymal neoplasms, and are also the subject of much debate and controversy regarding their nomenclature, histogenesis, criteria for diagnosis, prognostic manifestations, and classification[1,2]. Studies have revealed that some of these tumors may have tumor markers and features of neural, muscular or vascular endothelial differentiation. The term GIST has been adopted and defined as tumors arising from the stroma with no definite cell line of origin and varying patterns of differentiation[3]. Our GIST showed a mixed type of smooth muscle-neural origin. Some investigators emphasize the CD117 and CD34 expression in GISTs[1,4-6]. GISTs are now preferentially defined as tumors with c-kit (CD117) positive mesenchymal spindle cells or epithelioid neoplasms primarily in the gastrointestinal tract, omentum, and mesentery[4].

Statistically, GISTs are most common in the stomach (60-84.8%), followed by small intestine (10.5-30%), colon and rectum (3.5-5%), and esophagus (1.2-5%)[1,4]. The most important manifestation of stromal tumors is their

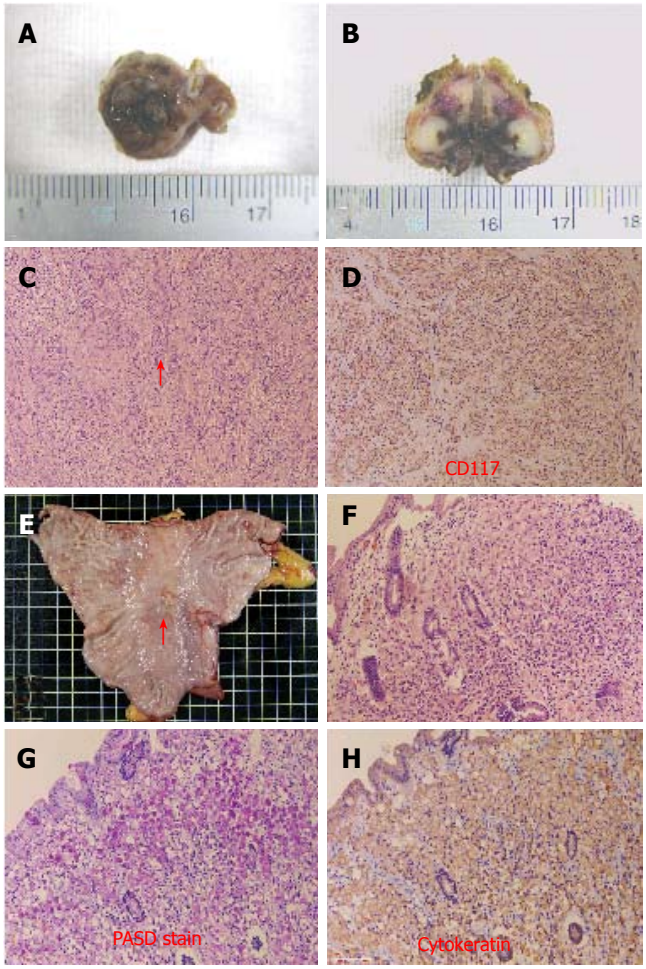

Figure 2 A The excised 1.1-cm fundal tumor showing gray fleshy and nodular cut surface; **B** and **C**: Its histologic picture demonstrating whorling bundles of spindle cells with a mitosis (arrow, H&E, × 200); **D**: staining of spindle cells for CD117 (IHC, × 200); **E**: subtotal gastrectomized specimen showing a 1.7 cm × 1.4 cm ulcerative mass at the angularis of the lesser curvature side; **F**: tumor showing signet ring cells within the lamina propria histologically (H&E, × 200); **G**: tumor stained for PAS-diesterase (×200); **H**: tumors stained for cytokeratin (IHC, ×200).

indolent, slow growing nature. The tumors are generally found within the deeper stroma and the submucosa, and are often incidentally found during imaging studies. Our small GIST protruded about half of its mass into the upper posterior wall of the gastric fundus and only a 0.4 cm polypoid mass could be found in the endoscopic field.

GISTs often present with non-specific symptoms, such as nausea, vomiting, abdominal pain, gastrointestinal bleeding, and metastatic diseases. Bleeding is considered as the most common presentation of the clinical course[2]. Symptoms depend on tumor size and location. In our case, the GIST was totally asymptomatic. Ulcerations seem relevant to the size of GISTs. The size and mitotic score are considered as important diagnostic criteria and prognostic predictive indicators[1,2]. This GIST was diagnosed as a potential malignancy with a very low risk according to the pathological findings of mitosis (2 per 50 HPF) with combined smooth muscle-neural differentiation and positive stains for CD117 and CD34[1,2,5-7].

Generally, the preoperative rate of diagnosis of submucosal tumors by an endoscopic biopsy or an imaging

study without skillful examination with an endoscopic ultrasonographic system and angiography is very low. Once a submucosal tumor is suspected, more careful endoscopic procedures, and repeat deep biopsy of the same area to reach the target tumor may have a higher diagnostic rate of histological findings, as our experience attests.

Patients with advanced GISTs which progress rapidly and result in organ destruction have poor prognosis. Therapeutic options for GISTs include surgery and treatment with STI-571 (Gleevec). STI-571 is a small molecule competitive inhibitor of the ATP binding site and demonstrates a high degree of specificity for inhibiting the activity of a small number of related tyrosine kinases: c-Ab1, Bcr-Ab1 (the molecular cause of chronic myeloid leukemia), platelet-derived growth factor receptors[8-10], and wild-type and mutant c-kit (stem cell factor receptor)[8,11,13]. This selective activity of STI-571 suggests that it has a relatively narrow target spectrum of anticancer activity. This highly selective molecule has a different therapeutic effect. Most malignant GISTs have mutant c-kit and some studies show that c-kit may be activated by mutation in three domains: extracellular, juxtamembrane and kinase portions (exon 9 or 11 or 13)[5,6,11,12]. STI-571 has the ability to inhibit signal transduction via c-kit and it is predictable that it should inhibit hematopoietic stem cells, resulting in neutropenia, anemia, thrombocytopenia, GI bleeding or intratumor hemorrhage[7].

Surgery is still the mainstay treatment for GISTs. Subtotal gastrectomy for the early gastric cancer with local resection for the small GIST was performed simultaneously for our patient with a small GIST concomitant with a IIc type of early gastric cancer.

In conclusion, we have reported a rare case of a small GIST combined with an early gastric cancer. It showed positive microscopic findings of *H pylori* microorganisms, a positive CLO test, and stainings for CD117 and CD34. More GIST cases are required for evaluating the relationship between *H pylori* infections and the etiologies of GIST concomitant with an early gastric cancer. Long-term observation for all GIST cases is greatly needed.

ACKNOWLEDGMENTS

We greatly appreciate the financial and technical support of Dalin Tzu Chi General Hospital, Dr. Takahira Ikeda and the president of Japan Jichi Medical University, Professor Fumimaro Takaku who kindly refined the manuscript.

