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[1]Passed away on October 20, 2007
[2]Passed away on June 14, 2008

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Pernicious anemia: New insights from a gastroenterological point of view

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Abstract

Pernicious anemia (PA) is a macrocytic anemia that is caused by vitamin B_{12} deficiency, as a result of intrinsic factor deficiency. PA is associated with atrophic body gastritis (ABG), whose diagnosis is based on histological confirmation of gastric body atrophy. Serological markers that suggest oxyntic mucosa damage are increased fasting gastrin and decreased pepsinogen I. Without performing Schilling's test, intrinsic factor deficiency may not be proven, and intrinsic factor and parietal cell antibodies are useful surrogate markers of PA, with 73% sensitivity and 100% specificity. PA is mainly considered a disease of the elderly, but younger patients represent about 15% of patients. PA patients may seek medical advice due to symptoms related to anemia, such as weakness and asthenia. Less commonly, the disease is suspected to be caused by dyspepsia. PA is frequently associated with autoimmune thyroid disease (40%) and other autoimmune disorders, such as diabetes mellitus (10%), as part of the autoimmune polyendocrine syndrome. PA is the end-stage of ABG. Long-standing *Helicobacter pylori* infection probably plays a role in many patients with PA, in whom the active infectious process has been gradually replaced by an autoimmune disease that terminates in a burned-out infection and the irreversible destruction of the gastric body mucosa. Human leucocyte antigen-DR genotypes suggest a role for genetic susceptibility in PA. PA patients should be managed by cobalamin replacement treatment and monitoring for onset of iron deficiency. Moreover, they should be advised about possible gastrointestinal long-term consequences, such as gastric cancer and carcinoids.

Key words: Pernicious anemia; Autoimmune diseases; Atrophic gastritis; Intrinsic factor; Autoantibodies; Parietal cells; Vitamin B_{12} deficiency; *Helicobacter pylori*

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INTRODUCTION

Pernicious anemia (PA) (also known as Biermer's disease[1] and Addisonian anemia[2]) is a macrocytic anemia due to vitamin B_{12} (cobalamin) deficiency, which, in turn, is the result of deficiency of intrinsic factor, a protein that binds avidly to dietary vitamin B_{12} and promotes its transport to the terminal ileum for absorption[3]. The deficiency of intrinsic factor is a consequence of the presence of atrophic body gastritis (ABG), which results in the destruction of the oxyntic mucosa, and thus, the loss of parietal cells, which normally produce chlorhydric acid as well as intrinsic factor[4]. The term PA is sometimes used as synonym for cobalamin deficiency or for macrocytic anemia, but to avoid ambiguity, PA should be reserved for conditions that result from impaired secretion of intrinsic factor and atrophy of oxyntic mucosa[5]. However, differential diagnosis may sometimes be challenging due to the limit of available diagnostic tools.

PA is considered an autoimmune disorder due to the frequent presence of gastric autoantibodies directed against intrinsic factor, as well as against parietal cells. PA is often considered a synonym of autoimmune

gastritis, because PA is thought to be the end stage of an autoimmune process that results in severe damage of the oxyntic gastric mucosa[6]. Recent experimental and clinical data strongly suggest an involvement of long-standing *Helicobacter pylori* (*H pylori*) infection in the pathogenesis of ABG and PA, but it is still under debate whether PA may be included among the long-term consequences of *H pylori* gastritis[7].

The present review focuses on novel aspects regarding the pathogenesis, clinical presentation, and diagnosis of PA, as well as the management of PA patients from a gastroenterological point of view.

PA: AN AUTOIMMUNE DISORDER OR AN INFECTIOUS DISEASE?

PA is the end-stage of ABG and is generally considered an autoimmune disease. The autoimmune origin of PA is based on the presence of parietal cell and/or intrinsic factor autoantibodies, and the frequent association with other autoimmune disorders, such as autoimmune thyroid disease (ATD), type 1 diabetes, and vitiligo[6,8].

ABG associated with PA is often called autoimmune gastritis or type A gastritis, which is defined as a type of chronic atrophic gastritis restricted to the body mucosa, characterized by a severe, diffuse atrophy of the oxyntic glands and hypochlorhydria, and a normal antral mucosa[4]. Another classical histological feature of ABG is the absence of *H pylori* on gastric mucosal biopsies[4]. It is now accepted that long-standing *H pylori* infection is able to induce atrophy of the gastric mucosa, and *H pylori* is considered the main causative agent of multifocal atrophic gastritis, in which the antrum is almost invariably involved[9]. Thus, ABG is generally considered a separate entity from *H pylori*-related atrophic gastritis, mainly because the prevalence of *H pylori* infection in patients with severe ABG and PA has been found to be low[10,11]. However, in the past few years, the question has been raised whether *H pylori* may be implicated in the pathogenesis of ABG, and, as a basic mechanism for the induction of gastric autoimmunity by *H pylori* infection, molecular mimicry has been proposed[7,12,13]. Molecular mimicry is defined as the possibility that sequence similarities between foreign and self-peptides are sufficient to result in the cross-activation of autoreactive T or B cells by pathogen-derived peptides. It is a phenomenon associated with some pathogens in which the antigens that evoke an immune response have enough similarity to the body's own proteins to cause an autoimmune reaction, such as in rheumatoid arthritis, mediated by cross-reactive T cells and/or circulating antibodies. In fact, gastric H^+/K^+-ATPase has been recognized as the major autoantigen in experimental and human ABG[14-16], and autoreactive gastric CD4+ T cells that recognize H^+/K^+-ATPase and *H pylori* antigens have been recently described in ABG[17,18]. Thus, PA and ABG seem to be an example of pathogen-induced, organ-specific autoimmunity, in which genetic susceptibility plays an important role in relation to the loss of immunological tolerance[18]. In fact, the immunological basis of molecular mimicry lies in the recognition by T-cell antigen receptors of antigenic peptides bound to human leucocyte antigen (HLA) molecules on the surface of antigen-presenting cells, and inappropriate activation of T cells may occur as a result of the upregulation of HLA molecules in genetically susceptible individuals[19]. A specific HLA-DR pattern was suggested in PA patients several years ago[20], and more recently, blocking experiments with anti-DR and anti-DQ antibodies have shown that DR antigen probably represents the HLA restriction element in ABG[17]. By using a DNA-based, sequence-specific oligonucleotide technology, we observed in our series of PA patients that the genotypes HLA-DRB1*03 and DRB1*04, which are known to be associated with other autoimmune disease (such as type 1 diabetes and ATD)[21], were significantly associated with PA, compared to a control group (unpublished data), which supports the idea that genetic susceptibility for autoimmunity may play a role in PA.

Table 1 shows the literature regarding *H pylori* infection and related gastric histological features in some PA patients[10,22-24]. The presence of *H pylori* infection was diagnosed by histology in up to 30% (median 11%), but by serology (IgG) in up to 51% (median 20.5%) of PA patients. It is well known that the diagnosis of *H pylori* infection may be difficult in patients with ABG. *H pylori* may disappear over time due to the hostile gastric microenvironment, and past infection may be demonstrated by serological positivity to *H pylori* in a large majority of patients with ABG or PA[10,25-27]. A recent study has reported that seropositivity against *H pylori* antigens may be demonstrated in a very high percentage of patients with ABG by using *ad hoc* immunoblotting[28]: in this study 47.8% of ABG patients had PA and all but two of them presented with seropositivity against *H pylori* antigens, including CagA and VacA. As far as regards histological features of the gastric body, in the vast majority of PA patients (> 70%) this disorder is associated with severe body atrophy and the presence of intestinal metaplasia. From data reported in Table 1, another interesting observation emerges: irrespective of the presence of *H pylori* infection, in about half of PA patients, the gastric antrum is involved, and about one-third have antral atrophic gastritis, whose presence is strongly related to *H pylori* infection[9]. This observation challenges the widely accepted notion that PA occurs exclusively in association with the classical histological feature of corpus-restricted atrophic gastritis. All these data taken together support the idea that long-standing *H pylori* infection probably plays an important role in many genetically susceptible PA patients. In these patients, the active infectious process has been gradually replaced by an autoimmune process directed by autoreactive gastric CD4+ T cells that recognize H^+/K^+-ATPase and *H pylori* antigens, which ends in a burned-out infection and the irreversible destruction of the gastric body mucosa. The failure to demonstrate *H pylori* infection in some of these individuals does not necessarily argue against the role of the bacterium in

Table 1 *H pylori* infection and related gastric histological features in a series of PA patients *n* (%)

First author/ publication year[Ref.]	No. of patients	Mean age (yr)	F:M ratio	Geographical origin	Severe body atrophy	Body intestinal metaplasia	Antral inflammation	Antral atrophic gastritis	Positive *H pylori* histology	Positive *H pylori* serology
Fong TL/1991[22]	28	59	1.2:0.8	USA[1]	ND	18 (64)	14 (50)	2 (7)	3 (11)	2 (7)
Haruma K/1995[23]	24	65	0.9:1.1	Japan	24 (100)	18 (75)	22 (92)	17 (71)	0 (0)	0 (0)
Sari R/2000[24]	30	60	0.9:1.1	Turkey	15 (50)	13 (43)	14 (47)	8 (27)	12 (30)	ND
Annibale B/2000[10]	81	62	0.9:1.1	Italy	56 (69)	70 (86)	43 (53)	27 (30)	8 (10)	41 (51)
Annibale B/ 2009[unpublished data]	177	60	1:1	Italy	124 (70)	161 (91)	81 (46)	40 (23)	19 (11)	61 (34)

[1]Hispanic, *n* = 20; white, *n* = 3; black, *n* = 5. ND: Not done.

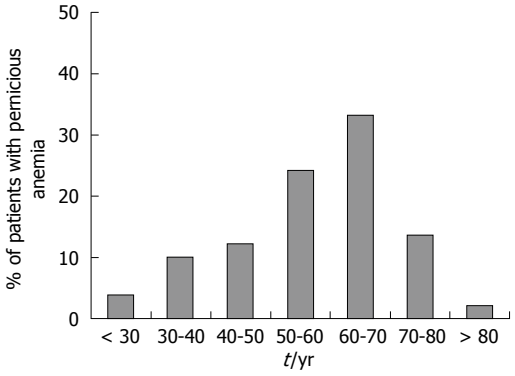

Figure 1 Age cohorts of a series of patients with PA (*n* = 177) consecutively diagnosed between 1992 and 2005 at an academic gastroenterology unit.

these patients, but more likely indicates that a point of no return may be reached beyond which the autoimmune process may no longer require the continued presence of the inducing pathogen[29].

PA is frequently described as a disease of adults > 60 years of age[8,30,31]. Among our unpublished series of 177 PA patients, about one half were < 60 years of age; in particular, 4% of patients were < 30 years and 10% were 30-40 years of age (Figure 1). Table 1 shows that the mean age of PA patients in published studies ranges from 59 to 62 years. These data challenge the common notion that PA is an exclusive disease of the elderly, and suggest that, in clinical practice, PA is probably under-diagnosed in elderly and younger patients[32]. Stratification by age cohorts (< 20 years to > 60 years) of ABG patients identified by hypergastrinemia and positive parietal cell antibodies has shown a regular and progressive increase in mean corpuscular volume and levels of ferritin and gastrin, and a decrease in vitamin B_{12} levels. However, the prevalence of *H pylori* infection has decreased from > 80% at age < 20 years to 12.5% at > 60 years[32]. This reminds us that: (1) iron deficiency is a complication of achlorhydria and may precede the development of PA[4]; (2) ABG patients frequently present with iron deficiency anemia[33-35]; and (3) iron deficiency may be present concomitantly with PA[36]. These findings further support the idea that PA seems to be a long-duration disease that is related to *H pylori*, gastric achlorhydria and atrophy, which begins many years before the establishment of clinical vitamin B_{12} deficiency.

CLINICAL PRESENTATION OF PA

The clinical presentation of PA is often insidious for various reasons. The onset and progression of PA are very slow. As a consequence, patients often are not aware of their symptoms related to anemia, because over time they have become used to them. In many such cases, the underlying disease may not be suspected until a complete red blood count has been performed. However, patients with PA may seek medical advice due to non-specific symptoms related to the presence of anemia *per se*, such as weakness, asthenia, decreased mental concentration, headache, and especially, in elderly patients, cardiological symptoms such as palpitations and chest pain[3,6]. Less frequently, patients with PA may present only with neurological symptoms, such as paresthesia, unsteady gait, clumsiness, and in some cases, spasticity. Indeed, vitamin B_{12} deficiency may cause peripheral neuropathy and lesions in the posterior and lateral columns of the spinal cord (subacute combined degeneration) and in the cerebrum, and these lesions progress from demyelinization to axonal degeneration and eventual neuronal death. It is particularly important to recognize these symptoms early, because the neurological lesions may not be reversed after replacement therapy with vitamin B_{12}[3,5]. Finally, the onset of PA may be observed in patients undergoing medical treatment for other autoimmune conditions frequently associated with PA, such as ATD, type 1 diabetes, and vitiligo, as part of the autoimmune polyendocrine syndromes[37].

Although the primary cause of PA is ABG, rarely the disease may result from gastrointestinal tract symptoms. The reason for the apparent paradox may lie in the fact that ABG is associated with hypochlorhydria, and symptoms of the upper gastrointestinal tract are often related to the presence of chlorhydric acid. However, hypochlorhydria itself may cause impaired gastric emptying, which eventually leads to dyspeptic symptoms such as epigastric discomfort, postprandial bloating and fullness, and early satiety[38]. In our experience, awareness and concern about upper gastrointestinal or neurological symptoms often are not sufficient to seek medical advice, since patients over time become used to these slowly and insidiously presenting complaints. Only 3% of PA patients presented directly to our gastroenterology unit for long-standing dyspepsia, and only 3% were referred from a neurologist. At the time of diagnosis of

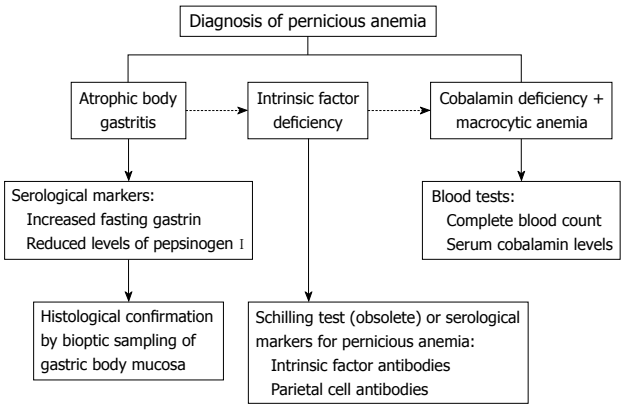

Figure 2 Diagnostic flow-chart for PA.

PA, dyspeptic symptoms were complained of by 28% of patients, and neurological symptoms were present in 19% (unpublished data).

An increased association of PA with other autoimmune diseases, such as type 1 diabetes (3%-4%)[39], vitiligo (2%-8%)[3], and in particular, ATD (3%-32%)[40] has been reported. Among our unpublished series of 177 PA patients, 41% had associated ATD and 10% presented with vitiligo or alopecia, which indicates that a subgroup of PA patients can be considered as having a type Ⅱ autoimmune polyendocrine syndrome. In a recent study, we have observed that ABG and ATD occur in a closely linked fashion, with ATD being present in about 40% of ABG patients[41]. These data suggest that, in patients with autoimmune disorders, in particular ATD, a possible association with PA should be suspected and excluded. The diagnosis of concomitant autoimmune thyroiditis and PA may have an important clinical implication, in particular, in those patients who require replacement therapy with thyroxine. Recently, it has been reported that patients with impaired acid secretion may present with thyroxine malabsorption that requires an increased dose of the drug[42], and in patients with PA, associated hypochlorhydria is always present, due to the loss of oxyntic mucosa[4].

Useful information about which patients may have PA can be derived also from epidemiological data. According to the older literature, PA is thought to be particularly common among individuals of Scandinavian, English or Irish ancestry, whereas it appears to be much less common in Caucasians of Italian or Greek origin[43]. However, more recently, the disease have been reported in black and Latin American subjects[30,44], and as shown in Table 1, series of PA patients have been diagnosed in the United States, Turkey and Italy, and even in Japan. The reason for the different distribution of PA among different ethnical groups is not known yet, but probably lies in their different genetic background, and in different awareness and diagnostic accuracy for this often overseen disorder. In the so-called high-risk groups, about nine new cases are detected per 100 000 population per year, and about 0.13% of the population is affected[31]. A more recent population survey has reported that 1.9% of persons aged > 60 years have undiagnosed PA[30].

A female preponderance ranging from 1.7 to 2.0:1 has been reported in white subjects[3]. This sex distribution has been confirmed in the more recent population survey of persons > 60 years old that was conducted in California, in which the prevalence of PA was 2.7% in women and 1.4% in men[30]. However, data reported in Table 1 concerning United States, Japanese, Turkish and Italian PA patients seem not to confirm the female preponderance described in older studies.

DIAGNOSIS OF PA

PA is defined as the presence of a hemoglobin concentration < 13 g/dL for men and < 12 g/dL for women[45], mean corpuscular volume ⩾ 100 fL[5], low levels of cobalamin (vitamin B_{12})[5], together with the concomitant presence of ABG and intrinsic factor deficiency (Figure 2). By definition, PA is associated with ABG, and strict diagnostic criteria for ABG are based on the histological confirmation of gastric body mucosal atrophy and enterochromaffin-like (ECL) cell hyperplasia, associated with hypochlorhydria to pentagastrin stimulation[4]. Increased levels of fasting gastrin and decreased levels of pepsinogen I are well accepted serological markers[46,47], which suggest the presence of oxyntic mucosa damage, which should be confirmed, however, by appropriate histological sampling of gastric body mucosa to diagnose ABG definitively.