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S- Editor Kumar M and Guo SY **L- Editor** Elsevier HK **E- Editor** Cao L

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World J Gastroenterol 2006 February 7; 12(5):818-821
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

A case of successful management with splenectomy of intractable ascites due to congenital dyserythropoietic anemia type II-induced cirrhosis

Themistoklis Vassiliadis, Vassilia Garipidou, Vassilios Perifanis, Konstantinos Tziomalos, Olga Giouleme, Kalliopi Patsiaoura, Michalis Avramidis, Nikolaos Nikolaidis, Sofia Vakalopoulou, Ioannis Tsitouridis, Antonios Antoniadis, Panagiotis Semertzidis, Anna Kioumi, Evangelos Premetis, Nikolaos Eugenidis

Themistoklis Vassiliadis, Vassilia Garipidou, Vassilios Perifanis, Konstantinos Tziomalos, Olga Giouleme, Michalis Avramidis, Nikolaos Nikolaidis, Sofia Vakalopoulou, Panagiotis Semertzidis, Nikolaos Eugenidis, 2nd Propedeutic Department of Internal Medicine, Aristotle University of Thessaloniki, Hippokration General Hospital, Thessaloniki, Greece
Kalliopi Patsiaoura, Department of Pathology, Hippokration General Hospital, Thessaloniki, Greece
Ioannis Tsitouridis, Department of Radiology, Papageorgiou General Hospital, Thessaloniki, Greece
Antonios Antoniadis, Organ Transplant Unit, Aristotle University of Thessaloniki, Hippokration General Hospital, Thessaloniki, Greece
Anna Kioumi, Department of Hematology, Papageorgiou General Hospital, Thessaloniki, Greece
Evangelos Premetis, Hematology Laboratory, Aghia Sophia Children's Hospital, Athens, Greece
Correspondence to: Konstantinos Tziomalos, 63 Solonos Street, Thessaloniki, 54248, Greece. ktziomalos@yahoo.com
Telephone: +30-2310823487 Fax: +30-2310992834
Received: 2005-06-22 Accepted: 2005-07-28

Abstract

The congenital dyserythropoietic anemias comprise a group of rare hereditary disorders of erythropoiesis, characterized by ineffective erythropoiesis as the predominant mechanism of anemia and by characteristic morphological aberrations of the majority of erythroblasts in the bone marrow. Congenital dyserythropoietic anemia type II is the most frequent type. All types of congenital dyserythropoietic anemias distinctly share a high incidence of iron loading. Iron accumulation occurs even in untransfused patients and can result in heart failure and liver cirrhosis. We have reported about a patient who presented with liver cirrhosis and intractable ascites caused by congenital dyserythropoietic anemia type II. Her clinical course was further complicated by the development of autoimmune hemolytic anemia. Splenectomy was eventually performed which achieved complete resolution of ascites, increase of hemoglobin concentration and abrogation of transfusion requirements.

Key words: Portosystemic shunt; Hemosiderosis; Splenectomy; Intractable ascites; Congenital dyserythropoietic anemia type II

Vassiliadis T, Garipidou V, Perifanis V, Tziomalos K, Giouleme O, Patsiaoura K, Avramidis M, Nikolaidis N, Vakalopoulou S, Tsitouridis I, Antoniadis A, Semertzidis P, Kioumi A, Premetis E, Eugenidis N. A case of successful management with splenectomy of intractable ascites due to congenital dyserythropoietic anemia type II-induced cirrhosis. *World J Gastroenterol* 2006; 12(5): 818-821

http://www.wjgnet.com/1007-9327/12/818.asp

INTRODUCTION

All types of congenital dyserythropoietic anemias (CDAs) distinctly share a high incidence of iron loading[1]. As in other forms of anemia with ineffective erythropoiesis, this is due to the upregulation of iron resorption[2]. Iron accumulation occurs even in untransfused patients and can result in diabetes mellitus, hypogonadotropic hypogonadism and more importantly, heart failure and liver cirrhosis[3-5].

We have reported about a patient who presented with liver cirrhosis and intractable ascites caused by CDA II. Her clinical course was further complicated by the development of autoimmune hemolytic anemia. Splenectomy was eventually performed, which achieved complete resolution of ascites, increase of hemoglobin concentration and abrogation of transfusion requirements.

CASE REPORT

A 58-year-old female presented to our department because of progressive abdominal distention. She had a history of CDA II that was diagnosed 15 years ago, and received blood transfusion only at the time of diagnosis and never again.

Physical examination revealed mild jaundice and abdominal distention. The edge of the liver extended 3 cm below the right costal margin and marked splenomegaly

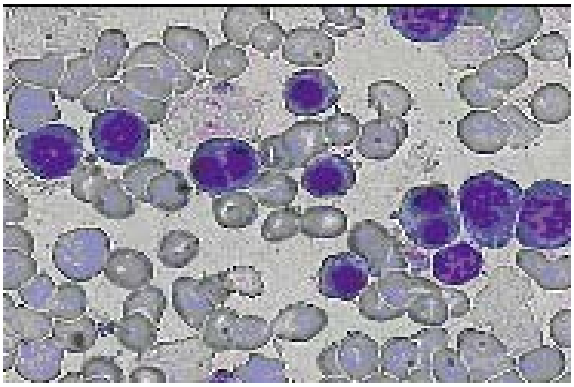

Figure 1 Peripheral blood smear showing binucleated normoblasts.

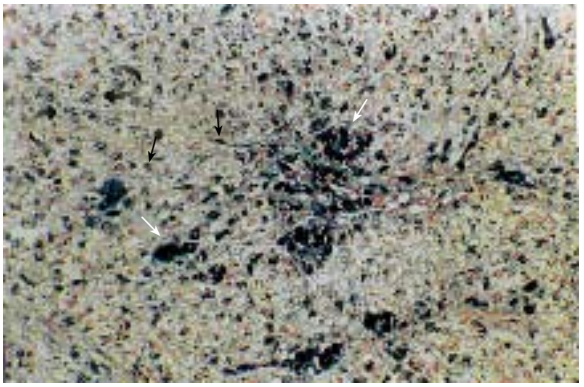

Figure 2 Liver biopsy showing marked siderosis of hepatocytes (curved arrows), Kupffer cells (arrowheads with ball) and portal macrophages (arrows) (Perl's stain, ×200).

was also found. Percussion of the abdomen revealed the presence of flank dullness and "shifting".

The results of laboratory tests were: WBC 7700/μL, Hb 9.8 g/dL, MCV 91.7 μm^3, MCH 32.1 pg, PLT 223 000/μL, PT 13.9, INR 1.07, PTT 28.0, glucose 120 mg/dL, urea 53 mg/dL, creatinine 0.9 mg/dL, AST 81 U/L, ALT 117 U/L, LDH 176 U/L, protein 7.0 g/dL, albumin 4.3 g/dL, total bilirubin 1.76 mg/dL, unconjugated bilirubin 1.44 mg/dL, ferritin 2 200 ng/mL. Folic acid was 7.4 ng/mL (normal range, >5.38 ng/mL) and vitamin B12 was 352 pg/mL (normal range, 211-911 pg/mL), thus ruling out megaloblastic anemia. Hemoglobin electrophoresis showed normal findings.

Analysis of the ascitic fluid obtained after abdominal paracentesis yielded the following results: absolute polymorphonuclear leukocyte count 100/mm^3, albumin 1.0 g/dL (serum-ascites albumin gradient 3.3 g/dL), glucose 128 mg/dL, LDH 43 U/L.

Peripheral blood smear showed anisopoikilocytosis, basophilic stippling and binucleated normoblasts (Figure 1). The Ham's test was negative, possibly because of a small amount of normal sera used. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) of erythrocyte membrane proteins showed that band 3 was narrower and had an accelerated migration rate. Bone marrow examination revealed marked erythroid hyperplasia, while approximately 30% of the mature erythroblasts were binucleate.

A liver biopsy documented cirrhosis with marked siderosis of hepatocytes as well as Kupffer cells and portal macrophages (Figure 2). Genetic testing for mutations of the HFE gene revealed heterozygosity for the H63D mutation, whereas the patient was negative for the C282Y mutation. Other causes of cirrhosis were thoroughly excluded, based on the medical history of the patient, negative virology for hepatitis B and C, normal immunologic tests (including testing for the presence of antinuclear antibodies, anti-smooth muscle cell antibodies and anti-mitochondrial antibodies) and liver biopsy findings.

Upper endoscopy revealed the presence of esophageal varices. An abdominal ultrasonographic study showed cholelithiasis. A cardiac ultrasonographic examination showed concentric hypertrophy and impaired diastolic function of the left ventricle, left atrium dilatation, mitral valve regurgitation and right ventricle dilatation with tricuspid valve regurgitation and pulmonary hypertension (right ventricular systolic pressure: 5 332 Pa). Magnetic resonance imaging was performed in order to determine heart and liver iron concentration using a unit (Magnetom Impact; Siemens, Germany) operating at a field strength of 1.0 Tesla. We used an electrocardiogram-triggered gradient-recalled-echo sequence for the evaluation of both myocardial and liver iron. Iron content measurement was based on a modification of the method of Bonkovsky, calculating the natural logarithm of the ratio of the mean signal intensity of the liver or heart respectively by that of the standard deviation of the mean of the signal intensity of the overlying air [ln signal intensity ratio, ln (SIR); normal values for both heart and liver, > 3.2][6]. The ln (SIR) values obtained for the heart and liver were 2.66 and 1.26 respectively.

Since the patient persistently refused to undergo iron chelation therapy with desferrioxamine, deferiprone (75 mg/kg per day in three divided doses) was initiated, spironolactone (200 mg once daily) and furosemide (80 mg once daily) were also prescribed but dose escalation was not possible because of deterioration in renal function. Since the patient also did not decrease her salt intake, large volume of paracentesis (approximately 8-10 L of ascitic fluid) had to be performed approximately every 10 d due to the development of refractory ascites.

Soon after her presentation, the patient began to require frequent blood transfusions. Direct antiglobulin test was positive due to the presence of IgG autoantibodies. Extensive investigation, including repeated immunologic tests to rule out autoimmune diseases and imaging studies to rule out occult malignancy, excluded secondary causes of autoimmune hemolytic anemia (AIHA). The patient was treated with prednisone and subsequently with intravenous immunoglobulin, both failed to produce a significant response. Therefore, we performed splenectomy in order to control both CDA and AIHA as well as proximal splenorenal shunt in order to relieve portal hypertension and ascites. Surgery and recovery were uneventful, but a Doppler examination performed immediately after the operation showed complete shunt occlusion.

During the 10-mo post-operative follow-up period, the

patient was transfusion-independent and ascites did not relapse although diuretics were discontinued. Hemoglobin concentration was persistently above 12.0 g/dL and unconjugated bilirubin was normalized. Nevertheless, the concentration of ferritin remained elevated at 1 800 ng/mL. A new cardiac ultrasonographic examination disclosed regression of the right ventricle dilatation and no signs of pulmonary hypertension (right ventricular systolic pressure: 1 999.5 Pa). Follow-up magnetic resonance imaging of the heart and liver showed heart siderosis within normal limits but no change in liver siderosis with her ln (SIR) values being 4.04 and 1.38, respectively.

DISCUSSION

The evolution of CDA II is doomed by iron overloading, which is also seen in patients with no need for transfusion and is mainly due to increased iron resorption. Lethal organ damage of the liver and myocardium is observed in untreated patients[7]. The rate of iron accumulation seems not to be affected by *HFE* gene polymorphism[8,9]. Our patient was heterozygous for the H63D mutation, which may contribute to increased hepatic iron levels but does not result in iron overload in the absence of the main mutation responsible for hereditary hemosiderosis (C282Y)[10-12].

Hemosiderosis in CDA type II is treated with desferrioxamine which is usually introduced as soon as ferritin reaches the concentration of 1 000 ng/mL[13,14]. Normal ferritin concentrations were reached in all the patients with satisfactory compliance[4] a full-stop Nevertheless, desferrioxamine must be given 5 to 7 d per week by a prolonged subcutaneous infusion[15]. On the other hand, deferiprone is an alternative chelator that is orally active and more effective than desferrioxamine in the removal of myocardial iron[16]. Indeed, heart siderosis returned to normal levels in our patient, even though the reduction in ferritin levels and liver iron overload was not so pronounced.

In CDA II, splenectomy leads to a moderate and sustained increase in hemoglobin concentration and decrease of hemolysis[4,8]. Though splenectomy has these benefits, recommendation for the operation is not uniform among the hematologists[8]. Furthermore, splenectomy does not prevent further iron loading, even in those patients with their hemoglobin concentrations becoming nearly normal as observed in our patient[4].

The concurrence of CDA II and AIHA has been described only once, thus suggesting chance occurrence[17]. In AIHA, splenectomy has the distinct advantage over other therapeutic options in that it has the potential for complete and long-term remission. Available data suggest that it triggers remission in more than 50% of patients[18]. Excellent responses or improvements are maintained during a mean follow-up period of 33 or 73 mo, respectively[19]. Accordingly, splenectomy should be considered in patients who do not respond adequately to corticosteroids[20].

In patients with refractory ascites, both transjugular intrahepatic portosystemic shunt (TIPS) and surgical portal systemic shunt have been used[21]. TIPS is a widely accepted percutaneous interventional procedure for treating complications of portal hypertension. An experienced skillful team, however, is necessary to ensure the technical success of TIPS and to avoid its potential procedural complications[22]. Shunt dysfunction is also a major problem, since over 50% of TIPS develop stenosis within 1 year and therefore, close shunt surveillance and frequent re-treatment are required with ensuing high costs[23-25]. Furthermore, hepatic encephalopathy is another important complication of cirrhotic patients with refractory ascites treated with TIPS, more than 40% of these patients develop encephalopathy[26,27]. Nevertheless, calibrated prosthesis can partly prevent TIPS complications[28].

The role of surgery in the treatment of portal hypertension remains a complex and highly controversial issue. Several factors must be considered when surgical options are to be entertained, including origin and extent of liver disease, response to prior medical treatment, and possibility of future liver transplantation[29]. Splenectomy was mandatory in our patient in order to control CDA and particularly AIHA. However, splenectomy alone is inappropriate for the treatment of portal hypertension and does not relieve ascites[30,31]. In contrast, portosystemic shunting is efficient in clearing ascites, but is associated with a high rate of encephalopathy and liver failure[29,32,33]. The indications for portosystemic shunting are therefore limited for the treatment of intractable ascites and portosystemic shunting should be performed only in patients with good liver function or when all other treatments fail[29,32]. Since our patient had preserved hepatic synthetic capacity, we chose to combine splenectomy with portosystemic shunting in order to relieve ascites and thus performed proximal splenorenal shunt. Compared to selective shunts, the central or proximal splenorenal shunt does not differ in operative mortality rates, 5-year survival rates, development of individual episodes of post-operative encephalopathy, or chronic post-operative encephalopathy[34]. Unfortunately, shunt occlusion may occur immediately after the operation. It is thus difficult to explain the complete remission of ascites and we can only postulate that a reduction in the blood flow into the portal system from the greatly enlarged spleen may result in a decrease in portal pressure. This reduction in portal pressure may also contribute to the amelioration of pulmonary hypertension as observed in our patient.

In conclusion, even in transfusion-independent patients with CDA II, iron overload can result in dramatic manifestations, posing major therapeutic difficulties. Therefore, ferritin levels should be controlled in all the patients with CDA II, because iron overload may approach risk levels at any age[1]. Iron depletion has to be started at the latest when ferritin approaches a level of 1 000 ng/mL to avoid the devastating complications of excessive iron accumulation[4].

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S- Editor Wang XL and Guo SY **L- Editor** Elsevier HK **E- Editor** Cao L

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World J Gastroenterol 2006 February 7; 12(5):822-824
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Five-year survival following a medial pancreatectomy for an invasive ductal carcinoma from the body of the pancreas

Hideki Abe, Kouichi Tsuneyama, Kazuhiro Tsukada, Masatoshi Makuuchi

Hideki Abe, Masatoshi Makuuchi, Hepatobiliarypancreatic Division, Department of Surgery, Graduate School of Medicine, University of Tokyo, Tokyo, Japan
Kouichi Tsuneyama, Department of Pathology, Toyama Medical and Pharmaceutical University, Toyama, Japan
Kazuhiro Tsukada, Second Department of Surgery, Toyama Medical and Pharmaceutical University, Toyama, Japan
Correspondence to: Hideki Abe, Hepatobiliarypancreatic Division, Department of Surgery, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 1138655 Japan. hidekiab-tky@umin.ac.jp
Telephone: +81-03-3815-5411-30352 Fax: +81-03-5684-3989
Received: 2005-01-09 Accepted: 2005-08-03

Abstract

We report a rare case of a patient who survived for 5 years after undergoing a medial pancreatectomy for invasive ductal carcinoma originating from the body of the pancreas. A 63-year-old woman was diagnosed as a small cancer of the pancreatic body, and surgery was performed. Even though the tumor was a carcinoma, its small size prompted us to perform a medial pancreatectomy with regional lymph nodes dissection. Additional chemoradiation was performed and, five years after surgery, the patient is well with no signs of recurrence. Medial pancreatectomy for invasive ductal carcinoma has not ever been reported. Furthermore, long-term survival after a lumpectomy for invasive ductal carcinoma has never been reported in the literatures. The current case suggests that long-term survival in patients with invasive ductal carcinoma of the pancreas may be associated with the pathological or biological features of pancreatic carcinoma.