As far as regards gastric mucosa histology, classical findings associated with PA are the presence of corpus-restricted atrophy with a spared antrum, as well as the presence of hyperplasia of ECL cells[4,6]. As shown in Table 1, in about 50% of PA patients, antral mucosa is not spared, and in about 27% of PA patients, a concomitant antral atrophic gastritis may be observed. These data strongly suggest that an extension of gastritis to the gastric antrum does not necessarily exclude the diagnosis of PA and the presence of gastric autoimmunity. The determination of ECL cell hyperplasia is helpful in the histological diagnosis of ABG, because the presence of this histological change may be considered an indirect confirmation of the presence of hypochlorhydria. This leads to hypergastrinemia, which in turn, is a trophic factor for ECL cells that leads to their hyperplasia, and eventually, to the development of gastric carcinoids[48].

Intrinsic factor deficiency can be proven by the now obsolete Schilling test. To confirm that the cobalamin deficiency is the result of intestinal malabsorption due to intrinsic factor deficiency, urinary excretion of orally administered vitamin B_{12} is low, and is increased by administration of vitamin B_{12} and intrinsic factor. Unfortunately, the availability of this test is vanishing due to problems related to its radioactive reagents. Therefore, in clinical practice, the presence of intrinsic factor deficiency may not be proven, and increasing reliance is placed on the detection of intrinsic factor antibodies for the diagnosis of PA, which are viewed as useful markers of this disease[49]. Earlier studies have reported positivity for intrinsic factor antibodies in 40%-60% of patients with PA[50,51], which rises to 60%-80% with increasing duration of disease[52].

Table 2 Differential diagnosis of PA: other causes of macrocytic anemia and cobalamin deficiency

Other causes of macrocytic anemia	Other causes of cobalamin deficiency
Folate deficiency due to decreased intake, impaired absorption or increased requirements	Gastric causes of impaired absorption/mal-digestion:
Drugs (e.g. methotrexate, azathioprine, 6-mercaptopurine, acyclovir, 5-fluorouracil, phenobarbital)	Gastrectomy
Accelerated erythropoiesis: hemolytic anemia, response to hemorrhage	Corpus-predominant *H pylori* gastritis
Liver disease (alcoholic, advance cirrhosis, poor dietary intake)	Long-term proton pump inhibitor therapy
Hypoplastic anemia, myelodysplastic syndrome	Intestinal causes of impaired absorption:
Chronic obstructive pulmonary disease	Ileal disease or resection
	Blind loop syndrome
	Fish tapeworm
	Severe pancreatic insufficiency
	Decreased intake due to vegetarianism

Recently, we reassessed the diagnostic performance of intrinsic factor and parietal cell antibodies in PA patients by using a novel ELISA[48], which yielded for intrinsic factor antibodies, a sensitivity and specificity of 37% and 100%, respectively, and for parietal cell antibodies, a sensitivity and specificity of 81.5% and 90.3%, respectively. The combined assessment of both autoantibodies significantly increased their diagnostic performance, which yielded 73% sensitivity for PA, while maintaining 100% specificity. Thus, our data show that, by combining the assessment of intrinsic factor and parietal cell autoantibodies, the diagnostic performance of these surrogate markers for PA may be notably improved. Beyond being a specific hallmark of PA, the positivity for intrinsic factor and parietal cell antibodies may be interpreted as an expression of oxyntic mucosal damage, because a positive correlation between the increasing histological score of body mucosa atrophy and the titer of both antibodies can be observed[27,35].

Accurate differential diagnosis of other causes of cobalamin deficiency is mandatory. As shown in Table 2, cobalamin deficiency may result from other causes of impaired absorption in the stomach or intestine, or by decreased intake due to vegetarianism. Among cases of mal-digestion, there are very rare cases related to severe pancreatic insufficiency, but more interesting is the recent evidence of mal-digestion of dietary cobalamin in patients with corpus-predominant *H pylori* gastritis, which leads to impaired acid secretion and consequent increased intragastric pH[53,54]. In fact, dietary cobalamin is bound to salivary proteins, which needs to be cleaved in the presence of chlorhydric acid before it can be bound to intrinsic factor and be absorbed in the terminal ileum[4]. In these cases of mal-digestion of dietary cobalamin, the Schilling test would be normal, which indicates that cobalamin deficiency is not due to intrinsic factor deficiency. Without performing a Schilling test, it may be challenging to discriminate between the presence of PA and mal-digestion of dietary cobalamin. However, from a practical point of view, the clinical management of these two groups of patients is similar. As observed[54,55], when atrophy of the gastric body mucosa is mild and active *H pylori* infection is present, in patients with mal-digestion of dietary cobalamin, a reversal of body mucosal atrophy, anemia and cobalamin deficiency following eradication treatment may be achieved. An accurate differential diagnosis should be carried out also for macrocytic anemia, which may underlie other causes such as folate deficiency and myelodysplastic syndrome (Table 2).

In this context, it should be kept in mind that, in order to diagnose vitamin B_{12} deficiency, total vitamin B_{12} measurement is used cost-effectively as the parameter of choice, but it has limited sensitivity and specificity, especially in persons with vitamin B_{12} concentrations in the lower reference range (< 400 pmol/L). As an alternative, modern biomarkers for early diagnosis of vitamin B_{12} deficiency, such as holotranscobalamin, also known as active B_{12}, and methyl malonic acid as a functional B_{12} marker, have been proposed[56]. Figure 2 shows a proposed diagnostic work-up when the presence of PA is suspected.

CLINICAL MANAGEMENT OF PATIENTS WITH PA

The clinical management of patients with PA has two different aspects: firstly, the treatment of cobalamin deficiency and the monitoring of onset of iron deficiency; and secondly, the surveillance of these patients, to detect early the long-term consequences of PA, such as gastric cancer and carcinoids.

Treatment of cobalamin deficiency and monitoring of iron deficiency

Cobalamin replacement treatment is able to correct the anemia, whereas the neurological complications may be corrected only if the replacement treatment is given soon after their onset. The therapeutic recommendations for PA with regard to dosage and administration of vitamin B_{12} substitution treatment are divergent[57]. In the United States, patients usually receive vitamin B_{12} injections of 1 mg/d in their first week of treatment; in the following month, they receive weekly injections and then monthly injections[58]. In Denmark, patients receive injections of 1 mg/wk cyanocobalamin during the first month and every 3 mo subsequently, or 1 mg hydroxycobalamin every other month[59]. According to our protocol, a higher dosage of cobalamin is used to prevent early relapse of cobalamin deficiency: firstly patients receive intramuscular injection of 5 mg/d cyanocobalamin for 5 d, which replenishes the cobalamin body stores; then, vitamin B_{12} stores are maintained by intramuscular injection of 5 mg cyanocobalamin every 3 mo.

Furthermore, according to our protocol, PA patients are monitored at least yearly by complete blood count, and serum cobalamin and ferritin levels, to monitor the replacement treatment and to detect early the eventual onset of iron deficiency. Also patients with ABG with iron deficiency anemia or without hematological alterations are monitored in the same way, to detect early the eventual onset of cobalamin deficiency. Finally, PA patients are monitored by at least a yearly clinical interview, to verify the onset of new symptoms that are suspicious of long-term consequences of PA, such as dysphagia, epigastric pain, dyspeptic symptoms, loss of body weight, and/or iron-deficiency, which require immediate gastroscopic investigation.

Long-term consequences of PA

Although PA is substantially a benign disorder for a large number of patients, it is epidemiologically and biologically linked to the development of intestinal-type gastric adenocarcinoma and gastric carcinoid type I [60,61]. Hypergastrinemia, secondary to hypochlorhydria in PA patients, is a well-known risk factor for ECL cell hyperplasia and gastric carcinoids[62,63], and it has been reported that one in 25 patients with PA develops gastric carcinoids[64]. Moreover, the crucial role of hypochlorhydria, as a consequence of atrophy of the oxyntic mucosa, in the development of gastric cancer, has been highlighted[65]. Hypochlorhydria leads to overgrowth of nitrosamine-producing bacteria with potential carcinogen activity[66]. Also ascorbic acid, the main redox agent in the gastric juice with protective action against reactive oxygen species, is reduced in the presence of atrophy of the oxyntic mucosa. It has been described previously that the level of ascorbic acid in the gastric juice is reduced in patients with ABG, with an indirect correlation between ascorbic acid level and intragastric pH[67].

In the literature, the annual incidence of gastric cancer in PA patients ranges from 0.1% to 0.5%[62,64,68]. A recent follow-up study of patients with ABG has reported an annual incidence risk of 0.14% for developing gastric cancer, during an observation period of 6.7 years[69]. To date, the need and cost-effectiveness of endoscopic/histological surveillance in patients with PA have not been established definitively[4]. One previous study[64] that has considered the relatively benign nature of gastric carcinoids in patients with PA has concluded that follow-up is indicated at 5-year intervals only in patients with ECL hyperplasia. As for gastric cancer, the same authors have concluded that the first gastroscopic follow-up after diagnosis of PA should be performed relatively soon, and that only PA patients with preneoplastic lesions and those with gastrointestinal symptoms should undergo endoscopic surveillance[64]. Another study has concluded that follow-up should be performed at 3-year intervals only in PA patients aged < 60 years[70]. A more recent study has compared the usefulness of 2- and 4-year follow-up in patients with ABG, and has shown that the first follow-up performed 4 years after the diagnosis seems to be safe and convenient for early detection of potentially neoplastic lesions[71]. As a result of the lack of other prospective data, and considering the risk for developing neoplastic lesions over time in some PA patients, in our unit, PA patients are monitored regularly by gastroscopy with antral and corporal biopsies at 4-year intervals.

CONCLUSION

PA is an often silent and under-diagnosed autoimmune disease, because its onset and progression are very slow and patients may become used to their complaints. Nevertheless, the clinical consequences of undiagnosed PA may be serious, including gastric neoplastic lesions. Thus, gastroenterologists should increase their awareness of this disorder, whose definite histological diagnosis may be preceded by reliable noninvasive serological screening.

REFERENCES

1 **Zittoun J**. [Biermer's disease] *Rev Prat* 2001; **51**: 1542-1546
2 **Meecham J**, Jones EW. Addison's disease and Addisonian anaemia. *Lancet* 1967; **1**: 535-538
3 **Wintrobe MM**, Lee GR, Boggs DR, Bithell TC, Foerster J, Athens JW, Lukens JN. Megaloblastic and nonmegaloblastic macrocytic anemias. In: Wintrobe MM, Lee GR, Boggs DR, Bithell TC, Foerster J, Athens JW, Lukens JN, editors. Clinical hematology. 8th ed. Philadelphia: Lea & Febiger, 1981: 559-604
4 **Lee EL**, Feldman M. Gastritis and other gastropathies. In: Feldman M, Friedman LS, Sleisenger MH, editors. Sleisenger & Fordtran's gastrointestinal and liver disease: pathophysiology, diagnosis, management. 7th ed. Philadelphia: Saunders, 2002: 810-827
5 **Babior BM**. Erythrocyte disorders: Anemias related to disturbance of DNA synthesis (megaloblastic anemias). In: Williams JW, Beutler E, Erslev AJ, Lichtman MA, editors. Hematology. 4th ed. New York: McGraw-Hill, 1998: 453-481
6 **Toh BH**, Gleeson PA, Whittingham S, van Driel IR. Autoimmune gastritis and pernicious anemia. In: Rose NR, Mackay IR, editors. The autoimmune diseases. 3rd ed. St. Louis, MO: Academic Press, 1998: 459-476
7 **D'Elios MM**, Appelmelk BJ, Amedei A, Bergman MP, Del Prete G. Gastric autoimmunity: the role of Helicobacter pylori and molecular mimicry. *Trends Mol Med* 2004; **10**: 316-323
8 **Toh BH**, van Driel IR, Gleeson PA. Pernicious anemia. *N Engl J Med* 1997; **337**: 1441-1448
9 **Peterson WL**, Graham DY. Helicobacter pylori. In: Feldman M, Friedman LS, Sleisenger MH, editors. Sleisenger & Fordtran's gastrointestinal and liver disease: pathophysiology, diagnosis, management. 7th ed. Philadelphia: Saunders, 2002: 732-746
10 **Annibale B**, Lahner E, Bordi C, Martino G, Caruana P, Grossi C, Negrini R, Delle Fave G. Role of Helicobacter pylori infection in pernicious anaemia. *Dig Liver Dis* 2000; **32**: 756-762
11 **Varis O**, Valle J, Siurala M. Is Helicobacter pylori involved in the pathogenesis of the gastritis characteristic of pernicious anaemia? Comparison between pernicious anaemia relatives and duodenal ulcer relatives. *Scand J Gastroenterol* 1993; **28**: 705-708
12 **Appelmelk BJ**, Faller G, Claeys D, Kirchner T, Vandenbroucke-Grauls CM. Bugs on trial: the case of Helicobacter pylori and autoimmunity. *Immunol Today* 1998; **19**: 296-299
13 **Field J**, Biondo MA, Murphy K, Alderuccio F, Toh BH. Experimental autoimmune gastritis: mouse models of human organ-specific autoimmune disease. *Int Rev Immunol*

2005; **24**: 93-110
14 **Toh BH**, van Driel IR, Gleeson PA. Autoimmune gastritis: tolerance and autoimmunity to the gastric H+/K+ ATPase (proton pump). *Autoimmunity* 1992; **13**: 165-172
15 **Ma JY**, Borch K, Mårdh S. Human gastric H,K-adenosine triphosphatase beta-subunit is a major autoantigen in atrophic corpus gastritis. Expression of the recombinant human glycoprotein in insect cells. *Scand J Gastroenterol* 1994; **29**: 790-794
16 **Claeys D**, Faller G, Appelmelk BJ, Negrini R, Kirchner T. The gastric H+,K+-ATPase is a major autoantigen in chronic Helicobacter pylori gastritis with body mucosa atrophy. *Gastroenterology* 1998; **115**: 340-347
17 **Amedei A**, Bergman MP, Appelmelk BJ, Azzurri A, Benagiano M, Tamburini C, van der Zee R, Telford JL, Vandenbroucke-Grauls CM, D'Elios MM, Del Prete G. Molecular mimicry between Helicobacter pylori antigens and H+, K+ --adenosine triphosphatase in human gastric autoimmunity. *J Exp Med* 2003; **198**: 1147-1156
18 **D'Elios MM**, Amedei A, Azzurri A, Benagiano M, Del Prete G, Bergman MP, Vandenbroucke-Grauls CM, Appelmelk BJ. Molecular specificity and functional properties of autoreactive T-cell response in human gastric autoimmunity. *Int Rev Immunol* 2005; **24**: 111-122
19 **Albert LJ**, Inman RD. Molecular mimicry and autoimmunity. *N Engl J Med* 1999; **341**: 2068-2074
20 **Ungar B**, Mathews JD, Tait BD, Cowling DC. HLA-DR patterns in pernicious anaemia. *Br Med J* (Clin Res Ed) 1981; **282**: 768-770
21 **Fernando MM**, Stevens CR, Walsh EC, De Jager PL, Goyette P, Plenge RM, Vyse TJ, Rioux JD. Defining the role of the MHC in autoimmunity: a review and pooled analysis. *PLoS Genet* 2008; **4**: e1000024
22 **Fong TL**, Dooley CP, Dehesa M, Cohen H, Carmel R, Fitzgibbons PL, Perez-Perez GI, Blaser MJ. Helicobacter pylori infection in pernicious anemia: a prospective controlled study. *Gastroenterology* 1991; **100**: 328-332
23 **Haruma K**, Komoto K, Kawaguchi H, Okamoto S, Yoshihara M, Sumii K, Kajiyama G. Pernicious anemia and Helicobacter pylori infection in Japan: evaluation in a country with a high prevalence of infection. *Am J Gastroenterol* 1995; **90**: 1107-1110
24 **Sari R**, Ozen S, Aydogdu I, Yildirim B, Sevinc A. The pathological examinations of gastric mucosa in patients with Helicobacter pylori-positive and -negative pernicious anemia. *Helicobacter* 2000; **5**: 215-221
25 **Valle J**, Kekki M, Sipponen P, Ihamäki T, Siurala M. Long-term course and consequences of Helicobacter pylori gastritis. Results of a 32-year follow-up study. *Scand J Gastroenterol* 1996; **31**: 546-550
26 **Karnes WE Jr**, Samloff IM, Siurala M, Kekki M, Sipponen P, Kim SW, Walsh JH. Positive serum antibody and negative tissue staining for Helicobacter pylori in subjects with atrophic body gastritis. *Gastroenterology* 1991; **101**: 167-174
27 **Annibale B**, Negrini R, Caruana P, Lahner E, Grossi C, Bordi C, Delle Fave G. Two-thirds of atrophic body gastritis patients have evidence of Helicobacter pylori infection. *Helicobacter* 2001; **6**: 225-233
28 **Annibale B**, Lahner E, Santucci A, Vaira D, Pasquali A, Severi C, Mini R, Figura N, Delle Fave G. CagA and VacA are immunoblot markers of past Helicobacter pylori infection in atrophic body gastritis. *Helicobacter* 2007; **12**: 23-30
29 **Rose NR**, Bona C. Defining criteria for autoimmune diseases (Witebsky's postulates revisited). *Immunol Today* 1993; **14**: 426-430
30 **Carmel R**. Prevalence of undiagnosed pernicious anemia in the elderly. *Arch Intern Med* 1996; **156**: 1097-1100
31 **Pedersen AB**, Mosbech J. Morbidity of pernicious anaemia. Incidence, prevalence, and treatment in a Danish county. *Acta Med Scand* 1969; **185**: 449-452
32 **Hershko C**, Ronson A, Souroujon M, Maschler I, Heyd J, Patz J. Variable hematologic presentation of autoimmune gastritis: age-related progression from iron deficiency to cobalamin depletion. *Blood* 2006; **107**: 1673-1679
33 **Annibale B**, Capurso G, Chistolini A, D'Ambra G, DiGiulio E, Monarca B, DelleFave G. Gastrointestinal causes of refractory iron deficiency anemia in patients without gastrointestinal symptoms. *Am J Med* 2001; **111**: 439-445
34 **Marignani M**, Delle Fave G, Mecarocci S, Bordi C, Angeletti S, D'Ambra G, Aprile MR, Corleto VD, Monarca B, Annibale B. High prevalence of atrophic body gastritis in patients with unexplained microcytic and macrocytic anemia: a prospective screening study. *Am J Gastroenterol* 1999; **94**: 766-772
35 **Annibale B**, Lahner E, Negrini R, Baccini F, Bordi C, Monarca B, Delle Fave G. Lack of specific association between gastric autoimmunity hallmarks and clinical presentations of atrophic body gastritis. *World J Gastroenterol* 2005; **11**: 5351-5357
36 **Carmel R**, Weiner JM, Johnson CS. Iron deficiency occurs frequently in patients with pernicious anemia. *JAMA* 1987; **257**: 1081-1083
37 **Eisenbarth GS**, Gottlieb PA. Autoimmune polyendocrine syndromes. *N Engl J Med* 2004; **350**: 2068-2079
38 **Tosetti C**, Stanghellini V, Tucci A, Poli L, Salvioli B, Biasco G, Paparo GF, Levorato M, Corinaldesi R. Gastric emptying and dyspeptic symptoms in patients with nonautoimmune fundic atrophic gastritis. *Dig Dis Sci* 2000; **45**: 252-257
39 **De Block CE**, De Leeuw IH, Van Gaal LF. Autoimmune gastritis in type 1 diabetes: a clinically oriented review. *J Clin Endocrinol Metab* 2008; **93**: 363-371
40 **Feldt-Rasmussen U**, Bech K, Bliddal H, Høier-Madsen M, Jørgensen F, Kappelgaard E, Nielsen H, Lanng Nielsen J, Ryder LP, Thomsen M. Autoantibodies, immune complexes and HLA-D in thyrogastric autoimmunity. *Tissue Antigens* 1983; **22**: 342-347
41 **Lahner E**, Centanni M, Agnello G, Gargano L, Vannella L, Iannoni C, Delle Fave G, Annibale B. Occurrence and risk factors for autoimmune thyroid disease in patients with atrophic body gastritis. *Am J Med* 2008; **121**: 136-141
42 **Centanni M**, Gargano L, Canettieri G, Viceconti N, Franchi A, Delle Fave G, Annibale B. Thyroxine in goiter, Helicobacter pylori infection, and chronic gastritis. *N Engl J Med* 2006; **354**: 1787-1795
43 **Friedlander RD**. Racial factor in pernicious anaemia. *Am J Med Sci* 1934; **187**: 634
44 **Carmel R**, Johnson CS. Racial patterns in pernicious anemia. Early age at onset and increased frequency of intrinsic-factor antibody in black women. *N Engl J Med* 1978; **298**: 647-650
45 **WHO/UNICEF/UNU**. Iron deficiency anaemia: assesment, prevention, and control. Geneva: World Health Organization, 2001 (WHO/NHD/01.3)
46 **Kekki M**, Samloff IM, Varis K, Ihamäki T. Serum pepsinogen I and serum gastrin in the screening of severe atrophic corpus gastritis. *Scand J Gastroenterol Suppl* 1991; **186**: 109-116
47 **Väänänen H**, Vauhkonen M, Helske T, Kääriäinen I, Rasmussen M, Tunturi-Hihnala H, Koskenpato J, Sotka M, Turunen M, Sandström R, Ristikankare M, Jussila A, Sipponen P. Non-endoscopic diagnosis of atrophic gastritis with a blood test. Correlation between gastric histology and serum levels of gastrin-17 and pepsinogen I: a multicentre study. *Eur J Gastroenterol Hepatol* 2003; **15**: 885-891
48 **Lahner E**, Norman GL, Severi C, Encabo S, Shums Z, Vannella L, Fave GD, Annibale B. Reassessment of intrinsic factor and parietal cell autoantibodies in atrophic gastritis with respect to cobalamin deficiency. *Am J Gastroenterol* 2009; **104**: 2071-2079
49 **Toh BH**, Alderuccio F. Parietal cell and intrinsic factor autoantibodies. In: Shoenfeld Y, Gershwin ME, Meroni PL, editors. Autoantibodies. 2nd ed. Amsterdam: Elsevier, 2007: 479-486
50 **Ungar B**, Whittingham S, Francis CM. Pernicious anaemia: incidence and significance of circulating antibodies to