Key words: Ductal carcinoma; Medial pancreatectomy; Pancreatic carcinoma

Abe H, Tsuneyama K, Tsukada K, Makuuchi M. Five-year survival following a medial pancreatectomy for an invasive ductal carcinoma from the body of the pancreas. *World J Gastroenterol* 2006; 12(5): 822-824

http://www.wjgnet.com/1007-9327/12/822.asp

INTRODUCTION

Invasive ductal carcinomas that originate from the body or tail of the pancreas are often unresectable because of unfortunate delays in tumor diagnosis. Most institutions continue to report resection rates for standard surgical procedures and 3-year or 5-year survival rates of no more than 20%[1]. Medial pancreatectomy is a segmental pancreatectomy for the treatment of benign lesions in the neck and body of the pancreas[2]. We report the extremely rare case of a patient who survived for 5 years after undergoing a medial pancreatectomy for invasive ductal carcinoma originating from the body of the pancreas.

CASE REPORT

A 63-year-old woman was admitted to our hospital for an evaluation of a dilatation in her main pancreatic duct. She had been diagnosed as having diabetes mellitus at the age of 53 years. Blood sugar control prompted her previous doctor to perform an abdominal computed tomography examination, which showed an apparent dilatation of the main pancreatic duct in the body and tail of the pancreas. Neither contrast-enhanced computed tomography, magnetic resonance imaging nor endoscopic ultrasonography revealed the cause of the dilatation, however, magnetic resonance cholangiopancreaography showed that the main pancreatic duct with beaded dilatation suddenly became occluded in the body of the pancreas. A clinical diagnosis of non-detectable, small cancer of the pancreatic body was made, and surgery was performed. An intraoperative ultrasonography examination depicted a round hypoechoic mass, measuring 1.0 cm in diameter, in the body of the pancreas. No infiltration to the vessels or metastatic lesions was detected. Even though the tumor was a carcinoma, its small size prompted us not to perform a Whipple procedure or a left pancreatectomy. Instead, a medial pancreatectomy, 4 cm along the long pancreatic axis, and an independent dissection of the lymph nodes around the common hepatic artery and proximal splenic artery were performed. The tail of the pancreas was anastomosed using a Roux loop. The tumor was histologically diagnosed as a well-differentiated adenocarcinoma, with a maximum diameter of 1.0 cm (Figure 1); one peripancreatic lymph node containing a metastasis was observed (Figure 2). When examined under low magnification, lymphocyte aggregation around the cancer stroma was observed, immunohistochemically, the tissue stained positive for both CD4 and CD8. The patient's postoperative course was uneventful; adjuvant chemoradiation was performed for 4 wk. The external-beam radiation doses were delivered using conventional

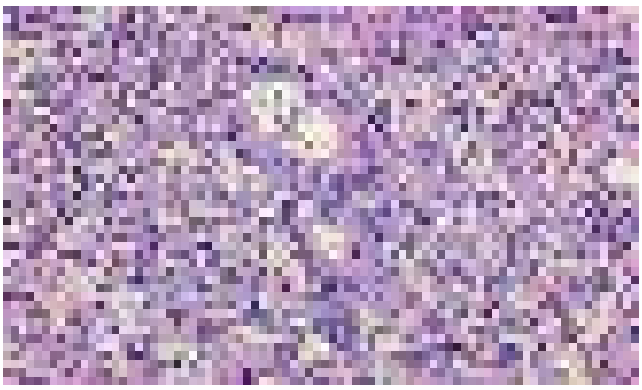

Figure 1 Under high magnification, the tumor is histologically diagnosed as a well-differentiated adenocarcinoma and is surrounded by lymphocyte aggregation.

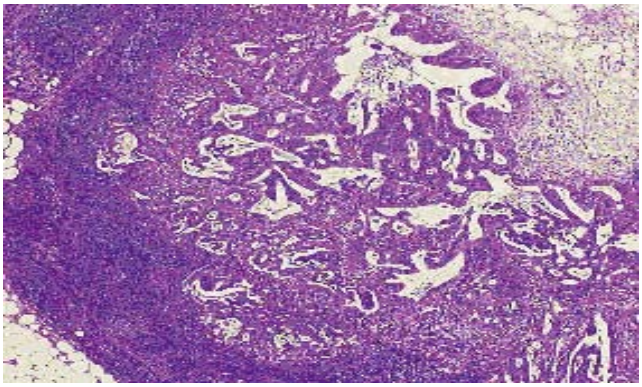

Figure 2 Cancer metastasis in a lymph node close to the pancreatic body.

fractionation: 2 Gy, 5 fractions a week. Concomitant chemotherapy was also performed on the first and fifth days of every week (10mg CDDP, 1KE OK432). In addition, she received a 120-h continuous infusion of 625 mg of 5-fluorouracil per week during the radiotherapy. The patient is well with no signs of recurrence five years after surgery.

DISCUSSION

Many surgeons perform extended radical surgeries, including an extended lymphadenectomy and retroperitoneal soft-tissue clearance, for various stages of pancreatic cancer; however, a survival benefit has not been demonstrated[3]. Even after a curative Whipple resection is performed, adjuvant treatments like radiotherapy and 5-FU treatment are often performed in the USA. So, Jeekel stated that a Whipple procedure was inadequate for pancreatic head cancer and regarded this procedure to be a lumpectomy rather than a curative procedure[4]. A medial pancreatectomy for invasive ductal carcinoma should also be regarded as a lumpectomy.

The main problem associated with performing a medial pancreatectomy is the need for careful patient selection[2]. In the present case, a very small tumor was visualized in the neck or right margin of the body of the pancreas. Preoperatively, evidence suggesting an invasive ductal carcinoma was minimal. The involvement of the main pancreatic duct enabled us to avoid enucleation. In conventional pancreatectomies, a pancreaticoduodenectomy or a near-total left pancreatectomy may be required. To avoid postoperative or late complications from a major pancreatic resection, we performed a medial pancreatectomy and independent lymph-node dissection in the present case. Medial pancreatectomy for invasive ductal carcinoma has not ever been reported. Furthermore, long-term survival after a lumpectomy for invasive ductal carcinoma has never been reported in the literatures, to the best our knowledge.

Large multivariate analyses of resected pancreatic adenocarcinoma have proven that lymph node metastasis serve as prognostic features, however, Fortner *et al* [5] reported that the presence of lymph node metastasis did not significantly influence survival after regional pancreatectomies for various stages of pancreatic cancer. In tumor biology, the existence of carcinoma cells in a lymph node is regarded as indicating metastatic invasion through the lymphatic ducts out of primary organ. In the present case, the peripancreatic lymph-node metastasis did not preclude long-term survival. Thus, long-term survival may not be precluded by the presence of lymph node metastasis, otherwise, extended radical surgery can not contribute to the treatment of metastatic invasion through the lymphatic ducts. The prognosis of patients with invasive ductal carcinoma of the pancreas who have survived for 5 years may be the same, regardless of whether an extended radical surgery or a lumpectomy is performed.

Many patients with even stage I diseases have recurrences after resection. Fortner *et al* [5] reported in 1996 that the 5-year survival rate after a regional pancreatectomy was 33% in 12 patients with ductal adenocarcinoma of the pancreatic head, in whom the tumor measured 2.5 cm or less in diameter. Some patients with advanced diseases, however, have survived longer than 5 years after their initial treatment. The pathological and biological features of the 5-year survivors varied.[6] In the present case, peritumoral lymphocyte aggregation was observed, and CD4+ and CD8+ T cells were noted using immnohistochemistry. Fukunaga *et al*[7] reported that the presence of CD4+ and CD8+ T cells in pancreatic adenocarcinoma serves as a good indicator of a favorable outcome after surgical treatment.

In summary, we report a patient with invasive ductal carcinoma from the body of the pancreas that survived for more than five years after undergoing a medial pancreatectomy. The current case suggests that complete surgical extirpation of early-stage carcinomas of the pancreas accompanied with adjuvant therapy can be long-term survival may be associated with the pathological or biological features of pancreatic carcinoma.

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S- Editor Guo SY **L- Editor** Elsevier HK **E- Editor** Cao L

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World J Gastroenterol 2006 February 7; 12(5):825-827
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Adult case of an omphalomesenteric cyst resected by laparoscopic-assisted surgery

Fumi Sawada, Rie Yoshimura, Kenichi Ito, Kazuhiko Nakamura, Hajime Nawata, Kazuhiro Mizumoto, Shuji Shimizu, Takahiro Inoue, Takashi Yao, Masazumi Tsuneyoshi, Atushi Kondo, Naohiko Harada

Fumi Sawada, Rie Yoshimura, Kenichi Ito, Kazuhiko Nakamura, Hajime Nawata, Naohiko Harada, Department of Medicine and Bioregulatory Science
Kazuhiro Mizumoto, Shuji Shimizu, Department of Surgery and Oncology
Takahiro Inoue, Takashi Yao, Masazumi Tsuneyoshi, Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Fukuoka, Japan
Atushi Kondo, Department of Gastroenterology, Aso Iizuka Hospital, Iizuka 820-8505, Fukuoka, Japan
Naohiko Harada, Department of Gastroenterology, Fukuoka Higashi Medical Center, Koga 811-3195, Fukuoka, Japan
Correspondence to: Naohiko Harada, MD, Department of Gastroenterology, Fukuoka Higashi Medical Center, Koga 811-3195, Fukuoka, Japan. haradan@fukuokae2.hosp.go.jp
Telephone: +81-92-943-2331 Fax: +81-92-943-8775
Received: 2005-06-10 Accepted: 2005-07-28

Abstract

This report describes an extremely rare adult case of an omphalomesenteric cyst resected by laparoscopic-assisted surgery. A 29-years-old Japanese man was referred and admitted to Kyushu University Hospital because of an abdominal mass and an elevated serum CEA (carcinoembryonic antigen) level (21.3 ng/mL) in August 2001. Abdominal CT and US demonstrated a cystic mass with septum and calcification. Laparoscopy showed a large mass to be attached to his abdominal wall, measuring 110 mm x 70 mm x 50 mm and filled with mucus. The mass was resected by laparoscopic-assisted surgery. The histological findings of its wall showed fibromuscular tissue, adipose tissue, calcification, and an intestinal structure. It was finally diagnosed to be an omphalomesenteric cyst.

Key words: Omphalomesenteric cyst; Laparoscopic-assisted surgery

Sawada F, Yoshimura R, Ito K, Nakamura K, Nawata H, Mizumoto K, Shimizu S, Inoue T, Yao T, Tsuneyoshi M, Kondo A, Harada N. Adult case of an omphalomesenteric cyst resected by laparoscopic-assisted surgery. *World J Gastroenterol* 2006; 12(5): 825-827

http://www.wjgnet.com/1007-9327/12/825.asp

INTRODUCTION

The omphalomesenteric duct remnant is one of the rare congenital anomalies associated with the primitive yolk stalk. Most omphalomesenteric duct remnants tend to be Meckel's diverticulum, however, the occurrence of an omphalomesenteric cyst is very rare. An omphalomesenteric duct remnant induces several symptoms, such as intestinal obstruction, abdominal pain, melena, and umbilical hernia. These symptoms tend to occur most frequently during the childhood years. In this report, we have described an extremely rare adult case of omphalomesenteric cyst resected by laparoscopic-assisted surgery.

CASE REPORT

A 29-year-old male patient visited Aso Iizuka Hospital with the complaint of an abdominal mass of one month duration in July 2001. The laboratory data revealed an elevated CEA level (21.3 ng/mL). Abdominal CT showed a cystic mass, suspected of being a mesenteric cyst. In August 2001, he was referred and admitted to Kyushu University Hospital for further management. Physical examinations showed no abnormalities except for a fist-sized, elastic soft mass in his umbilical region. The laboratory data demonstrated elevations in the ALT (32I U/L), CEA (7.9 ng/mL, normal range <3.2 ng/mL), and CA19-9 (62.6 U/mL, normal range <37.0 U/mL) levels. However, the serum CA125 and alpha fetoprotein (AFP) levels were both within the normal limits. Abdominal US and CT showed a cystic mass with septum and calcification, measuring 100 mm x 80 mm x50 mm in size (Figure 1). MRI (T2-WI) showed a cystic mass with septum, which was suspected to be a mesenteric cyst (Figure 2). A barium small-bowel enema showed no abnormality except for extraductal oppression. Angiography showed an avascular area in the mass. Our preoperative diagnosis was mesenteric cyst.

Laparoscopic-assisted surgery was performed for the purpose of making a precise diagnosis and resection, because the possibility of a CEA-producing malignant tumor first had to be ruled out. A laparoscope was inserted through a 1 cm-trocar site at the median upper abdomen. Laparoscopy showed the mass to be attached to his abdominal wall at the median hypogastrium (Figure 3), and two ligaments, considered to be remnants of the umbilical arteries, at its attached portion. The mass had no communication with his small bowel. There was no evidence

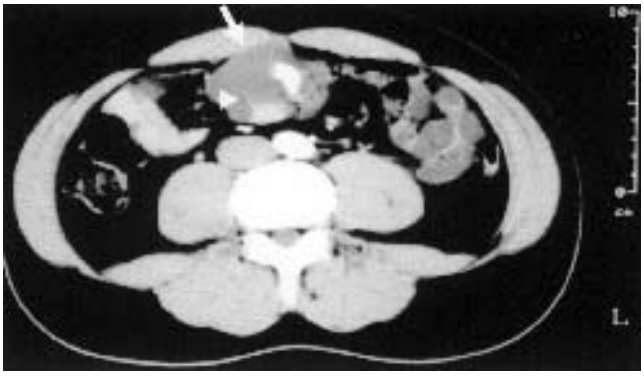

Figure 1 Abdominal CT demonstrating a cystic mass (arrow), measuring 100 mm x 80 mm x 50 mm in size, with septum (arrow head) and calcification in the umbilical region.

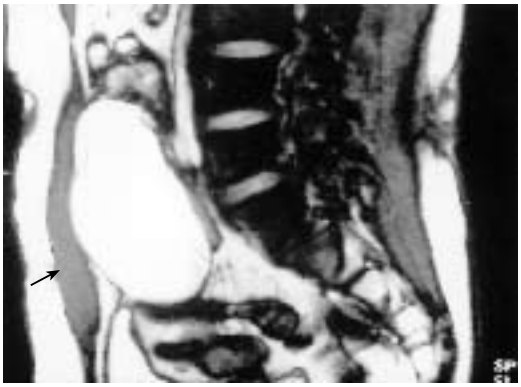

Figure 2 MRI (T2-WI) showing a cystic mass with septum.

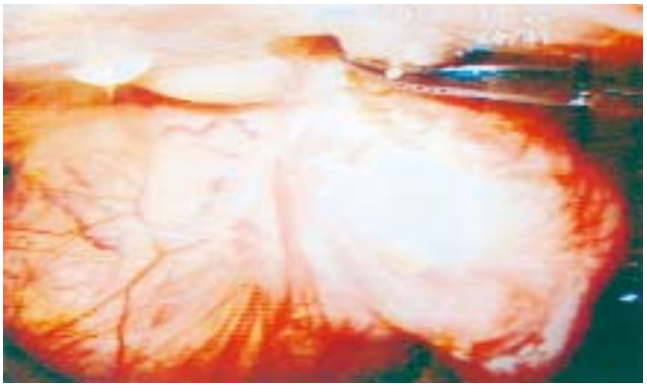

Figure 3 Laparoscopy showing the mass to be attached to abdominal wall.