intrinsic factor and to parietal cells. *Australas Ann Med* 1967; **16**: 226-229
51 **Davidson RJ**, Atrah HI, Sewell HF. Longitudinal study of circulating gastric antibodies in pernicious anaemia. *J Clin Pathol* 1989; **42**: 1092-1095
52 **Carmel R**. Reassessment of the relative prevalences of antibodies to gastric parietal cell and to intrinsic factor in patients with pernicious anaemia: influence of patient age and race. *Clin Exp Immunol* 1992; **89**: 74-77
53 **Carmel R**, Perez-Perez GI, Blaser MJ. Helicobacter pylori infection and food-cobalamin malabsorption. *Dig Dis Sci* 1994; **39**: 309-314
54 **Cohen H**, Weinstein WM, Carmel R. Heterogeneity of gastric histology and function in food cobalamin malabsorption: absence of atrophic gastritis and achlorhydria in some patients with severe malabsorption. *Gut* 2000; **47**: 638-645
55 **Annibale B**, Capurso G, Delle Fave G. Consequences of Helicobacter pylori infection on the absorption of micronutrients. *Dig Liver Dis* 2002; **34** Suppl 2: S72-S77
56 **Herrmann W**, Obeid R. Causes and early diagnosis of vitamin B12 deficiency. *Dtsch Arztebl Int* 2008; **105**: 680-685
57 **Hvas AM**, Nexo E. Diagnosis and treatment of vitamin B12 deficiency--an update. *Haematologica* 2006; **91**: 1506-1512
58 **Pruthi RK**, Tefferi A. Pernicious anemia revisited. *Mayo Clin Proc* 1994; **69**: 144-150
59 **Bastrup-Madsen P**, Helleberg-Rasmussen I, Nørregaard S, Halver B, Hansen T. Long term therapy of pernicious anaemia with the depot cobalamin preparation betolvex. *Scand J Haematol* 1983; **31**: 57-62
60 **Correa P**. Human gastric carcinogenesis: a multistep and multifactorial process--First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention. *Cancer Res* 1992; **52**: 6735-6740
61 **Wu MS**, Chen CJ, Lin JT. Host-environment interactions: their impact on progression from gastric inflammation to carcinogenesis and on development of new approaches to prevent and treat gastric cancer. *Cancer Epidemiol Biomarkers Prev* 2005; **14**: 1878-1882
62 **Sjöblom SM**, Sipponen P, Miettinen M, Karonen SL, Jrvinen HJ. Gastroscopic screening for gastric carcinoids and carcinoma in pernicious anemia. *Endoscopy* 1988; **20**: 52-56
63 **Annibale B**, Azzoni C, Corleto VD, di Giulio E, Caruana P, D'Ambra G, Bordi C, Delle Fave G. Atrophic body gastritis patients with enterochromaffin-like cell dysplasia are at increased risk for the development of type I gastric carcinoid. *Eur J Gastroenterol Hepatol* 2001; **13**: 1449-1456
64 **Kokkola A**, Sjöblom SM, Haapiainen R, Sipponen P, Puolakkainen P, Järvinen H. The risk of gastric carcinoma and carcinoid tumours in patients with pernicious anaemia. A prospective follow-up study. *Scand J Gastroenterol* 1998; **33**: 88-92
65 **Uemura N**, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, Taniyama K, Sasaki N, Schlemper RJ. Helicobacter pylori infection and the development of gastric cancer. *N Engl J Med* 2001; **345**: 784-789
66 **Eisenbrand G**, Adam B, Peter M, Malfertheiner P, Schlag P. Formation of nitrite in gastric juice of patients with various gastric disorders after ingestion of a standard dose of nitrate--a possible risk factor in gastric carcinogenesis. *IARC Sci Publ* 1984; 963-968
67 **Annibale B**, Capurso G, Lahner E, Passi S, Ricci R, Maggio F, Delle Fave G. Concomitant alterations in intragastric pH and ascorbic acid concentration in patients with Helicobacter pylori gastritis and associated iron deficiency anaemia. *Gut* 2003; **52**: 496-501
68 **Schafer LW**, Larson DE, Melton LJ 3rd, Higgins JA, Zinsmeister AR. Risk of development of gastric carcinoma in patients with pernicious anemia: a population-based study in Rochester, Minnesota. *Mayo Clin Proc* 1985; **60**: 444-448
69 **Lahner E**, Bordi C, Cattaruzza MS, Iannoni C, Milione M, Delle Fave G, Annibale B. Long-term follow-up in atrophic body gastritis patients: atrophy and intestinal metaplasia are persistent lesions irrespective of Helicobacter pylori infection. *Aliment Pharmacol Ther* 2005; **22**: 471-481
70 **Sjöblom SM**, Sipponen P, Järvinen H. Gastroscopic follow up of pernicious anaemia patients. *Gut* 1993; **34**: 28-32
71 **Lahner E**, Caruana P, D'Ambra G, Ferraro G, Di Giulio E, Delle Fave G, Bordi C, Annibale B. First endoscopic-histologic follow-up in patients with body-predominant atrophic gastritis: when should it be done? *Gastrointest Endosc* 2001; **53**: 443-448

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EDITORIAL

Costimulatory molecule programmed death-1 in the cytotoxic response during chronic hepatitis C

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Abstract

Hepatitis C virus (HCV)-specific $CD8^+$ T cells play an important role in the resolution of HCV infection. Nevertheless, during chronic hepatitis C these cells lack their effector functions and fail to control the virus. HCV has developed several mechanisms to escape immune control. One of these strategies is the up-regulation of negative co-stimulatory molecules such us programmed death-1 (PD-1). This molecule is up-regulated on intrahepatic and peripheral HCV-specific cytotoxic T cells during acute and chronic phases of the disease, whereas PD-1 expression is low in resolved infection. PD-1 expressing HCV-specific $CD8^+$ T cells are exhausted with impairment of several effector mechanisms, such as: type-1 cytokine production, expansion ability after antigen encounter and cytotoxic ability. However, PD-1 associated exhaustion can be restored by blocking the interaction between PD-1 and its ligand (PD-L1). After this blockade, HCV-specific $CD8^+$ T cells reacquire their functionality. Nevertheless, functional restoration depends on PD-1 expression level. High PD-1-expressing intrahepatic HCV-specific $CD8^+$ T cells do not restore their effector abilities after PD-1/PD-L1 blockade. The mechanisms by which HCV is able to induce PD-1 up-regulation to escape immune control are unknown. Persistent TCR stimulation by a high level of HCV antigens could favour early PD-1 induction, but the interaction between HCV core protein and gC1q receptor could also participate in this process. The PD-1/PD-L1 pathway modulation could be a therapeutic strategy, in conjunction with the regulation of others co-stimulatory pathways, in order to restore immune response against HCV to succeed in clearing the infection.

Key words: Chronic hepatitis; Exhaustion; Hepatitis C virus core; Hepatitis C virus; Programmed death-1; Programmed death-1 ligand

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INTRODUCTION

Hepatitis C virus (HCV) is a hepatotropic non-cytopathic positive-strand RNA virus which belongs to the *Flaviviridae* family. HCV infection is a major public problem, affecting more than 200 million people worldwide[1]. Only around a quarter of acute HCV infections resolve within a few months, while in the majority of cases the virus establishes a persistent infection, and a significant proportion of cases progress to fibrosis, cirrhosis, liver failure or even hepatocellular carcinoma[2-4]. Nowadays standard

anti-HCV therapy resolves about 50% of chronic infections[5-7], therefore new therapeutic strategies should be designed to control this disease. HCV-specific cytotoxic T lymphocytes (CTLs) play a major role in viral control during acute infection[8]. Nevertheless, during persistent infection HCV-specific CTL effector functions are significantly impaired, and this situation is a major cause of host inability to eliminate the persistent virus[9,10]. Appropriate activation of primed virus-specific CTLs in the infected site depends on the engagement between T cell receptor (TCR) and HLA-I/epitope complex plus interaction among positive co-stimulatory molecules and their ligands[11,12]. Virus-specific CTLs, after developing their effector function, express negative co-stimulatory molecules to switch-off their activity. The appropriate virus-specific CTL response development correlates with the adequate balance between positive and negative co-stimulatory signals (Table 1)[13,14]. Programmed death-1 (PD-1) is one of the negative co-stimulatory molecules. Engagement of PD-1 and its ligand (PD-L1) delivers a negative signal to the TCR activation pathway, avoiding proliferation, and interleukin (IL)-2 production, which leads to T cell anergy[15,16]. Evidence that PD-1 suppresses activation of the immune response comes from studies in which mice deficient in PD-1 developed autoimmune diseases, such as systemic lupus erythematosus, dilated cardiomyopathy, rheumatoid arthritis and type I diabetes mellitus, due to the uncontrolled persistent T cell activation against different epitopes[17,18]. The PD-1 induced exhaustion on virus-specific T cells was first described by Barber *et al*[19], in a murine model of lymphocytic choriomeningitis virus (LCMV) infection. The authors demonstrated that the majority of LCMV-specific $CD8^+$ T cells were anergic during the chronic phase of infection in association with PD-1 up-regulation. Mice treated with anti-PD-L1 monoclonal antibodies restored the LCMV-specific cyototoxic response and facilitated viral control. These experimental data suggested that the PD-1/PD-L1 pathway could play a major role in the development of persistent infections by non-cytopathic viruses, and different groups started to research the role of this pathway in different chronic viral infections in humans, such as hepatitis B virus (HBV), HCV and human immunodeficiency virus (HIV) infections. Bearing in mind these data, the PD-1/PD-L1 pathway could be an effective escape mechanism and its blockade could be a therapeutic target to reverse T-cell dysfunction. In this editorial, the current state of knowledge about the role of PD-1 expression on specific cytotoxic responses during HCV infection is reviewed.

STRUCTURE AND EXPRESSION OF PD-1 AND PD-L1

PD-1 is a 55 kDa glycoprotein which belongs to the CD28 immunoglobulin superfamily of transmembrane proteins[20]. PD-1 shares a 23% homology with CTLA-4, which is another member of this family, although PD-1 has lost the MYPPPY motif for binding to B7 molecules[20], and the cysteine residue necessary for homodimerization[21]. PD-1 is expressed on activated T cells, B cells and myeloid cells (Table 1)[20]. The PD-1 structure consists of two regions; the extracellular region is formed by a single IgV-like domain and its cytoplasmic region contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM)[22,23]. Following antigen stimulation, PD-1 recruits the protein tyrosine phosphatase src homology 2 domain-containing tyrosine phosphatase 2 (SHP-2) to ITSM but not to the ITIM motif, and subsequently, SHP-2 dephosphorylates effector molecules downstream of the TCR-induced nuclear regulatory pathway[24,25]. The direct result of PD-1 mediated inhibition of T cell proliferation is cell cycle arrest in G0/G1 and the inhibition of IL-2 production[15,16] (Figure 1). The ligands for PD-1 (PD-L1 and PD-L2) are type I transmembrane proteins with IgV and IgC-like domains in the extracellular region[26,27]. PD-L1 is expressed on resting and activated B and T cells, and on non-lymphoid cells such as pancreas, placenta and heart, while PD-L2 is induced on dendritic cells (DC) and macrophages[26-30] (Table 1). Interestingly, PD-L1 can be up-regulated on hepatocytes by α-interferon (IFN) and γ-IFN, and also by activated lymphocytes, and by direct viral infection (perhaps also through IFN pathways)[31-33]. PD-1 plays an important physiological role in regulating the cellular immune response, tuning-down the cellular effector functions after T cells have developed their tasks. This physiological function of PD-1 can be damaged by persistent viruses inducing a tolerogenic-like status on specific T cells to avoid immune viral control.