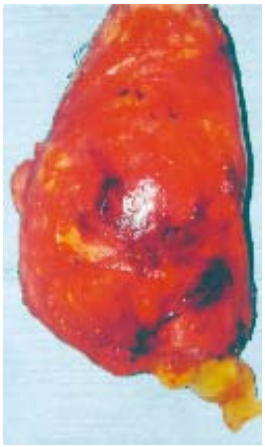

Figure 4 Mass measuring 110 mm x 70 mm x 50 mm in size.

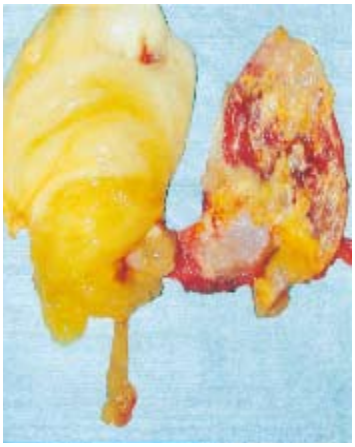

Figure 5 Cystic mass filled with mucus.

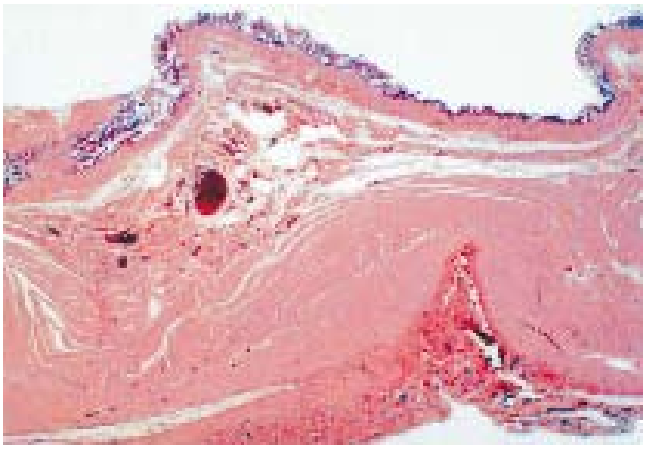

Figure 6 Histological features showing cystic wall to be composed of fibromuscular tissue, adipose tissue, focal calcification, and an intestinal structure, lined by an intestinal type mucosa with goblet cells, accompanied by ulceration with a regenerative epithelium.

of a persistent urachus. Next, an additional two ports were made with one at the right upper abdomen and another at the right lateral abdomen. The mass was then resected from his abdominal wall using laparoscopic scissors and electrocautery. The mass was removed through a 5-cm median incision made at his lower abdomen. The mass measured 110 mm x 70 mm x 50 mm in size (Figure 4), and weighed 160 g. This cystic mass was filled with mucus (Figure 5). The histological findings demonstrated the cystic wall to be composed of fibromuscular tissue, adipose tissue, focal calcification and an intestinal type mucosa with goblet cells, accompanied by ulceration with a regenerative epithelium (Figure 6). The histological findings showed no evidence of malignancy. It was finally diagnosed to be an omphalomesenteric cyst. Six months after the operation, the serum CEA and CA19-9 levels both became normal (0.7 ng/mL and 12.46 U/mL, respectively).

DISCUSSION

Omphalomesenteric duct remnants (vitelline duct anomalies) have been reported to be congenital anomalies associated with the primitive yolk stalk. The omphalomesenteric duct is the embryonic structure connecting the primary yolk sac to the embryonic midgut that normally closes off spontaneously at about 5-9 wk of gestation[1]. The failure of such closure may result in various lesions occurring in the omphalomesenteric duct remnants, such as Meckel's diverticulum, complete umbilical enteric fistula, incomplete umbilical sinus, omphalomesenteric cyst, or umbilical mucosal polyp[2]. Most of omphalomesenteric duct remnants (67%) tend to develop into Meckel's diverticulum, however,

omphalomesenteric cyst is also rarely observed[3]. Vane *et al*[1] reported only three cases of omphalomesenteric cyst in 217 children with omphalomesenteric duct remnants. The etiology of omphalomesenteric cyst is a persistent lesion of the intermediate duct with closure at both ends, leaving a cyst which can attach itself to the umbilicus, bowel, or both[4].

Omphalomesenteric duct remnants may persist in approximately 2% of infants[4,5]. Vane *et al*[1] reported the male-female ratio in a 217 remnant series to be 2:1. Yamada *et al*[6] reported the male-female ratio to be 2.8:1 in a Japanese series of 65 cases. The male-female ratio of omphalomesenteric cyst was reported to be 4:1 which correlates with the previously reported male predominance in other omphalomesenteric duct anomalies[7].

Common symptoms of omphalomesenteric duct remnants include abdominal pain, rectal bleeding, intestinal obstruction, umbilical drainage, and umbilical hernia[3], and all these symptoms appear to be age-dependent. In addition, most of the symptoms usually appear before the age of 4 years[1]. Eighty-five percent of infants younger than 1 month and 77% of children aged 1 month to 2 years had a symptomatic presentation[1]. It was also reported that 40% of the children with this anomaly had symptomatic lesions, while this anomaly is usually asymptomatic in adults[1]. Adult cases of omphalomesenteric cyst are extremely rare. A surgical resection is necessary for symptomatic omphalomesenteric duct remnants, but not necessary for asymptomatic omphalomesenteric duct remnants[8,9]. In our case, although asymptomatic omphalomesenteric cyst was incidentally found, it was removed because the possibility of a CEA-producing malignant tumor and small-bowel obstruction had to be ruled out[1]. The elevated tumor markers (CEA, CA19-9) all returned to normal levels after the resection. However, its histological findings showed no evidence of malignancy. The etiology of such elevated tumor markers remains to be elucidated.

In our case, the omphalomesenteric cyst was resected safely by laparoscopic-assisted surgery. Recent advances in diagnostic modalities have resulted in an increased incidental discovery of asymptomatic congenital anomalies. In such cases, the use of laparoscopic surgery is considered to be an effective, safe and less invasive treatment.

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S- Editor Kumar M and Guo SY **L- Editor** Elsevier HK **E- Editor** Li HY

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World J Gastroenterol 2006 February 7; 12(5):828-829
World Journal of Gastroenterology ISSN 1007-9327

LETTERS TO THE EDITOR

Clinical practice guidelines for hepatocellular carcinoma: the first evidence based guidelines from Japan

Masatoshi Makuuchi, Norihiro Kokudo

Masatoshi Makuuchi, Norihiro Kokudo, Department of Surgery, Hepato-Biliary-Pancreatic Surgery Division, University of Tokyo, Tokyo, Japan
Correspondence to: Masatoshi Makuuchi, Department of Surgery, Hepato-Biliary-Pancreatic Surgery Division, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. makuuchi-tky@umin.ac.jp
Telephone: +86-358008841
Received: 2005-07-07 Accepted: 2005-08-25

Key words: Hepatocellular carcinoma; Surgery

Makuuchi M, Kokudo N. Clinical practice guidelines for hepatocellular carcinoma: the first evidence based guidelines from Japan. *World J Gastroenterol* 2006; 12(5):828-829

http://www.wjgnet.com/1007-9327/12/828.asp

TO THE EDITOR

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer death in Japan, ranked 3rd in males and 5th in females. Thanks to recent progress, there are several definitive treatment modalities available for HCC, including surgery (liver resection and transplantation), ablation therapy, and transarterial chemoembolization (TACE). It is fortunate for both patients and doctors to have multiple treatment options, however, there have been very few evidence-based guidelines for decision-making[1]. Supported by the Japanese Ministry of Health, Labour and Welfare, we have compiled the "Clinical practice guidelines for hepatocellular carcinoma"[2]. This set of guidelines covers 6 fields for HCC, including prevention, diagnosis and surveillance, surgery, chemotherapy, TACE, and ablation therapy. We have surveyed 7 192 publications on HCC extracted mainly from MEDLINE (1966-2002), and selected 334 articles to form 58 pairs of research questions and recommendations. For convenience in practical use, we have also created algorithms for the surveillance and treatment of HCC.

The algorithm for the treatment of HCC was based on the evidence from the selected articles above and modified according to the current status in Japan, where liver resection for HCC is safe with less than 1% mortality, and cadaveric donors for liver transplantation are extremely difficult to obtain Figure 1. This algorithm was devised on the basis of three factors, namely, degree of liver damage[3], number of tumors, and diameter of the tumors. In patients with the severity of the liver damage categorized into class A or B: (1) If there is only one tumor, liver resection is recommended, irrespective of the diameter of the tumor. Ablation therapy may also be selected if the severity of liver damage is class B and the diameter of the tumor is not more than 2 cm; (2) From 2 to 3 tumors of no more than 3 cm in diameter, liver resection or ablation therapy is recommended; (3) From 2 to 3 tumors with a diameter of 3 cm or more, liver resection or TACE is recommended; (4) For 4 or more tumors, TACE or he-

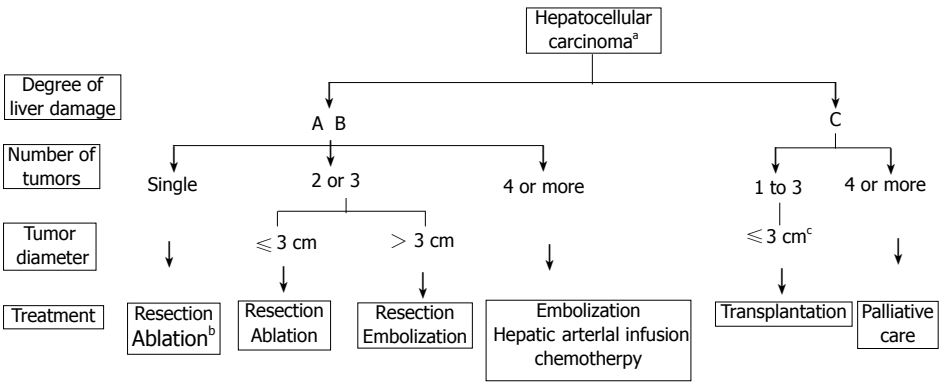

Figure 1 Treatment algorithm for hepatocellular carcinoma (cited from Ref.2 with permission). [a]Presence of vascular invasion or extrahepatic metastasis to be indicated separately. [b]Selected when the severity of liver damage is class B and tumor diameter is no greater than 2 cm. [c]Tumor diameter should be no greater than 5 cm when there is only one tumor.

patic arterial infusion chemotherapy is recommended. For patients with class C liver damage: (1) If the tumor condition is within the so-called Milan criteria[4], liver transplantation is recommended; (2) If the number of tumors is 4 or more, palliative treatment is recommended. For patients with class A liver damage accompanied by vascular invasion, liver resection may be selected, and for patients with extra-hepatic metastasis, chemotherapy may be selected.

English translation for this guideline will be released on the website of The Japan Society of Hepatology (http://www.jsh.or.jp/) soon.

Co-investigators for "Clinical practice guidelines for hepatocellular carcinoma" (in alphabetical order): Shigeki Arii, MD, Shunji Futagawa, MD, Yuji Itai, MD, Shuichi Kaneko, MD, Seiji Kawasaki, MD, Ken-ichi Kobayashi, MD, Hiroshi Matsuyama, PhD, Masatoshi Okazaki, MD, Kiwamu Okita, MD, Masao Omata, MD, Yukihisa Saida, MD, Tadatoshi Takayama, MD, Yoshio Yamaoka, MD.

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S- Editor Guo SY **L- Editor** Elsevier HK **E- Editor** Cao L

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World J Gastroenterol 2006 February 7; 12(5): 830-831
World Journal of Gastroenterology ISSN 1007-9327

LETTERS TO THE EDITOR

VEGF in hepatocellular carcinoma and surrounding cirrhotic liver tissues

Muriel Mathonnet, Bernard Descottes, Denis Valleix, François Labrousse, Yves Denizot

Muriel Mathonnet, Service de Chirurgie Digestive, Endocrinienne et Générale, CHU Dupuytren, Limoges, France
Bernard Descottes, Denis Valleix, Service de Chirurgie Viscérale et Transplantation, CHU Dupuytren, Limoges, France
François Labrousse, Service d'Anatomo-Pathologie, CHU Dupuytren, Limoges, France
Yves Denizot, UMR CNRS 6101, Faculté de Médecine, Limoges, France
Correspondence to: Yves Denizot, UMR CNRS 6101, Faculté de Médecine, 2 rue Dr. Marcland, 87025 Limoges, France. yves.denizot@unilim.fr
Telephone: +33-555-435896 Fax: +33-555-435897
Received: 2005-05-17 Accepted: 2005-08-25

Key words: Vascular endothelial growth factor; Hepatocellular carcinoma; Cirrhotic liver tissues

Mathonnet M, Descottes B, Valleix D, Labrousse F, Denizot Y. VEGF in hepatocellular carcinoma and surrounding cirrhotic liver tissues. *World J Gastroenterol* 2006; 12(5): 830-831

http://www.wjgnet.com/1007-9327/12/830.asp

TO THE EDITOR

We read with a great interest the recent work of Deli *and colleagues*.[1] in the *World Journal of Gastroenterology* reporting vascular endothelial growth factor (VEGF) expression in hepatocellular carcinoma (HCC) and cirrhotic liver tissues. This well-documented work shows that VEGF was significantly higher in surrounding cirrhotic liver tissues than in HCC. Authors assessed VEGF expression using immunohistochemistry. The immunohistochemical staining is an efficient tool to assess the percentage of cells stained positively for VEGF but is not really efficient to estimate their true VEGF content. Evaluation of the VEGF protein by an enzyme-linked immunosorbent assay (ELISA) has been reported, by us and others[2-5], to be an efficient tool in order to assess tissue VEGF expression. We have, thus, tested whether the ELISA method might be an efficient tool in order to confirm data reporting higher amounts of VEGF in surrounding cirrhotic liver tissues than in HCC. Deli *and colleagues*.[1] also correctly pointed out that basic fibroblast growth factor (bFGF) has been reported to act cooperatively on VEGF expression[6]. We have, thus, also assessed bFGF tissue levels in order to search for a putative link between VEGF and bFGF levels in cirrhotic tissues.

The procedure of the present study followed the rules edited by the French National Ethics. Between January 1999 and December 2004, 12 consecutive (10 males and 2 females, mean age 67 ± 4 years, range 36-83 years) patients with an HCC developed in a cirrhosis underwent resection in the Department of Surgery of Limoges' CHU. Specimens for the HCC tissue and the surrounding cirrhotic liver tissue were obtained during the surgical procedure and frozen at -80 °C until used. Cirrhosis was present in all cases with the cause being alcoholism or hepatitis C. Histologically, there was 5 well-differentiated HCC, 4 moderately differentiated HCC, and 3 poorly differentiated HCC. Tissue samples were homogenized in potassium phosphate buffer and VEGF and bFGF dosages were performed by specific ELISA assays (DuoSet®, R&D Systems Europe) according to the manufacturer's recommendations. Results in pg per mg of wet weight are reported as means ± SEM of 12 patients. Differences in mean content of VEGF and bFGF among tumour and the surrounding cirrhotic tissue were analysed by the Student's *t*-test for paired data. A $P < 0.05$ was considered statistically significant. Correlations between bFGF and VEGF values were assessed using linear regression analysis. A $P < 0.05$ and $r > 0.5$ were considered statistically significant.

As shown in Figure 1A, VEGF contents were significantly elevated ($P = 0.01$) in the surrounding cirrhotic liver tissues (18.9 ± 2.1 pg/mg, range 2-33 pg/mg) than in HCC tissues (13.6 ± 1.9, range 2-25 pg/mg). As shown in Figure 1B, bFGF contents were significantly elevated ($P = 0.02$) in the surrounding cirrhotic liver tissues (55.4±6.0 pg/mg, range 15-94 pg/mg) than in HCC tissues (35.2 ± 8.5, range 4-63 pg/mg). While bFGF and VEGF levels were not correlated in HCC ($r = 0.02$, $P = 0.66$), bFGF and VEGF were strongly correlated in cirrhotic liver tissues ($r = 0.85$, $P = 0.0005$).