PD-1 EXPRESSION IN THE LIVER

The liver is characterised by being an immunotolerant organ prepared to deal with intense contact with antigens from the gut, and PD-1/PD-L1 is expressed in resident and infiltrating liver cells to carry out this task[34]. The liver is also the primary site for HCV replication and disease pathogenesis[35], and HCV can take advantage of the PD-1/PD-L1 pathway to impair the HCV-specific response reaching the infected liver in order to escape immune control. The liver is exposed to antigens and microbiologically-derived molecules which cause a unique microenvironment that requires liver immunological properties to induce tolerance rather than immunity[36-38]. Hepatic tolerance contributes to the common ineffectiveness of immune response against HCV which often results in chronic viral persistence[39]. When naive T cells reach the liver from the bloodstream they are activated by resident antigen presenting cells and are prone to become anergic, and this process could take part in the interaction between PD-1 and PD-L1 (Figure 2)[34,40]. On the other hand, primed effector HCV-specific T cells, reaching the infected liver, are also conducted through anergy by several mechanisms. One of them is PD-1 up-regulation on T cells in the liver[41,42], and the expression of its ligand on resident liver cells, such as hepatocytes, Kuppfer cells and sinusoidal endothelial cells (Figure 2)[31]. Usually, PD-L1 is constitutively expressed in non-lymphoid tissues such as heart,

Table 1 Summary of CD28 family co-stimulatory and co-inhibitory pathways

Receptor	Expression	Ligand	Ligand expression	T cell response regulation
CD28	T cells (naive and some memory)	CD80	B, T and DC, macrophages	Positive
PD-1	Activate T cells, B cells, macrophages	PD-L1	B, T and DC, macrophages, non-lymphoid cells	Negative
		PD-L2	(pancreas, placenta, heart)	
CTLA-4	Activate T cells and regulatory T cells	CD80	B, T and DC, macrophages	Negative
		CD86		
BTLA	B and T cells	PD-L2	Macrophages, DC	Negative
		B7-H3	T and B cells, NK	
		B7-H4		
ICOS	T cells (memory and effector)	ICOS-L	B, T cells, macrophages and DC	Positive

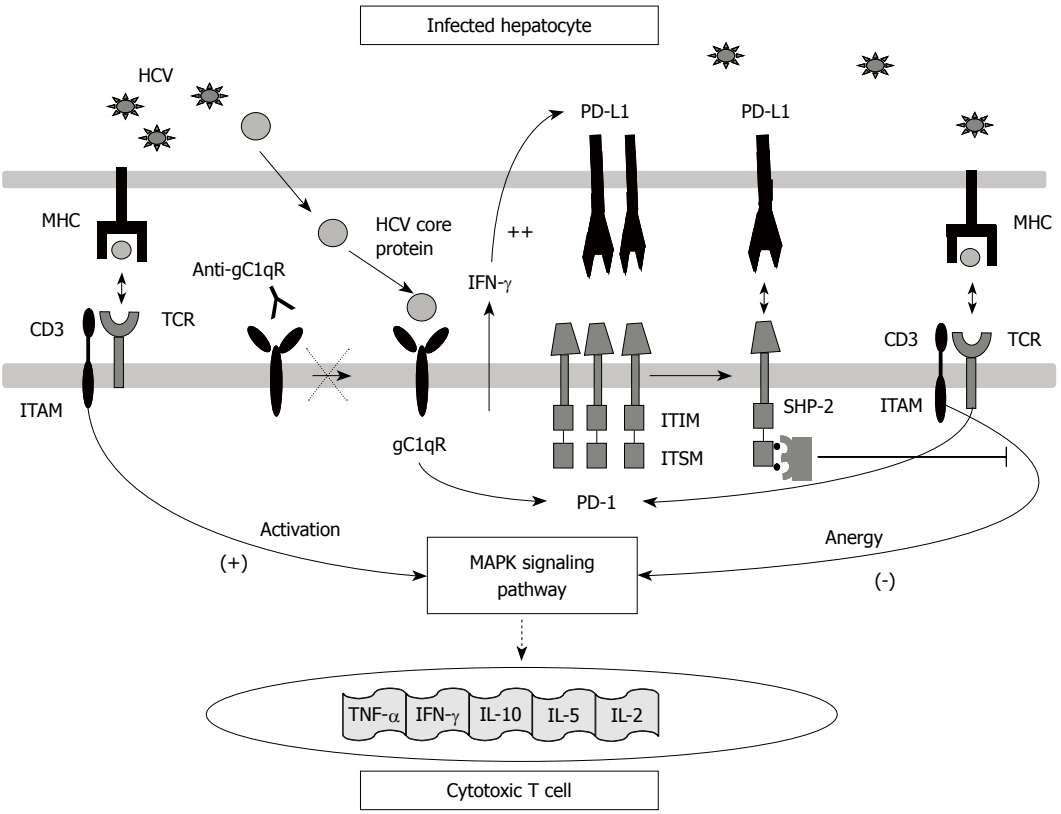

Figure 1 Programmed death-1 (PD-1) structure and interactions. Interaction between the PD-1 molecule expressed on T cells and its ligand PD-L1 expressed on antigen-presenting cells leads to immunoreceptor tyrosine-based switch motif (ITSM) motif phosphorylation in its cytoplasmic tyrosines which are recognized by src homology 2 domain-containing tyrosine phosphatase 2 (SHP-2). All of these interactions cause T cell anergy due to T cell receptor (TCR)-dependent MAP Kinase-pathway signalling inhibition which avoids interleukin (IL)-2 gene transduction. PD-1 expression is induced by TCR activation but could also be favoured by HCV-core protein through interaction with gC1qR. PD-L1 is up-regulated on antigen presenting cells by the effect of γ-interferon produced during HCV infection by activated lymphocytes.

lung, placenta, kidney, and liver[43-45], but during chronic HCV infection, this molecule is up-regulated on parenchymal liver cells, as previously commented (Figure 3A). The regulation of HCV-specific effector CTLs is also controlled by intrahepatic CD4+CD25+FoxP3+ cells (regulatory T cells, Treg). These cells have an important role in maintaining the balance between tolerance and immunity in HCV infection[46-48]. The PD-1/PD-L1 pathway is also important in modulating the regulatory activity of these Treg cells. The PD-1/PD-L1 interaction on intrahepatic Treg suppresses their regulatory activity, favouring CTL response[49-51]. Nevertheless, when it is necessary to down-modulate HCV-specific CTL response in order to avoid liver damage, PD-1/PD-L1 engagement is not produced between Tregs and intrahepatic PD-L1 expressing cells, due to PD-L1 down-regulation on resident liver cells, allowing Tregs to down-modulate HCV-specific CTLs effector functions (Figure 2)[52]. The important role of PD-1 in liver pathogenesis during HCV chronic infection is evident, as it is shown by the high PD-1 expression on total intrahepatic T cells[53-56], indicating that some non-specific HCV-dependent stimulus is acting in liver infiltrating T cells to favour PD-1 up-regulation. Previous reports suggest that this factor could be HCV-core protein and this will be discussed later. In addition to this non-specific stimulation on T cells, PD-1 expression is also induced by persistent specific TCR stimulation. PD-1 expression is higher on intrahepatic than in peripheral HCV-specific

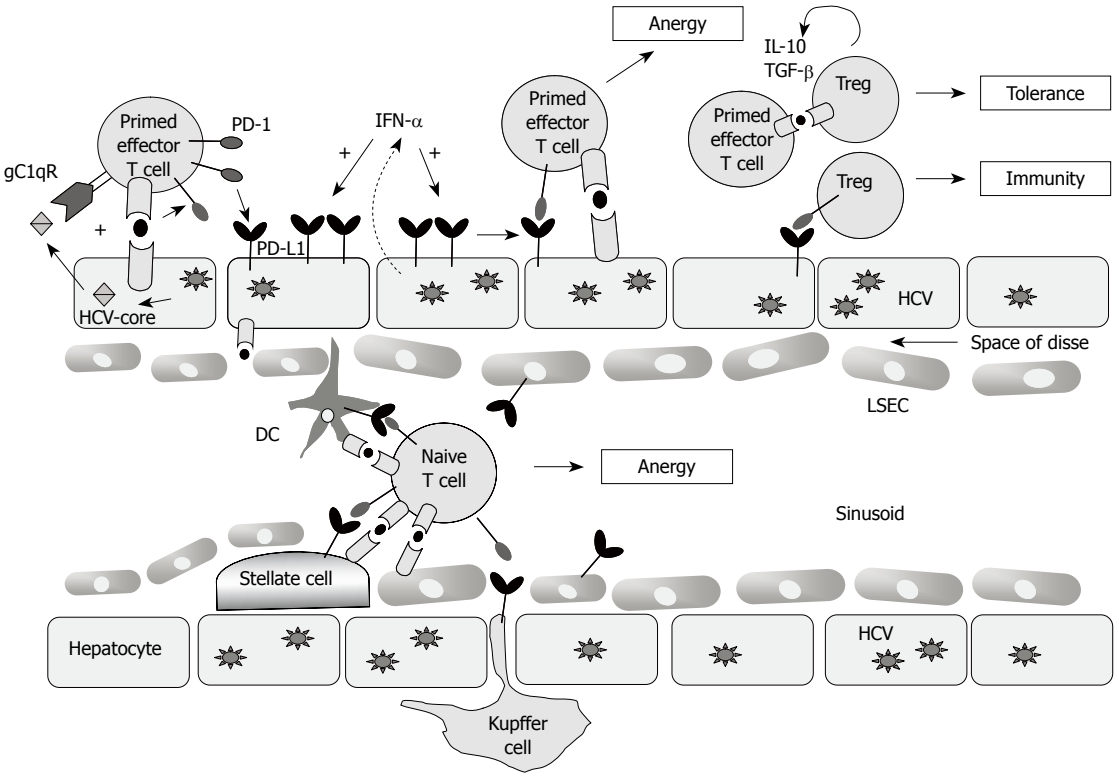

Figure 2 Liver microenvironment. Circulating HCV-specific $CD8^+$ T cells migrating through the hepatic sinusoid interact with resident liver cells [Kupffer cells, dendritic cells (DC), hepatocytes, liver sinusoidal endothelial cells (LSECs), stellate cells] that could act as antigen presenting cells. These cells up-regulate PD-L1 expression during persistent HCV infection and interact with the PD-1 molecule expressed on HCV-specific $CD8^+$ T cells. This interaction leads to T cell anergy. PD-1 up-regulation is produced by TCR stimulation in addition to the interaction between HCV-core protein and the complement receptor (gC1qR). In this micro-environment the regulatory T cells (Treg) also participate, whose activity is also regulated by the PD-1/PD-L1 pathway.

$CD8^+$ cells[41] (Figure 3B). These data suggest that the intense TCR activation produced in the liver in conjunction with the high level of HCV-core protein leads to the highest PD-1 expression on HCV-specific $CD8^+$ cells. This high PD-1 up-regulation on intrahepatic specific $CD8^+$ cells is exquisitely HCV specific, so that PD-1 expression on other virus-specific $CD8^+$ T cells is not up-regulated during chronic HCV infection[41]. Therefore, liver environment conditions produce a huge PD-1 up-regulation on HCV-specific CTLs during persistent infection, and this could impair viral control by the cellular immune response through anergy induction[41].

DIFFERENTIAL PD-1 EXPRESSION IN ACUTE, CHRONIC AND RESOLVED HEPATITIS C VIRUS INFECTION

During the initial phase of acute infection, HCV-specific $CD8^+$ T cells are dysfunctional irrespective of the final outcome of the disease, and this impairment persists when infection becomes chronic[10]. In contrast, effector and memory $CD8^+$ T cells generated after acute onset are highly functional in cases of resolving infection[57,58]. One of the possible mechanisms responsible for impairment of virus-specific CTL response could be the exhaustion of these cells caused by PD-1 up-regulation. The exhaustion of virus-specific $CD8^+$ T cells has been observed in different human infections such as HIV, HBV and HCV infections[56,59-65]. In HCV infection, during the early period of primo-infection irrespective of the final outcome, PD-1 is up-regulated on all HCV-specific $CD8^+$ T cells[53,66]. However, after the acute stage of the disease PD-1 expression is modulated depending on the progression. Therefore, during self-limited infection HCV-specific $CD8^+$ cells down-regulate PD-1 expression, and acquire a $CD127^+$ phenotype which correlates with appropriate effector functions (Figure 4)[67]. CD127 is the IL-7 receptor (IL-7R) which plays an essential role in mature lymphocyte survival through a pathway activated by the interaction with IL-7[67]. However, in persistent infection HCV-specific $CD8^+$ cells remain CD127 negative, and maintain high levels of PD-1 expression[66,68] (Figure 4). Therefore, $PD\text{-}1^+$ $CD127^-$ expressing HCV-specific $CD8^+$ cells during persistent infection are not only anergic, but also prone to apoptosis after antigen encounter due to the absence of CD127 expression. Furthermore, PD-1 up-regulation on peripheral and intrahepatic HCV-specific $CD8^+$ cells during the acute and chronic phases of infection is correlated with the apoptosis susceptibility of these cells[55]. As a result, the majority of high PD-1 expressing HCV-specific $CD8^+$ cells could follow an apoptotic process[69], indicating that PD-1 is involved in anergy induction but could also be implicated in specific T cell deletion. Probably, both mechanisms are damaged by HCV infection to escape cellular immune response.

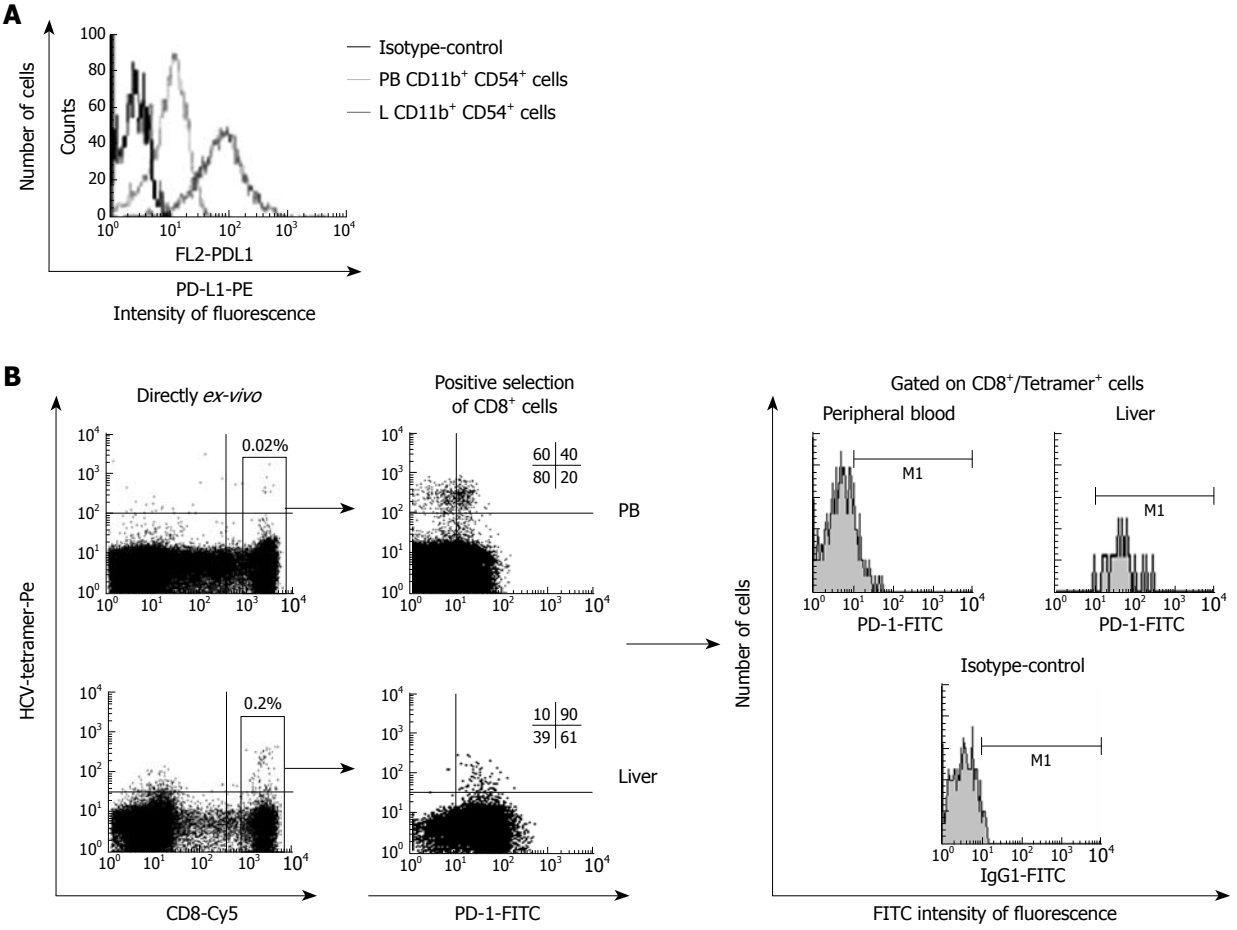

Figure 3 PD-L1 expression on Kupffer cells and PD-1 expression on HCV-specific CD8$^+$ T cells. A: PD-L1-FITC FACS® histograms gated on CD11b$^+$ CD54$^+$ cells from liver (L) and peripheral blood (PB), showing a higher PD-L1 expression on intrahepatic Kuppfer cells; B: FACS® dot-plots and histograms of peripheral blood and intrahepatic T cells stained with CD8-Cy mAb, HCV-tetramers-PE and PD-1-FITC mAb. Gated Tetramer$^+$/CD8$^+$ cells are presented in the histograms for PD-1-FITC expression. PD-1 is up-regulated on intrahepatic HCV-specific CD8$^+$ cells.