VEGF and bFGF amounts are elevated in cirrhotic liver tissue as compared with HCC ones. VEGF results obtained with the ELISA method corroborate those of Deli *and colleagues*.[1] concerning VEGF expression using immunohistochemistry. Immunohistochemistry and ELISA are, thus, two complementary methods that can be used in concert and bring concordant results. However, in our opinion, the main advantage of the ELISA method is the possibility to test several factors into the same tissue extract in order to highlight potential links between factors such as those found between VEGF and bFGF in the cirrhotic tissues (but not in HCC ones). As suggested by Deli

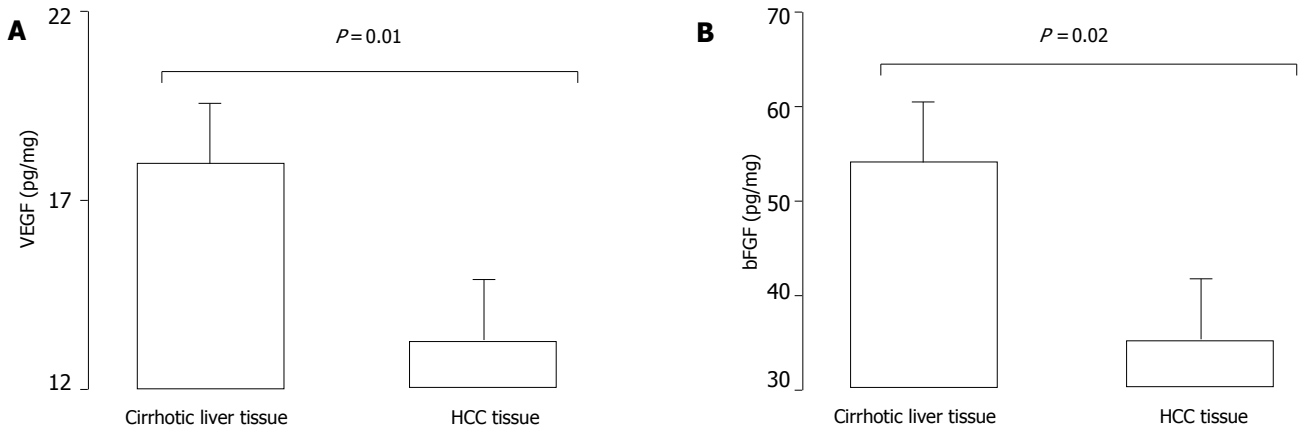

Figure 1 VEGF and bFGF in HCC and surrounding cirrhotic liver tissues. Specimens of HCC and surrounding cirrhotic tissues were obtained during the surgical procedure. VEGF **(A)** and bFGF **(B)** amounts were determined by ELISA. Results are reported as means±SEM of 12 patients. Statistical analysis was performed using the Student's *t* test for paired samples.

and colleagues, VEGF (but also bFGF) may play an important role in the angiogenesis of liver cirrhosis. The higher expression of VEGF and bFGF suggested a possible role of angiogenesis in HCC carcinogenesis. Anti-angiogenic therapy potentially targeting VEGF and bFGF pathways could promise new strategies for the treatment of HCC.

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ACKNOWLEDGMENTS

Acknowledgments to Reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those were published and those were rejected in this issue) during the last editing period of time.

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Department of Gastroenterology, Saiseikai Niigata Second Hospital, Teraji 280-7, Niigata, Niigata 950-1104, Japan

Kei Ito, MD,
Department of Gastroenterology, Sendai City Medical Center, 5-22-1, Tsurugaya, Miyagino-ku, Sendai City 983-0824, Japan

Seigo Kitano, Professor,
Department of Surgery I, Oita University Faculty of Medicine, 1-1 Idaigaoka Hasama-machi, Oita 879-5593, Japan

Robert J Korst, MD,
Department of Cardiothoracic Surgery, Weill Medical College of Cornell University, Room M404, 525 East 68th Street, New York 10032, United State

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Hepato-Billiary-Pancreatic Surgery, Osaka City University Graduate School of Medicine, 1-4-3 Asahimachi, Abeno-ku, Osaka 545-8585, Japan

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Jia-Yu Xu, Professor,
Shanghai Second Medical University, Rui Jin Hospital, 197 Rui Jin Er Road, Shanghai 200025, China

Eric M Yoshida, MD, Department of Medicine, University of British Columbia, 100-2647 Willow Street, Vancouver V5Z 3P1, Canada

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MAJOR MEETINGS COMING UP

Digestive Disease Week
107th Annual Meeting of AGA, The American Gastroenterology Association
May 20-25, 2006
Loas Angeles Converntion Center, California
www.ddw.org

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

10 th World Congress of the International Society for Diseases of the Esophagus (ISDE 2006)
February 22-25, 2006
Adelaide
isde@sapmea.asn.au
www.isde.net

EASL 2006 - The 41st Annual Meeting
April 26-30, 2006
Vienna, Austria

International Gastrointestinal Fellows Initiative
February 22-24, 2006
Banff, Alberta
CAGOffice@cag-acg.org
www.cag-acg.org

Canadian Digestive Disease Week
February 24-27, 2006
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European Multidisciplinary Colorectal Cancer Congress 2006
February 12-14, 2006
Berlin
info@congresscare.com
www.colorectal2006.org

ILTS 12th Annual International Congress
May 3-6, 2006
Milan
www.ilts.org

World Congress on Gastrointestinal Cancer
June 14-17, 2006
Barcelona, Spain
c.chase@imedex.com

5th International Congress of The African Middle East Association of Gastroenterology
February 24-26, 2006
Sharjah
infoevent@infomedweb.com
www.infomedweb.com

Digestive Disease Week 2006
May 20-25, 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
May 25-26, 2006
Los Angeles, CA
www.asge.org/education

EVENTS AND MEETINGS IN 2006

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September 3-8, 2006
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EASL 2006 - The 41st Annual Meeting
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Prague Hepatology Meeting 2006
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European Multidisciplinary Colorectal Cancer Congress 2006
February 12-14, 2006
Berlin
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www.colorectal2006.org

World Congress on Controversies in Obesity, Diabetes and Hypertension (CODHy)
October 25-28, 2006
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ILTS 12th Annual International Congress
May 3-6, 2006
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XXX pan-american congress of digestive diseases
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47	CM	cm	centimeter
48	MM	mm	millimeter
49	Kg, KG	kg	kilogram
50	Gm, gr	g	gram
51	nt	N	newton
52	l	L	liter
53	db	dB	decibel
54	rpm	r/min	rotation per minute
55	bq	Bq	becquerel, a unit symbol
56	amp	A	ampere
57	coul	C	coulomb
58	HZ	Hz	
59	w	W	watt
60	KPa	kPa	kilo-pascal
61	p	Pa	pascal
62	ev	EV	volt (electronic unit)
63	Jonle	J	joule
64	J/mmol	kJ/mol	kilojoule per mole
65	$10\times10\times10cm^3$	10 cm×10 cm×10 cm	
66	N·km	KN·m	moment
67	$x\pm s$	mean±SD	In figures, tables or text narration
68	Mean±SEM	mean±SE	In figures, tables or text narration
69	*im*	im	intramuscular injection
70	*iv*	iv	intravenous injection
71	Wang et al	Wang *et al.*	
72	EcoRI	*Eco*RI	*Eco* in italic and RI in positive. Restriction endonuclease has its prescript form of writing.
73	Ecoli	*E.coli*	Bacteria and other biologic terms have their specific expression.
74	Hp	*H pylori*	
75	Iga	*Iga*	writing form of genes
76	*igA*	IgA	writing form of proteins
77	-70 kDa	~70 ku	