CORRELATION BETWEEN PD-1 EXPRESSION AND EFFECTOR FUNCTION IMPAIRMENT ON HCV-SPECIFIC CTLs

Once differential PD-1 expression on HCV-specific CD8$^+$ T cells between chronic and resolved patients has been described, the next point to address is to analyse whether this difference translates into different quality of HCV-specific CTLs effector functions. Cytotoxic T-cell exhaustion represents a spectrum of effector defects that are correlated with the level of PD-1 expression. Recent reports show that patients with HCV chronic infection, whose CTLs display high PD-1 expression, have impaired CTL capacity to synthesise type-1 cytokines, such us γ-IFN, α-tumor necrosis factor (TNF) and IL-2, in addition to cytolytic molecules, such as perforin and granzyme B, after direct *ex-vivo* specific *in-vitro* challenge[41,54]. One of the variables determining viral control has been suggested to be the ability of virus-specific CD8$^+$ cells to clonally expand after antigen encounter[45]. HCV-specific CD8$^+$ T cells during persistent infection also displayed impaired proliferation ability after specific stimulation, which correlated with PD-1 expression level[70,71]. Because of the role of the PD-1/PD-L1 pathway in proliferation impairment, subsequent works were aimed at trying to enhance HCV-specific CD8$^+$ T cell proliferation by modulating this pathway. Blocking the interaction between PD-1 and its ligand increased the proliferation ability of peripheral HCV-specific CD8$^+$ cells from some chronic HCV patients, characterised by high PD-1 expression, but did not occur in others, suggesting the presence of another anti-proliferative mechanism not yet described[54,63]. During HCV-specific CTL exhaustion, not all effector functions are altered at the same time; proliferative potential and IL-2 production are lost at an early phase, whereas cytokine production and cytolytic function are lost later[72]. This progressive impairment could be related to the level of PD-1 up-regulation. Interestingly, the exhaustion of CTLs during chronic HCV infection is highly antigen-specific and related to the level of antigenemia, not being present in either CTLs against other specifities or HCV-specific CTLs from patients with resolved infection[41,66]. In these two situations PD-1 is not up-regulated on specific CTLs. As commented before, intrahepatic HCV-specific CD8$^+$ T cells are highly PD-1 positive and they do not expand after antigen encounter and do not produce either γ-IFN or perforin, whereas intrahepatic specific CTLs against other

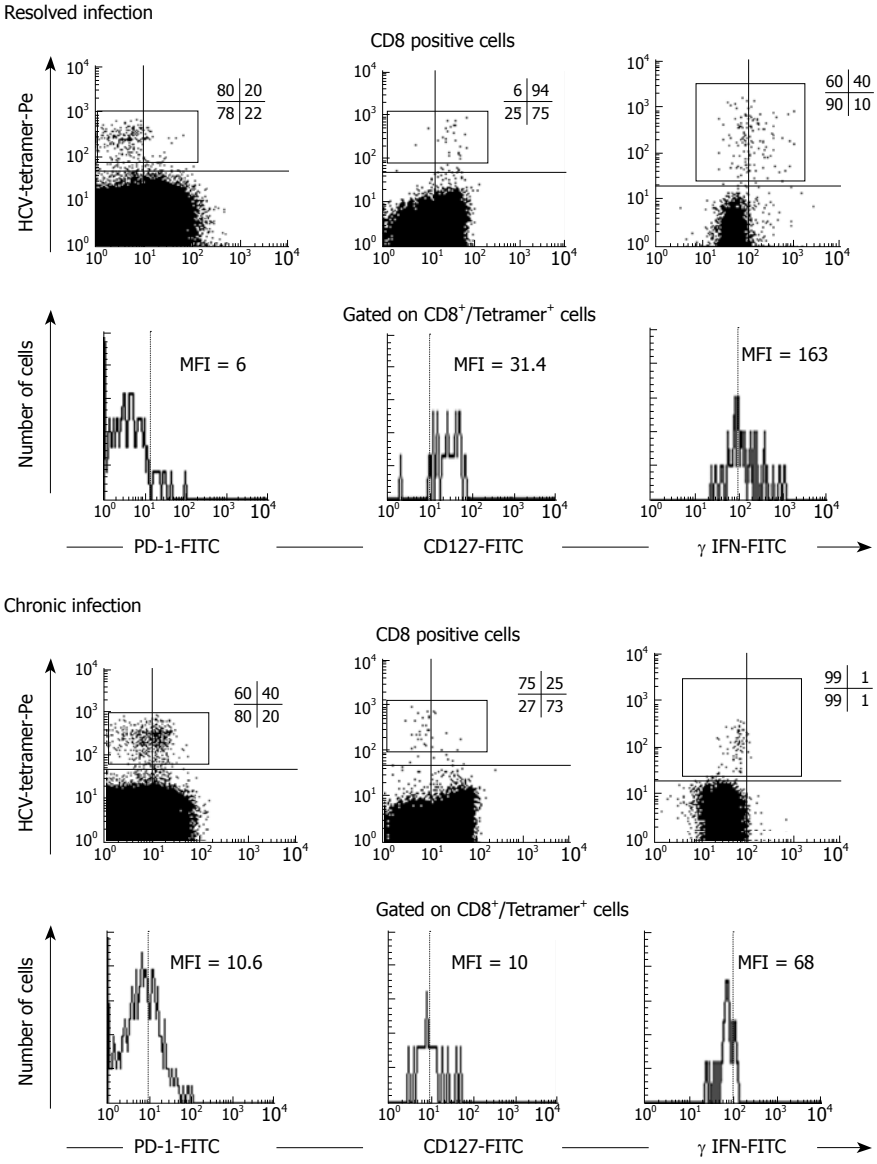

Figure 4 Direct *ex-vivo* PD-1, CD127 expression and IFN-γ production. FACS® dot-plots and histograms of peripheral blood T cells from two representative patients, one with persistent HCV infection and the other with resolved HCV infection. T cells are stained with PD-1-FITC, CD127-FITC, γ-IFN-FITC and CD8-Cy mAbs and HCV-tetramers. In persistent infection, HCV-specific $CD8^+$ cells maintain a $PD\text{-}1^+/CD127^-/IFN\text{-}\gamma^-$ phenotype while the resolved infection case displays an opposite phenotype $PD\text{-}1^-/CD127^+/IFN\text{-}\gamma^+$.

viruses, such as influenza virus-specific $CD8^+$ T cells, expand efficiently and present a high level of perforin expression, but interestingly they are PD-1 negative[41]. High PD-1 expressing intrahepatic HCV-specific CTLs do not respond to anti-PD-L1 treatment[41]. Therefore, when PD-1 expression is extremely up-regulated, treatment with anti-PD-L1 antibodies can not counteract the HCV-specific CTLs exhaustion, induced by the PD-1/PD-L1 pathway.

HCV-SPECIFIC CTL FUNCTIONAL RESTORATION AFTER PD-1/PD-L1 INTERACTION BLOCKADE

Previous studies developed an LCMV infection animal model, and specific CTL function restoration during persistent infection after treatment with anti-PD-L1 monoclonal antibodies was shown[19,73]. This finding could have clinical implications in the treatment of persistent viral infections, as will be discussed later. In HCV infection, the *in-vitro* blockade of the PD-1/PD-L1 pathway with anti-PDL-1 antibodies increases proliferation capacity after antigen encounter in peripheral PD-1 expressing HCV-specific CTLs from chronic patients (Figure 5). *In-vitro* treatment with anti-PD-L1 antibodies also restored γ-IFN, perforin, CD107a, IL-2 and IL-13 production after antigen specific stimulation[41,54,74]. However, this PD-1/PD-L1 pathway blockade is not efficient on intrahepatic HCV-specific $CD8^+$ T cells, which are characterised by a higher PD-1 expression, as previously discussed. These cells failed to proliferate and produce perforin, γ-IFN and CD107a after specific stimulation in the presence of anti-PD-L1 antibodies[41]. All these findings suggest that PD-1 expression level correlates inversely with HCV-specific $CD8^+$ T cells functional restoration by PD-1/PD-L1 blockade. PD-1/PD-L1 blockade may increase the functionality of peripheral HCV-specific $CD8^+$ T cells with intermediate PD-1 expression, whereas this blockade did not enhance the effector functions of intrahepatic $PD\text{-}1^{high}$ expressing HCV-specific $CD8^+$ T cells. High antigenic stimulation in the liver induces other negative co-stimulatory molecules, such as CTLA-4[41],

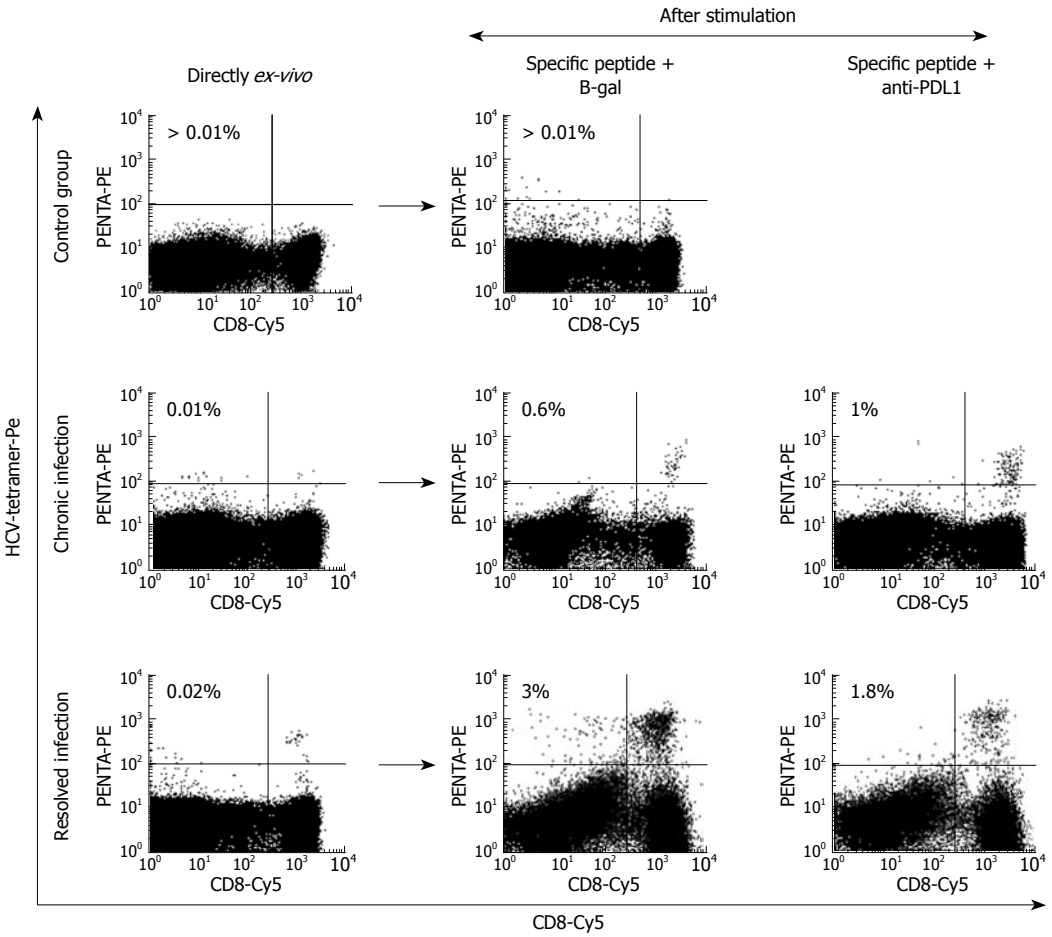

Figure 5 Proliferation restoration after PD-1 blockade. FACS® dot-plots of peripheral blood T cells from two representative patients, one with persistent HCV infection and the other with resolved HCV infection, and a control case, after specific stimulation in the presence or absence of anti-PD-L1 mAb. T cells were stimulated for 10 d with the HCV–specific peptide plus IL-2. After stimulation, T cells were stained with CD8-Cy mAbs and HCV-tetramers-PE. PD-1/PD-L1 pathway blockade by anti-PD-L1 antibodies increases the HCV-specific cell proliferation in the chronic patient that had a high level of PD-1 expression.

which could maintain the anergic status, despite blockage of the PD-1/PD-L1 pathway. Therefore, the functional restoration of intrahepatic HCV-specific CTLs could be obtained by the combined blocking of different negative co-stimulatory molecules. In fact, a previous report has shown that combined PD-1 and CTLA-4 blockade induces a restoration of intrahepatic HCV-specific $CD8^+$ T cell function in chronically HCV infected patients[75]. Obviously, the modulation of different co-stimulatory molecules on HCV-specific T cells, such as CD137[76], OX40[77] and ICOS[78] should be tested in combination with PD-1/PD-L1 blockade in order to restore HCV-specific CTL effector functions[79,80]. Nevertheless, blocking the engagement between PD-1 and PD-L1 is not enough in many cases to restore peripheral HCV-specific CTL functionality in chronic patients, even in combination with the blockade of other negative co-stimulatory molecules. It is reasonable to assume that these cells, exposed to high persistent antigenic stimulus, are prone to apoptosis. Previous data on HBV chronic infection showed an up-regulation of the pro-apoptotic molecule Bim on HBV-specific CTLs[81]. In this chronic infection, only $CD127^+$ (IL-7R) cells maintained the ability to expand after antigen encounter. These $CD127^+$ cells could be protected from apoptosis due to the antiapoptotic molecule Mcl-1, induced by IL-7. Otherwise, $CD127^-$ HBV-specific CTLs would die due to apoptosis after antigen encounter, mediated by the Bim pathway. Bearing in mind these data on HBV infection, it is possible that the benefit observed by blocking the PD-1/PD-L1 interaction may occur only in specific T cells protected against apoptosis by CD127 expression. This phenotype is quite rare in patients with long-standing HCV infection, and this could explain why not all PD-1 expressing HCV-specific CTLs respond to anti-PD-L1 treatment. This theoretical scenario should be tested in the near future.

HCV CORE PROTEIN INDUCES PD-1 UP-REGULATION

The PD-1 up-regulation on intrahepatic total T cells[53,56] suggests that something other than TCR stimulation is involved in the PD-1 expression regulation during HCV infection. HCV-core protein binding to the complement receptor gC1q (gC1qR) is responsible for impairing T cell proliferation ability[82] through down-regulation of the high affinity IL-2 receptor[83]. A recent report suggests that this process could be mediated by PD-1 expression

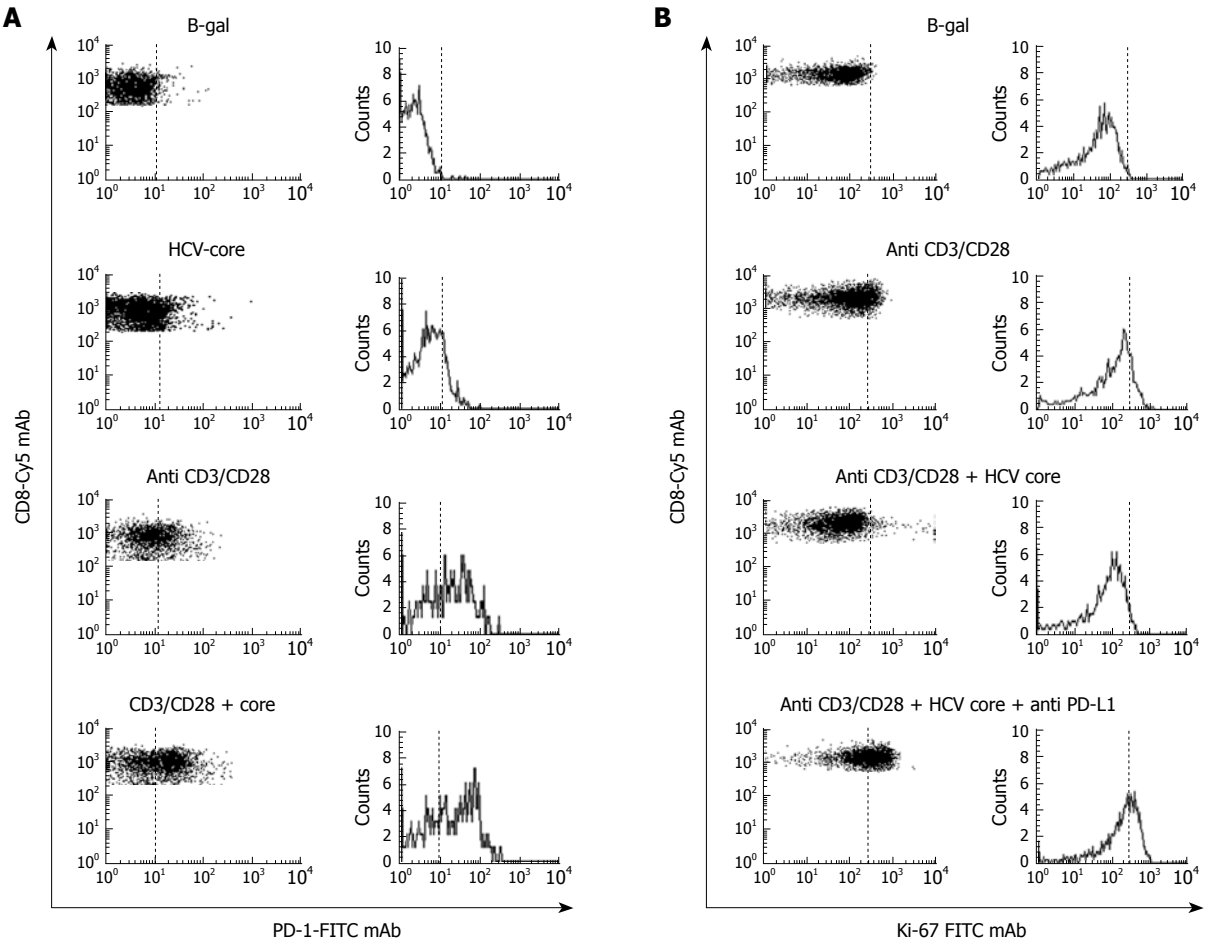

Figure 6 PD-1 up-regulation induced by HCV-core protein. A: FACS® dot-plots and histograms of peripheral blood $CD8^+$ cells stained with PD-1-FITC and CD8-Cy mAbs from a healthy subject. $CD8^+$ cells were stimulated with B-galactosidase, HCV-core protein, CD3-CD28 mAb, and HCV-core protein plus CD3-CD28 mAbs. PD-1 expression was highly up-regulated on $CD8^+$ cells after non-specific stimulation by anti CD3/CD28 mAbs in the presence of HCV core protein; B: FACS® dot-plots and histograms of peripheral blood $CD8^+$ cells stained with Ki-67 FTIC and CD8-Cy mAbs from the same healthy subject to test proliferation ability of $CD8^+$ cells after incubation with B-galactosidase, CD3-CD28 mAb, HCV-core protein plus CD3-CD28 mAb and HCV-core protein plus CD3-CD28 and anti-PD-L1 mAbs. HCV-core protein decreased the proliferation induced by CD3-CD28 mAb stimulation. This proliferation impairment induced by HCV-core protein was resolved by anti-PD-L1 mAb treatment.

induction[84]. In fact, in an intrahepatic HCV-core protein expressing mouse model, liver infiltration by PD-1 expressing cytotoxic T cells unable to clear the virus has been shown. However, the liver from HCV-core non-expressing mice was infiltrated by non-PD-1 expressing specific-CTLs which could control the viral infection[85]. These data suggest that HCV-core protein could play a role in early PD-1 induction on T cells, mainly in the liver environment where this protein is richly expressed[86,87]. At least *in-vitro*, PD-1 and PD-L1 expression are up-regulated on activated T cells in the presence of HCV-core protein[84]. PD-1 up-regulation induced by HCV-core protein translated into impairment of T cell proliferation ability[84]. However, this dysfunction could be partially restored by blocking the PD-1/PD-L1 pathway with anti-PD-L1 antibodies (Figures 1 and 6) and by blocking the interaction between HCV-core protein and gC1qR[84]. Probably the interaction between HCV-core protein and gC1qR co-operate with the continuous TCR stimulation to produce an early PD-1 up-regulation in order to induce a premature anergy on HCV-specific CTLs as an efficient HCV escape mechanism.

PD-1/PD-L1 BLOCKADE AS A THERAPUTIC TOOL

As previously commented, a defective virus-specific cytotoxic T cell response is one of the most important causes of host inability to eliminate a persistent viral infection. Several studies have highlighted the role of the PD-1/PD-L1 pathway in the development of anergy on virus-specific $CD8^+$ T cells, and how PD-1/PD-L1 blockade could enhance virus-specific $CD8^+$ T cell functionality *in-vitro*[41,74,88-92]. Recently, several works have been carried out to analyse whether modulation of the PD-1/PD-L1 pathway could improve T cell response against persistent viral infections either directly, using anti PD-L1 antibodies alone, or in combination with a therapeutic vaccine. Therapeutic vaccine usually fails to induce a vigorous T cell response due to the tolerogenic-like status of HCV-specific T cells[93,94]. This scenario could be positive if negative co-stimulatory molecules, such as PD-1, were blocked when the therapeutic vaccine is administered in order to enhance the specific immune response against the supplied epitopes. In the chronic LCMV infection

animal model, the administration of a therapeutic vaccine in combination with PD-1/PD-L1 interaction blockade enhances expansion and improves the function of LCMV-specific $CD8^+$ T cells. In addition, this combinatorial therapeutic vaccination accelerates viral control compared with either therapeutic vaccine or PD-1 blockade alone[95]. Moreover, the effect of anti-PD-L1 antibodies alone could also be effective in controlling persistent viral infection by restoring specific CTL response. The administration of anti-PD-L1 monoclonal antibodies during simian immunodeficiency virus (SIV) chronic infection in macaques resulted in a rapid expansion and restoration of SIV-specific $CD8^+$ T cells[96,97]. Although these results seem to be quite promising, the blockade of negative costimulatory pathways could lead to the development of autoimmune diseases[17,18], which could prevent the use of this strategy as a therapeutic tool in humans. Therefore, more research is necessary in this field before blockade of the PD-1/PD-L1 pathway is suitable for the treatment of chronic HCV infection.

CONCLUSION

In summary, the PD-1/PD-L1 pathway displays an important role in the induction of anergy on HCV-specific cytotoxic T cells, and could be important in the development of HCV persistent infection. Blocking the PD-1/PD-L1 interaction, probably in association with the modulation of other co-stimulatory molecules, could be an interesting strategy to restore HCV-specific CTL response in patients unresponsive to standard anti-HCV treatment.

REFERENCES

1 **Lauer GM**, Walker BD. Hepatitis C virus infection. *N Engl J Med* 2001; **345**: 41-52
2 **Bowen DG**, Walker CM. Adaptive immune responses in acute and chronic hepatitis C virus infection. *Nature* 2005; **436**: 946-952
3 **Liang TJ**, Heller T. Pathogenesis of hepatitis C-associated hepatocellular carcinoma. *Gastroenterology* 2004; **127**: S62-S71
4 **Afdhal NH**. The natural history of hepatitis C. *Semin Liver Dis* 2004; **24** Suppl 2: 3-8
5 **Fried MW**, Shiffman ML, Reddy KR, Smith C, Marinos G, Gonçales FL Jr, Häussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975-982
6 **Manns MP**, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958-965
7 **Hayashi N**, Takehara T. Antiviral therapy for chronic hepatitis C: past, present, and future. *J Gastroenterol* 2006; **41**: 17-27
8 **Grüner NH**, Gerlach TJ, Jung MC, Diepolder HM, Schirren CA, Schraut WW, Hoffmann R, Zachoval R, Santantonio T, Cucchiarini M, Cerny A, Pape GR. Association of hepatitis C virus-specific CD8+ T cells with viral clearance in acute hepatitis C. *J Infect Dis* 2000; **181**: 1528-1536
9 **Spangenberg HC**, Viazov S, Kersting N, Neumann-Haefelin C, McKinney D, Roggendorf M, von Weizsäcker F, Blum HE, Thimme R. Intrahepatic CD8+ T-cell failure during chronic hepatitis C virus infection. *Hepatology* 2005; **42**: 828-837
10 **Gruener NH**, Lechner F, Jung MC, Diepolder H, Gerlach T, Lauer G, Walker B, Sullivan J, Phillips R, Pape GR, Klenerman P. Sustained dysfunction of antiviral CD8+ T lymphocytes after infection with hepatitis C virus. *J Virol* 2001; **75**: 5550-5558
11 **Mueller DL**, Jenkins MK, Schwartz RH. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Annu Rev Immunol* 1989; **7**: 445-480
12 **Bretscher P**. The two-signal model of lymphocyte activation twenty-one years later. *Immunol Today* 1992; **13**: 74-76
13 **Nurieva R**, Thomas S, Nguyen T, Martin-Orozco N, Wang Y, Kaja MK, Yu XZ, Dong C. T-cell tolerance or function is determined by combinatorial costimulatory signals. *EMBO J* 2006; **25**: 2623-2633
14 **Frauwirth KA**, Thompson CB. Activation and inhibition of lymphocytes by costimulation. *J Clin Invest* 2002; **109**: 295-299
15 **Carter L**, Fouser LA, Jussif J, Fitz L, Deng B, Wood CR, Collins M, Honjo T, Freeman GJ, Carreno BM. PD-1:PD-L inhibitory pathway affects both CD4(+) and CD8(+) T cells and is overcome by IL-2. *Eur J Immunol* 2002; **32**: 634-643
16 **Latchman Y**, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, Iwai Y, Long AJ, Brown JA, Nunes R, Greenfield EA, Bourque K, Boussiotis VA, Carter LL, Carreno BM, Malenkovich N, Nishimura H, Okazaki T, Honjo T, Sharpe AH, Freeman GJ. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol* 2001; **2**: 261-268
17 **Nishimura H**, Nose M, Hiai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* 1999; **11**: 141-151
18 **Nishimura H**, Okazaki T, Tanaka Y, Nakatani K, Hara M, Matsumori A, Sasayama S, Mizoguchi A, Hiai H, Minato N, Honjo T. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science* 2001; **291**: 319-322
19 **Barber DL**, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, Freeman GJ, Ahmed R. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 2006; **439**: 682-687
20 **Agata Y**, Kawasaki A, Nishimura H, Ishida Y, Tsubata T, Yagita H, Honjo T. Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. *Int Immunol* 1996; **8**: 765-772
21 **Zhang X**, Schwartz JC, Guo X, Bhatia S, Cao E, Lorenz M, Cammer M, Chen L, Zhang ZY, Edidin MA, Nathenson SG, Almo SC. Structural and functional analysis of the costimulatory receptor programmed death-1. *Immunity* 2004; **20**: 337-347
22 **Grakoui A**, John Wherry E, Hanson HL, Walker C, Ahmed R. Turning on the off switch: regulation of anti-viral T cell responses in the liver by the PD-1/PD-L1 pathway. *J Hepatol* 2006; **45**: 468-472
23 **Okazaki T**, Honjo T. The PD-1-PD-L pathway in immunological tolerance. *Trends Immunol* 2006; **27**: 195-201
24 **Okazaki T**, Maeda A, Nishimura H, Kurosaki T, Honjo T. PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine. *Proc Natl Acad Sci USA* 2001; **98**: 13866-13871
25 **Chemnitz JM**, Parry RV, Nichols KE, June CH, Riley JL. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. *J Immunol* 2004; **173**: 945-954
26 **Ishida M**, Iwai Y, Tanaka Y, Okazaki T, Freeman GJ, Minato

N, Honjo T. Differential expression of PD-L1 and PD-L2, ligands for an inhibitory receptor PD-1, in the cells of lymphohematopoietic tissues. *Immunol Lett* 2002; **84**: 57-62
27 **Yamazaki T**, Akiba H, Iwai H, Matsuda H, Aoki M, Tanno Y, Shin T, Tsuchiya H, Pardoll DM, Okumura K, Azuma M, Yagita H. Expression of programmed death 1 ligands by murine T cells and APC. *J Immunol* 2002; **169**: 5538-5545
28 **Liang SC**, Latchman YE, Buhlmann JE, Tomczak MF, Horwitz BH, Freeman GJ, Sharpe AH. Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses. *Eur J Immunol* 2003; **33**: 2706-2716
29 **Wiendl H**, Mitsdoerffer M, Schneider D, Chen L, Lochmüller H, Melms A, Weller M. Human muscle cells express a B7-related molecule, B7-H1, with strong negative immune regulatory potential: a novel mechanism of counterbalancing the immune attack in idiopathic inflammatory myopathies. *FASEB J* 2003; **17**: 1892-1894
30 **Salama AD**, Chitnis T, Imitola J, Ansari MJ, Akiba H, Tushima F, Azuma M, Yagita H, Sayegh MH, Khoury SJ. Critical role of the programmed death-1 (PD-1) pathway in regulation of experimental autoimmune encephalomyelitis. *J Exp Med* 2003; **198**: 71-78
31 **Mühlbauer M**, Fleck M, Schütz C, Weiss T, Froh M, Blank C, Schölmerich J, Hellerbrand C. PD-L1 is induced in hepatocytes by viral infection and by interferon-alpha and -gamma and mediates T cell apoptosis. *J Hepatol* 2006; **45**: 520-528
32 **Maier H**, Isogawa M, Freeman GJ, Chisari FV. PD-1:PD-L1 interactions contribute to the functional suppression of virus-specific CD8+ T lymphocytes in the liver. *J Immunol* 2007; **178**: 2714-2720
33 **Meier A**, Bagchi A, Sidhu HK, Alter G, Suscovich TJ, Kavanagh DG, Streeck H, Brockman MA, LeGall S, Hellman J, Altfeld M. Upregulation of PD-L1 on monocytes and dendritic cells by HIV-1 derived TLR ligands. *AIDS* 2008; **22**: 655-658
34 **Willberg C**, Barnes E, Klenerman P. HCV immunology--death and the maiden T cell. *Cell Death Differ* 2003; **10** Suppl 1: S39-S47
35 **Bartenschlager R**, Lohmann V. Replication of hepatitis C virus. *J Gen Virol* 2000; **81**: 1631-1648
36 **Knolle PA**, Gerken G. Local control of the immune response in the liver. *Immunol Rev* 2000; **174**: 21-34
37 **Knolle PA**, Limmer A. Neighborhood politics: the immunoregulatory function of organ-resident liver endothelial cells. *Trends Immunol* 2001; **22**: 432-437
38 **Crispe IN**. Hepatic T cells and liver tolerance. *Nat Rev Immunol* 2003; **3**: 51-62
39 **Griffith TS**, Brunner T, Fletcher SM, Green DR, Ferguson TA. Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science* 1995; **270**: 1189-1192
40 **Isogawa M**, Furuichi Y, Chisari FV. Oscillating CD8(+) T cell effector functions after antigen recognition in the liver. *Immunity* 2005; **23**: 53-63
41 **Nakamoto N**, Kaplan DE, Coleclough J, Li Y, Valiga ME, Kaminski M, Shaked A, Olthoff K, Gostick E, Price DA, Freeman GJ, Wherry EJ, Chang KM. Functional restoration of HCV-specific CD8 T cells by PD-1 blockade is defined by PD-1 expression and compartmentalization. *Gastroenterology* 2008; **134**: 1927-1937, 1937.e1-e2
42 **Iwai Y**, Terawaki S, Ikegawa M, Okazaki T, Honjo T. PD-1 inhibits antiviral immunity at the effector phase in the liver. *J Exp Med* 2003; **198**: 39-50
43 **Freeman GJ**, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, Fitz LJ, Malenkovich N, Okazaki T, Byrne MC, Horton HF, Fouser L, Carter L, Ling V, Bowman MR, Carreno BM, Collins M, Wood CR, Honjo T. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* 2000; **192**: 1027-1034
44 **Dong H**, Zhu G, Tamada K, Chen L. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat Med* 1999; **5**: 1365-1369
45 **Nowak MA**, Bangham CR. Population dynamics of immune responses to persistent viruses. *Science* 1996; **272**: 74-79
46 **Boettler T**, Spangenberg HC, Neumann-Haefelin C, Panther E, Urbani S, Ferrari C, Blum HE, von Weizsäcker F, Thimme R. T cells with a CD4+CD25+ regulatory phenotype suppress in vitro proliferation of virus-specific CD8+ T cells during chronic hepatitis C virus infection. *J Virol* 2005; **79**: 7860-7867
47 **Cabrera R**, Tu Z, Xu Y, Firpi RJ, Rosen HR, Liu C, Nelson DR. An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology* 2004; **40**: 1062-1071
48 **Rushbrook SM**, Ward SM, Unitt E, Vowler SL, Lucas M, Klenerman P, Alexander GJ. Regulatory T cells suppress in vitro proliferation of virus-specific CD8+ T cells during persistent hepatitis C virus infection. *J Virol* 2005; **79**: 7852-7859
49 **Gavin MA**, Clarke SR, Negrou E, Gallegos A, Rudensky A. Homeostasis and anergy of CD4(+)CD25(+) suppressor T cells in vivo. *Nat Immunol* 2002; **3**: 33-41
50 **Raimondi G**, Shufesky WJ, Tokita D, Morelli AE, Thomson AW. Regulated compartmentalization of programmed cell death-1 discriminates CD4+CD25+ resting regulatory T cells from activated T cells. *J Immunol* 2006; **176**: 2808-2816
51 **Polanczyk MJ**, Hopke C, Vandenbark AA, Offner H. Treg suppressive activity involves estrogen-dependent expression of programmed death-1 (PD-1). *Int Immunol* 2007; **19**: 337-343
52 **Franceschini D**, Paroli M, Francavilla V, Videtta M, Morrone S, Labbadia G, Cerino A, Mondelli MU, Barnaba V. PD-L1 negatively regulates CD4+CD25+Foxp3+ Tregs by limiting STAT-5 phosphorylation in patients chronically infected with HCV. *J Clin Invest* 2009; **119**: 551-564
53 **Kasprowicz V**, Schulze Zur Wiesch J, Kuntzen T, Nolan BE, Longworth S, Berical A, Blum J, McMahon C, Reyor LL, Elias N, Kwok WW, McGovern BG, Freeman G, Chung RT, Klenerman P, Lewis-Ximenez L, Walker BD, Allen TM, Kim AY, Lauer GM. High level of PD-1 expression on hepatitis C virus (HCV)-specific CD8+ and CD4+ T cells during acute HCV infection, irrespective of clinical outcome. *J Virol* 2008; **82**: 3154-3160
54 **Golden-Mason L**, Palmer B, Klarquist J, Mengshol JA, Castelblanco N, Rosen HR. Upregulation of PD-1 expression on circulating and intrahepatic hepatitis C virus-specific CD8+ T cells associated with reversible immune dysfunction. *J Virol* 2007; **81**: 9249-9258
55 **Radziewicz H**, Ibegbu CC, Fernandez ML, Workowski KA, Obideen K, Wehbi M, Hanson HL, Steinberg JP, Masopust D, Wherry EJ, Altman JD, Rouse BT, Freeman GJ, Ahmed R, Grakoui A. Liver-infiltrating lymphocytes in chronic human hepatitis C virus infection display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression. *J Virol* 2007; **81**: 2545-2553
56 **Golden-Mason L**, Klarquist J, Wahed AS, Rosen HR. Cutting edge: programmed death-1 expression is increased on immunocytes in chronic hepatitis C virus and predicts failure of response to antiviral therapy: race-dependent differences. *J Immunol* 2008; **180**: 3637-3641
57 **Zhang Z**, Jin B, Zhang JY, Xu B, Wang H, Shi M, Wherry EJ, Lau GK, Wang FS. Dynamic decrease in PD-1 expression correlates with HBV-specific memory CD8 T-cell development in acute self-limited hepatitis B patients. *J Hepatol* 2009; **50**: 1163-1173
58 **Ye P**, Weng ZH, Zhang SL, Zhang JA, Zhao L, Dong JH, Jie SH, Pang R, Wei RH. Programmed death-1 expression is associated with the disease status in hepatitis B virus infection. *World J Gastroenterol* 2008; **14**: 4551-4557
59 **Peng G**, Li S, Wu W, Tan X, Chen Y, Chen Z. PD-1 upregulation is associated with HBV-specific T cell dysfunction in chronic hepatitis B patients. *Mol Immunol* 2008; **45**: 963-970
60 **Trautmann L**, Janbazian L, Chomont N, Said EA, Gimmig S,

Bessette B, Boulassel MR, Delwart E, Sepulveda H, Balderas RS, Routy JP, Haddad EK, Sekaly RP. Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. *Nat Med* 2006; **12**: 1198-1202

61 **Day CL**, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, Mackey EW, Miller JD, Leslie AJ, DePierres C, Mncube Z, Duraiswamy J, Zhu B, Eichbaum Q, Altfeld M, Wherry EJ, Coovadia HM, Goulder PJ, Klenerman P, Ahmed R, Freeman GJ, Walker BD. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 2006; **443**: 350-354

62 **Li YG**, Chen LE, Chen GF, Wang FS. [Expressions and significance of B7-H1 and programmed death-1 in lymphocytes from patients with chronic hepatitis B virus infection] *Zhonghua Ganzangbing Zazhi* 2007; **15**: 738-741

63 **Penna A**, Pilli M, Zerbini A, Orlandini A, Mezzadri S, Sacchelli L, Missale G, Ferrari C. Dysfunction and functional restoration of HCV-specific CD8 responses in chronic hepatitis C virus infection. *Hepatology* 2007; **45**: 588-601

64 **Boni C**, Fisicaro P, Valdatta C, Amadei B, Di Vincenzo P, Giuberti T, Laccabue D, Zerbini A, Cavalli A, Missale G, Bertoletti A, Ferrari C. Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection. *J Virol* 2007; **81**: 4215-4225

65 **Rutebemberwa A**, Ray SC, Astemborski J, Levine J, Liu L, Dowd KA, Clute S, Wang C, Korman A, Sette A, Sidney J, Pardoll DM, Cox AL. High-programmed death-1 levels on hepatitis C virus-specific T cells during acute infection are associated with viral persistence and require preservation of cognate antigen during chronic infection. *J Immunol* 2008; **181**: 8215-8225

66 **Urbani S**, Amadei B, Tola D, Massari M, Schivazappa S, Missale G, Ferrari C. PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion. *J Virol* 2006; **80**: 11398-11403

67 **Boettler T**, Panther E, Bengsch B, Nazarova N, Spangenberg HC, Blum HE, Thimme R. Expression of the interleukin-7 receptor alpha chain (CD127) on virus-specific CD8+ T cells identifies functionally and phenotypically defined memory T cells during acute resolving hepatitis B virus infection. *J Virol* 2006; **80**: 3532-3540

68 **Bengsch B**, Spangenberg HC, Kersting N, Neumann-Haefelin C, Panther E, von Weizsäcker F, Blum HE, Pircher H, Thimme R. Analysis of CD127 and KLRG1 expression on hepatitis C virus-specific CD8+ T cells reveals the existence of different memory T-cell subsets in the peripheral blood and liver. *J Virol* 2007; **81**: 945-953

69 **Radziewicz H**, Ibegbu CC, Hon H, Osborn MK, Obideen K, Wehbi M, Freeman GJ, Lennox JL, Workowski KA, Hanson HL, Grakoui A. Impaired hepatitis C virus (HCV)-specific effector CD8+ T cells undergo massive apoptosis in the peripheral blood during acute HCV infection and in the liver during the chronic phase of infection. *J Virol* 2008; **82**: 9808-9822

70 **Wedemeyer H**, He XS, Nascimbeni M, Davis AR, Greenberg HB, Hoofnagle JH, Liang TJ, Alter H, Rehermann B. Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. *J Immunol* 2002; **169**: 3447-3458

71 **Folgori A**, Spada E, Pezzanera M, Ruggeri L, Mele A, Garbuglia AR, Perrone MP, Del Porto P, Piccolella E, Cortese R, Nicosia A, Vitelli A. Early impairment of hepatitis C virus specific T cell proliferation during acute infection leads to failure of viral clearance. *Gut* 2006; **55**: 1012-1019

72 **Keir ME**, Francisco LM, Sharpe AH. PD-1 and its ligands in T-cell immunity. *Curr Opin Immunol* 2007; **19**: 309-314

73 **Yao S**, Chen L. Reviving exhausted T lymphocytes during chronic virus infection by B7-H1 blockade. *Trends Mol Med* 2006; **12**: 244-246

74 **Jeong HY**, Lee YJ, Seo SK, Lee SW, Park SJ, Lee JN, Sohn HS, Yao S, Chen L, Choi I. Blocking of monocyte-associated B7-H1 (CD274) enhances HCV-specific T cell immunity in chronic hepatitis C infection. *J Leukoc Biol* 2008; **83**: 755-764

75 **Nakamoto N**, Cho H, Shaked A, Olthoff K, Valiga ME, Kaminski M, Gostick E, Price DA, Freeman GJ, Wherry EJ, Chang KM. Synergistic reversal of intrahepatic HCV-specific CD8 T cell exhaustion by combined PD-1/CTLA-4 blockade. *PLoS Pathog* 2009; **5**: e1000313

76 **Arribillaga L**, Sarobe P, Arina A, Gorraiz M, Borrás-Cuesta F, Ruiz J, Prieto J, Chen L, Melero I, Lasarte JJ. Enhancement of CD4 and CD8 immunity by anti-CD137 (4-1BB) monoclonal antibodies during hepatitis C vaccination with recombinant adenovirus. *Vaccine* 2005; **23**: 3493-3499

77 **Zabaleta A**, Arribillaga L, Llopiz D, Dotor J, Lasarte JJ, Prieto J, Borrás-Cuesta F, Esteban JI, Quer J, Vayreda F, Sarobe P. Induction of potent and long-lasting CD4 and CD8 T-cell responses against hepatitis C virus by immunization with viral antigens plus poly(I:C) and anti-CD40. *Antiviral Res* 2007; **74**: 25-35

78 **Nanji SA**, Hancock WW, Anderson CC, Adams AB, Luo B, Schur CD, Pawlick RL, Wang L, Coyle AJ, Larsen CP, Shapiro AM. Multiple combination therapies involving blockade of ICOS/B7RP-1 costimulation facilitate long-term islet allograft survival. *Am J Transplant* 2004; **4**: 526-536

79 **Blackburn SD**, Shin H, Haining WN, Zou T, Workman CJ, Polley A, Betts MR, Freeman GJ, Vignali DA, Wherry EJ. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol* 2009; **10**: 29-37

80 **Golden-Mason L**, Palmer BE, Kassam N, Townshend-Bulson L, Livingston S, McMahon BJ, Castelblanco N, Kuchroo V, Gretch DR, Rosen HR. Negative immune regulator Tim-3 is overexpressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4+ and CD8+ T cells. *J Virol* 2009; **83**: 9122-9130

81 **Lopes AR**, Kellam P, Das A, Dunn C, Kwan A, Turner J, Peppa D, Gilson RJ, Gehring A, Bertoletti A, Maini MK. Bim-mediated deletion of antigen-specific CD8 T cells in patients unable to control HBV infection. *J Clin Invest* 2008; **118**: 1835-1845

82 **Kittlesen DJ**, Chianese-Bullock KA, Yao ZQ, Braciale TJ, Hahn YS. Interaction between complement receptor gC1qR and hepatitis C virus core protein inhibits T-lymphocyte proliferation. *J Clin Invest* 2000; **106**: 1239-1249

83 **Sundström S**, Ota S, Dimberg LY, Masucci MG, Bergqvist A. Hepatitis C virus core protein induces an anergic state characterized by decreased interleukin-2 production and perturbation of mitogen-activated protein kinase responses. *J Virol* 2005; **79**: 2230-2239

84 **Yao ZQ**, King E, Prayther D, Yin D, Moorman J. T cell dysfunction by hepatitis C virus core protein involves PD-1/PDL-1 signaling. *Viral Immunol* 2007; **20**: 276-287

85 **Lukens JR**, Cruise MW, Lassen MG, Hahn YS. Blockade of PD-1/B7-H1 interaction restores effector CD8+ T cell responses in a hepatitis C virus core murine model. *J Immunol* 2008; **180**: 4875-4884

86 **Maillard P**, Krawczynski K, Nitkiewicz J, Bronnert C, Sidorkiewicz M, Gounon P, Dubuisson J, Faure G, Crainic R, Budkowska A. Nonenveloped nucleocapsids of hepatitis C virus in the serum of infected patients. *J Virol* 2001; **75**: 8240-8250

87 **Masalova OV**, Atanadze SN, Samokhvalov EI, Petrakova NV, Kalinina TI, Smirnov VD, Khudyakov YE, Fields HA, Kushch AA. Detection of hepatitis C virus core protein circulating within different virus particle populations. *J Med Virol* 1998; **55**: 1-6

88 **Xu B**, Zhang Z, Shi Y, Chen XY, Wang FS. [PD-1 up-regulation influenced apoptosis of HBV-specific CD8 T cells in patients with acute resolved hepatitis B] *Zhonghua Yixue Zazhi* 2009; **89**: 1158-1161

89 **Boasso A**, Hardy AW, Landay AL, Martinson JL, Anderson SA, Dolan MJ, Clerici M, Shearer GM. PDL-1 upregulation on monocytes and T cells by HIV via type I interferon: restricted expression of type I interferon receptor by CCR5-expressing leukocytes. *Clin Immunol* 2008; **129**: 132-144

90 **Iglesias-Chiesa MC**, Crabtree-Ramírez B, Reyes-Terán G. [A new therapeutic strategy to control HIV? The PD1 molecule and its role in inhibiting cellular immune responses] *Gac Med Mex* 2008; **144**: 75-78

91 **Trautmann L**, Chomont N, Sékaly RP. [Inhibition of the PD-1 pathway restores the effector function of HIV-specific T cells] *Med Sci* (Paris) 2007; **23**: 24-25

92 **Urbani S**, Amadei B, Tola D, Pedrazzi G, Sacchelli L, Cavallo MC, Orlandini A, Missale G, Ferrari C. Restoration of HCV-specific T cell functions by PD-1/PD-L1 blockade in HCV infection: effect of viremia levels and antiviral treatment. *J Hepatol* 2008; **48**: 548-558

93 **von Herrath MG**, Berger DP, Homann D, Tishon T, Sette A, Oldstone MB. Vaccination to treat persistent viral infection. *Virology* 2000; **268**: 411-419

94 **Dikici B**, Kalayci AG, Ozgenc F, Bosnak M, Davutoglu M, Ece A, Ozkan T, Ozeke T, Yagci RV, Haspolat K. Therapeutic vaccination in the immunotolerant phase of children with chronic hepatitis B infection. *Pediatr Infect Dis J* 2003; **22**: 345-349

95 **Ha SJ**, Mueller SN, Wherry EJ, Barber DL, Aubert RD, Sharpe AH, Freeman GJ, Ahmed R. Enhancing therapeutic vaccination by blocking PD-1-mediated inhibitory signals during chronic infection. *J Exp Med* 2008; **205**: 543-555

96 **Velu V**, Titanji K, Zhu B, Husain S, Pladevega A, Lai L, Vanderford TH, Chennareddi L, Silvestri G, Freeman GJ, Ahmed R, Amara RR. Enhancing SIV-specific immunity in vivo by PD-1 blockade. *Nature* 2009; **458**: 206-210

97 **Finnefrock AC**, Tang A, Li F, Freed DC, Feng M, Cox KS, Sykes KJ, Guare JP, Miller MD, Olsen DB, Hazuda DJ, Shiver JW, Casimiro DR, Fu TM. PD-1 blockade in rhesus macaques: impact on chronic infection and prophylactic vaccination. *J Immunol* 2009; **182**: 980-987

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REVIEW

Metabolic syndrome and risk of subsequent colorectal cancer

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Abstract

The metabolic syndrome and visceral obesity have an increasing prevalence and incidence in the general population. The actual prevalence of the metabolic syndrome is 24% in US population and between 24.6% and 30.9% in Europe. As demonstrated by many clinical trials (NAHANES Ⅲ, INTERHART) the metabolic syndrome is associated with an increased risk of both diabetes and cardiovascular disease. In addition to cardiovascular disease, individual components of the metabolic syndrome have been linked to the development of cancer, particularly to colorectal cancer. Colorectal cancer is an important public health problem; in the year 2000 there was an estimated total of 944 717 incident cases of colorectal cancer diagnosed world-wide. This association is sustained by many epidemiological studies. Recent reports suggest that individuals with metabolic syndrome have a higher risk of colon or rectal cancer. Moreover, the clusters of metabolic syndrome components increase the risk of associated cancer. The physiopathological mechanism that links metabolic syndrome and colorectal cancer is mostly related to abdominal obesity and insulin resistance. Population and experimental studies demonstrated that hyperinsulinemia, elevated C-peptide, elevated body mass index, high levels of insulin growth factor-1, low levels of insulin growth factor binding protein-3, high leptin levels and low adiponectin levels are all involved in carcinogenesis. Understanding the pathological mechanism that links metabolic syndrome and its components to carcinogenesis has a major clinical significance and may have profound health benefits on a number of diseases including cancer, which represents a major cause of mortality and morbidity in our societies.

Key words: Metabolic syndrome; Colorectal cancer; Insulin resistance; Obesity; Adipokines; Inflammatory cytokines

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INTRODUCTION

The concept of metabolic syndrome has existed for at least 80 years and was first described by Kylin[1], a Swedish physician, as a clustering of hypertension, hyperglycemia and gout, and later on by Vague[2] who added to the previous description the presence of abdominal obesity.

While the concept of metabolic syndrome has been accepted for a long time, there was no largely recognized international definition until 1998.

The first proposal came in 1998 from a consultation group for the definition of diabetes for World Health Organisation. This definition was then modified by the European Group for Study of Insulin Resistance in 1999, the National Cholesterol Education Program Adult Treatment Panel Ⅲ (ATP Ⅲ) in 2001 and revised in 2005, and the International Diabetes Foundation (IDF) in 2005. These definitions agree on the different components of metabolic syndrome but differ in details (Table 1)[3].

More recently, the use of the term of "metabolic syndrome" has been questioned by the American

Diabetes Association and the European Association for the Study of Diabetes for several reasons: (1) Criteria are ambiguous or incomplete (for example it is unclear if the blood pressure definition is systolic blood pressure ⩾ 130 mmHg and diastolic ⩾ 85 mmHg or whether it is either ⩾ 130 mmHg or > 85 mmHg); (2) The value of including diabetes in the definition is questionable and the role of insulin resistance as unifying etiology is uncertain. Furthermore it is still unclear the extent to which an elevated cardiovascular (CVD) risk is due to insulin resistance itself *vs* isolated hyperinsulinemia; (3) There is no clear basis for including/excluding other CVD risk factors; the CVD risk associated with the syndrome appears to be no greater than the sum of its parts; (4) The treatment of the syndrome is not different from the treatment for each of its components.

A recent review of the ATP Ⅲ definition broadened the etiological basis of the syndrome from insulin resistance alone to include "obesity and disorders of adipose tissue"[4].

The actual prevalence of obesity is 30.5% and that of associated metabolic syndrome is 24% in the US population[3,5].

In Europe, the age- and sex-adjusted prevalence of metabolic syndrome was 24.6% using the 2005 ATP Ⅲ definition and 30.9% using the International Diabetes Federation definition, according to the MADRIC study (MADrid Rlsego Cardiovascular Study) performed on 1344 participants[6]. In this study, the authors found a good overall agreement between the ATP Ⅲ and IDF definitions, much closer in women than in men ($\kappa = 0.92 \pm 0.07$ *vs* $\kappa = 0.66 \pm 0.06$). The prevalence of metabolic syndrome was greater according to the IDF definition than according to ATP Ⅲ, because the former definition has a lower threshold of abdominal obesity.

A cross-sectional analysis of 10206 participants aged 20-89 years in the Nord-Trøndelag Health Study 1995-97 (HUNT 2) in Norway, found a prevalence of IDF-defined metabolic syndrome of 29.6%, compared to 25.9% using the 2005 ATP Ⅲ criteria[7].

In a meta-analysis, Cameron *et al*[8] found a variable prevalence of metabolic syndrome in urban populations from 8% (India) to 24% (USA) in men, and from 7% (France) to 43% (Iran) in women.

It is well known that the metabolic syndrome is associated with an increased risk of both diabetes and cardiovascular disease.

Many clinical studies outlined the interrelation between the metabolic syndrome and cardiovascular risk[9].

Applying the ATP Ⅲ criteria to 10537 NHANES Ⅲ participants resulted in a significant association between the metabolic syndrome with prevalent myocardial infarction and stroke in a multivariate analysis: myocardial infarction [OR: 2.01, 95% confidence intervals (CI): 1.53-2.64], stroke (OR: 2.16, 95% CI: 1.48-3.16), and myocardial infarction/stroke (OR: 2.05, 95% CI: 1.64-2.57)[10].

The INTERHART study performed on 15152 cases and 14820 controls in nearly 52 countries found a significant association between abnormal lipids, smoking, hypertension, diabetes, abdominal obesity, psychosocial factors, consumption of fruits, vegetables, and alcohol, and regular physical activity and the risk of myocardial infarction. Collectively, these nine risk factors accounted for 90% of the population risks in men and 94% in women[11].

In addition to CVD, individual components of the metabolic syndrome have been linked to the development of cancer[12].

Colorectal cancer is an important health problem since one million new cases are diagnosed world-wide each year with half million related deaths[13]. The incidence rate of colon cancer according to Five Continents cancer registries varies from 3% in Africa (Algeria) up to 40% in North America. In Europe the incidence of colon cancer ranges from 12.1% in Belarus up to 30.5% in Italy[14].

There is evidence that body composition and hormonal factors contribute to colorectal cancer etiology. In this paper we will highlight this association supported by epidemiological data and pathophysiological mechanisms arising from prospective human research studies.

EPIDEMIOLOGY

In an analysis of nearly 58000 individuals who participated in the National Health Interview Survey (2002-2003), Garow *et al*[15] identified 1200 individuals with metabolic syndrome, 350 of them being diagnosed with colorectal cancer. After controlling for age, race, gender, obesity, smoking and alcohol use the individuals with metabolic syndrome had a 75% increased risk for colon or rectal cancer.

In a large prospective study of more than 900000 US adults (404576 men and 495477 women) conducted by Calle *et al*[16], there were 57145 deaths from cancer during a follow up period of 16 years. The authors also studied the relationship between the relative risk (RR) of death and body mass index (BMI). For all cancer there was a trend in increasing death rate with BMI. For colorectal cancer the RR of death varied from 1.34 (95% CI: 0.94-1.34) for a BMI of 25-29.9, to 1.90 (95% CI: 1.46-2.47) and 4.52 (95% CI: 2.94-6.94) for an BMI between 30.0-34.9 and 35.0-39.9, respectively[16].

Recent studies also provide information concerning the association between colorectal cancer incidence and the number of metabolic syndrome components, especially BMI, waist circumference (WC), lipid levels, plasma glucose and glycosylated hemoglobin (HbA1c). In an analysis of 14109 participants from the ARIC study (Atherosclerosis Risk in Communities), 194 incident colorectal cancers were identified. In this study baseline metabolic syndrome (> 3 components *vs* 0 components) had a positive association with age-adjusted and gender-adjusted colorectal cancer incidence (RR: 1.49, 95% CI: 1.0-2.4). There was a dose-response association between colorectal cancer incidence and the number of metabolic syndrome components present at baseline (*P* for trend = 0.006) after multivariate adjustment[17].

In another study, Trevisan *et al*[18], used information

Table 1 Comparison of definitions of metabolic syndrome

WHO 1999	ATP Ⅲ 2001	IDF 2005
Diabetes or impaired fasting glycemia or impaired glucose tolerance or insulin resistance Plus 2 or more of the following: Obesity: BMI > 30 or waist-to-hip ratio > 0.9 (male) or 0.85 (female) Dyslipidemia: triglycerides > 150 mg/dL or HDL cholesterol < 35 mg/dL (male) or < 39 mg/dL (female) Hypertension: blood pressure > 140/90 mmHg Microalbuminuria: albumin excretion > 20 μg/min	Three or more of the following: Central obesity: waist circumference > 102 cm (male), > 88 cm (female) Hypertriglyceridemia: triglycerides > 150 mg/dL Low HDL cholesterol: < 40 mg/dL (male), < 50 mg/dL (female) Hypertension: blood pressure > 130/85 mmHg Fasting plasma glucose > 100 mg/dL	Increased waist circumference > 94 cm cm in men and > 80 cm in women plus any 2 of the following: Hypertriglyceridemia: triglycerides > 150 mg/dL Low HDL cholesterol: < 40 mg/dL (male), < 50 mg/dL (female) Hypertension: blood pressure > 130/85 mmHg Fasting plasma glucose > 100 mg/dL

from the Risk Factors and Life Expectancy study, which pooled data from nine epidemiological studies conducted in Italy between 1978 and 1987, including 21 311 men and 15 991 women. In this study, low high density lipoprotein (HDL) and high triglyceride levels, hypertension and plasma glucose levels were also analyzed as individual components of the metabolic syndrome. For the presence of the cluster of metabolic abnormalities, the calculated hazard ratios and 95% CIs were 2.99 (1.27-7.01) when both sexes were combined. When analyzing the individual components, only glucose level was associated with an increased risk of death from colorectal cancer, and only in men and women combined (RR: 1.8, 95% CI: 1.05-3.09). The results of this study suggest that the effects of the individual components of metabolic syndrome are additive, because the RR of death from colorectal cancer was increased in cluster analysis compared with glucose alone.

The association between plasma glucose levels reflected by HbA1c and the incidence of colorectal cancer was outlined in a prospective analysis from the European Prospective Investigation into Cancer and Nutrition (EPIC) study[19]. Among 9605 participants in this study, aged between 45 and 79 years, there were 67 incident colorectal cancers. In this study population, the RR of colorectal cancer for men and women combined was 2.94 (95% CI: 0.80-10.85), age and sex adjusted for an HbA1c ⩾ 7%, compared with RR, 1.13 (95% CI: 0.56-2.30), for HbA1c of 5.0%-5.9%. For the same HbA1c levels of > 7%, the age adjusted RR was higher in men than in women [RR: 4.94 (95% CI: 0.89-27.35) in men, and 1.58 (95% CI: 0.19-13.14) in women]. The association of higher HbA1c levels and increased colorectal cancer risk was also present in the CLUE Ⅱ cohort[20].

Conversely, to evaluate the association between metabolic syndrome and colorectal cancer, Stocks *et al*[21] evaluated the presence of metabolic syndrome components (C-peptide, HbA1c, leptin, adiponectin, BMI, hypertension and fasting glucose) in 306 individuals with known colorectal cancer. The presence of hypertension, obesity and hyperglycemia, correlated with a RR for three *vs* null factors of 2.57 (95% CI: 1.20-5.52, *P* trend = 0.00021).

The relationship between BMI and colon cancer was also studied in the recent EPIC study[22], which was based on 984 cases of colon cancer. A 55% increased risk of colon cancer was observed between the high and low quintiles of BMI in men, but no significant association was observed in women.

Some recent studies considered anthropometric measures of adipose distribution in addition to BMI in relation to the risk of colon cancer of adenoma. In most of these studies, the association between WC or waist-to-hip ratio and colon cancer risk was stronger than that between BMI and cancer risk. Moore *et al*[23], in a retrospective analysis of 7566 subjects from the Framingham cohort, found 306 cases of incident colorectal cancer. The authors demonstrated a two-fold increased risk of colorectal cancer for a WC of > 99 cm in women and 101 cm in men; the risk increased linearly with increasing WC[23]. One Japanese study[24] of 51 consecutive patients aged ⩾ 40 years, suggests that visceral adipose tissue rather than whole body adipose tissue correlates better with the risk of colorectal adenoma. Furthermore, in this study, low adiponectin level is a factor associated with the development of colorectal adenoma. It is known that adiponectin levels decrease in obesity, especially abdominal obesity in association with insulin resistance; thus, the results of this study offer an insight to understanding the relationship of colorectal carcinogenesis with abdominal obesity and insulin resistance which will be discussed later on this paper.

The fact that the metabolic syndrome is a risk factor for both CVD and colorectal cancer raised the question if there is any association between CVD and colorectal cancer. This correlation was found to be positive in several studies. In a pilot study of 63 patients with colorectal cancer, Hamoudi and Dumitrascu demonstrated a statistical association between CVD and colorectal cancer in men[25].

The relationship between individual components of metabolic syndrome and the risk of colorectal cancer was also separately analyzed by several studies. Colangelo *et al*[26] found a 35% increased risk of colorectal cancer associated with high blood pressure. The results were confirmed by another study[17]. Both studies also underlined that the clustering of metabolic syndrome components significantly increased the risk of associated colorectal cancer.

High circulating triacylglycerols were associated in a large prospective study with a non-significant two-

fold elevation in risk of colorectal cancer in men, but no clear association was observed in women[17]. In another prospective study, there was a 40% increased risk of colorectal cancer for men and women in the top quartile of triacylglycerol levels, although this association was not significant[27].

The association between C-peptide levels as a marker of hyperinsulinemia and colorectal cancer risk was also examined by several studies. In a case control study in the Physicians' Health Study, an increased concentration of plasma C-peptide was statistically significantly associated with an increased risk of colorectal cancer in men (RR for the highest *vs* lowest quintile of plasma C-peptide = 2.7, 95% CI: 1.2-6.2, P trend = 0.047), after adjusting for age, smoking status, fasting, BMI and alcohol consumption. The results of this study also suggest that elevated insulin production, as reflected by elevated concentrations of plasma C-peptide, may predict the risk of developing colorectal cancer, independently of BMI, factors related to insulin resistance, or levels of insulin growth factor (IGF)-1 and insulin growth factor binding protein (IGFBP)-3[28]. The interrelation between a high concentration of plasma C-peptide and colorectal adenoma was also demonstrated in women in a series of 380 patients with a multivariable relative risk (MVRR) top *vs* bottom quartile, 1.63, 95% CI: 1.01-2.66, $P = 0.01$, even after including BMI and physical activity in the statistical model[29].

The findings of all these studies suggest that the clusters of the metabolic syndrome components may be predictors for developing colorectal cancer and for colorectal cancer mortality. The understanding of the underlying physiopathology that links the metabolic syndrome and cancer may play a key role in developing new strategies for prevention and treatment.

PHYSIOPATHOLOGICAL LINKS BETWEEN METABOLIC SYNDROME AND COLORECTAL CANCER

Obesity, insulin resistance and insulin growth factors and binding proteins

It has been hypothesized that insulin resistance is the most important underlying mechanism of the metabolic syndrome in close relationship to abdominal obesity. Insulin has been shown to affect growth of normal and neoplastic epithelial cells and to have mitogenic actions *in vitro* and in experimental models, either directly or indirectly through IGF-1[17]. At high concentrations, insulin can bind to IGF-1 receptors (IGF1Rs) or can act directly to promote IGF-1 biosynthesis, enhancing IGF-1 bioavailability and inhibiting the production of IGFBP-1, IGFBP-2 and IGFBP-3[30].

IGF-1 is an important mitogen required for the progression through the cell cycle and has autocrine, paracrine and endocrine actions on cell proliferation and apoptosis[31], increasing the risk of cellular transformation by enhancing cell turnover. In addition, IGF-1 increases the production of vascular endothelial growth factor (VEGF), an angiogenic factor that can support cancer growth[32].

It has been shown that normal colorectal epithelia and colon cancer cells have both insulin and IGF1Rs[17]. Tissue homeostasis in the normal colonic crypt relies on a balance between proliferation, differentiation and apoptosis, with apoptosis occurring at the top of the colonic crypt as the culmination of a differentiation pathway.

The link between IGF-1 and IGFBP-3 levels and the increased risk of colorectal adenoma and cancer came first to attention in acromegalic patients, characterized by chronically elevated growth hormone (GH) levels. GH excess leads to hepatic and peripheral insulin resistance and thus to hyperinsulinemia, a common feature of acromegaly and metabolic syndrome, that causes IGF-1 hypersecretion and low IGFBP-3 levels[33].

The relationship between IGF-1 and IGFBP-3 levels and colorectal cancer was examined by Giovannucci *et al*[34] on 32 826 women from Nurses' Health Study. Controlling for IGFBP-3 level, relative to women in the lowest tertile of IGF-1, those in the highest tertile were at elevated risk of intermediate/late-stage colorectal neoplasia adenoma (MVRR: 2.78, 95% CI: 0.76-9.76) and cancer (RR: 2.18, 95% CI: 0.94-5.08). Controlling for IGF-1 level, relative to women in the lowest tertile of IGFBP-3, women in the highest tertile of IGFBP-3 were at lower risk of intermediate/late-stage colorectal adenoma (RR: 0.28, 95% CI: 0.09-0.85) and cancer (RR: 0.28, 95% CI: 0.10-0.83). Neither IGF-1 nor IGFBP-3 had any appreciable relation with early-stage adenoma. These analyses indicate that high levels of circulating IGF-1 and particularly low levels of IGFBP-3 are associated independently with an elevated risk of large or tubulo-villous/villous colorectal adenoma and cancer. These results are concordant with those obtained previously in Physicians' Health Study[35].

The role of IGFBP-3 in colorectal cancer was independently analyzed by Williams *et al*[36] IGFBP-3 has been shown to enhance p53-dependent apoptosis after DNA damage. Therefore, loss of IGFBP-3 could contribute to the development of colonic adenomas that retain wild-type p53 function through suppression of p53-dependent apoptotic signals, allowing aberrant cell survival and tumor formation. Furthermore there is disruption in both adenoma and carcinoma tissue. This pattern is similar to that of TGF-β distribution in normal, adenoma and carcinoma tissue[37]. Because it is known that TGF-β is a potent growth inhibitor for colonic epithelium[36], this similarity suggests that IGFBP-3 may have an important role in the regulation of differentiation and apoptosis in human colonic epithelium[37].

The role of insulin resistance and hyperinsulinemia in colorectal cancer was directly assessed by Schoen *et al*[27] in a study performed on 5849 participants in the Cardiovascular Health Study cohort. The authors identified 102 cases of colorectal cancer. Fasting insulin was not related to an increased risk (RR = 1.2), whereas 2 h insulin was related to a significantly increased risk (RR = 2.0).

Giovannucci *et al*[38] found that BMI was not significantly

associated with an increased risk of distal colon adenoma irrespective of size, while WC and waist hip ratio were strong risk factors for large distal colon adenomas with diameter ⩾ 1 cm but were unrelated to small adenomas with diameter < 1 cm. The association of WC with an increased risk of cancer has been reported to be slightly stronger for distal colon cancer.

There are also several studies which determined the relationship between C-peptide (an indicator of insulin production) and the risk of colorectal cancer. As mentioned before, in the Physicians' Health Study, men with C-peptide in the top *vs* the bottom quintile had a 2.7-fold significantly higher risk of colorectal cancer after control for BMI and exercise; this RR increased to 3.4 after the analysis was controlled for indicators of the metabolic syndrome[28]. In a prospective study of 14275 women in New York State, a 3-fold higher risk of colorectal cancer was observed in those in the top quartile of C-peptide, and a 4-fold higher risk was observed for colon cancer alone[39].

Adipokines and inflammatory cytokines

Adipose tissue is a complex endocrine organ, responsible for the secretion and synthesis of hormones, cytokines and other signaling proteins, collectively termed as adipokines. Adipokines are a diverse group of signaling molecules that play roles in such processes as appetite and energy balance, inflammation, insulin resistance/sensitivity, angiogenesis, lipid metabolism, cell proliferation and atherosclerosis. Many of these functions are related to either the metabolic syndrome or cancer, and they may serve as a link between these two pathologies[40].

Adiponectin

Adiponectin, a 30-kDa complement C1q-related protein, is a key regulator of insulin sensitivity and inflammation and modulates several physiologic processes, such as metabolism of glucose and fatty acids. In contrast to other adipokines such as leptin, adiponectin circulating levels are decreased in obese individuals and in those with diabetes[41]. Decreased plasma adiponectin concentrations are associated with insulin resistance, type 2 diabetes and atherosclerosis. In addition, it was recently shown that adiponectin may play a role in the development and progression of various types of malignancies. Accumulating evidence suggests that adiponectin is an important regulator of cell proliferation. Adiponectin may act either directly on cancer cells or indirectly by regulating whole-body insulin sensitivity[42].

Mechanisms that may link adiponectin with carcinogenesis

In obesity, reduced adiponectin levels lead to the development of insulin resistance and compensatory, chronic hyperinsulinaemia. Increased insulin levels results in increased levels of bioavailable IGF-1. Insulin and IGF-1 signal through the insulin receptors and IGF1R, promote cellular proliferation and inhibit apoptosis in many tissue types up-regulating the secretion of VEGF, contributing thus to carcinogenesis[39]. Adiponectin has also been shown to inhibit both the production of TNF-α in macrophages and its action in endothelial cells, thus promoting carcinogenesis through the altered effect of TNF-α on tumor cell proliferation and angiogenesis[43].

Adiponectin can also protect from carcinogenesis through more direct effects.

Specifically, adiponectin has been found to be an important negative regulator of hematopoiesis and the immune system. Moreover, adiponectin may inhibit activation of nuclear factor-κB (NF-κB), a transcription factor that upregulates VEGF[44].

Several signalling molecules such as 50-AMP-activated protein kinase (AMPK), NF-κB, peroxisome proliferators activated receptor (PPAR)-α and p38 mitogen-activated protein kinase are known to mediate adiponectin-induced metabolic effects. AMPK might inhibit the growth and/or survival of cancer cells[45]. Finally, adiponectin may also regulate angiogenesis negatively (independently of AMPK) through induction of apoptosis in vascular endothelial cells by activating the caspase cascade, a group of apoptotic enzymes[46].

The relationship between circulating adiponectin levels and colorectal cancer was demonstrated by several clinical and experimental studies.

Ferroni *et al*[47] demonstrated in a study involving 60 patients with non metastatic colorectal cancer that low adiponectin levels are inversely correlated with increases in tumor stage and were independent predictors of recurrent disease. Low adiponectin levels were found in 52% of relapsing patients, compared with 26% of non-relapsing patients[47].

Similar results were obtained by Wei *et al*[48] in a prospective case-control study of 18225 men enrolled in the Health Professional Follow-up Study. Over the approximately 8 years of follow-up, the authors noted 25 cases of colorectal cancer in the 3645 men in the highest category of adiponectin compared with 54 cases of colorectal cancer in the 3645 men in the lowest quintile of adiponectin.

Leptin

Leptin is a 16 kDa glycoprotein which is expressed almost exclusively (> 95%) by adipocytes. Initial interest in leptin focused on its role in obesity but recently leptin, has been associated with the inflammatory response, insulin signaling, and carcinogenesis.

Insulin and leptin interact at multiple levels within a complex network of adipose tissue signaling pathways, providing several mechanisms that could link leptin to colon cancer.

Of particular importance for cancer is the influence of leptin on suppressors of cytokine signaling 1 and 3 which in turn limits insulin signaling[49].

Although data directly linking leptin to colon cancer are limited, some studies have shown increased risk of colon and colorectal cancer with high serum leptin levels.

Data from a cohort study in Norway detected an almost 3-fold increased risk of colon cancer among people with high leptin levels, independently of BMI[50].

Another study found that men in the highest tertile of leptin concentrations had a 3.3-fold (95% CI: 1.2-8.7) increased adenoma risk compared with those in the lowest tertile[51]. The association between leptin concentration and colorectal cancer was also evaluated in women, in a case-control study conducted in Japan, suggesting that leptin increases substantially the risk of female colorectal cancer, independent of BMI[52].

Inflammatory cytokines and colorectal cancer

Accumulating evidence suggests that systemic inflammation might be a plausible mechanism for colon carcinogenesis. Studies have shown that genetic variations in inflammation-related genes, such as interleukin (IL)-6, IL-8, and IL-10, are associated with susceptibility to colorectal cancer and adenomas.

IL-6 appears to enhance tumorigenesis by a paracrine and autocrine mechanism, to stimulate cell growth and inhibit apoptosis. Also IL-6 concentrations reflected disease status and were commonly associated with metastatic disease[53].

TNF-α activates NF-κB (by phosphorylation of its inhibitor IκB), which increases production of NO, a substrate for reactive oxygen species (ROS) formation, and stimulates other inflammatory cytokines[54]. With respect to cancer, ROS can damage DNA by several processes including DNA base modification, deletions, frame shifts, strand breaks, DNA-protein cross-links, and chromosomal rearrangements. DNA damage can occur in genes that are important in cell proliferation (such as ras), or cell survival (such as p53), which can then trigger cancer progression[55].

There are several studies which demonstrated the correlation between high levels of IL-6, TNF-α, C-reactive proteins (CRP) and colorectal carcinogenesis. Moreover, a Greek study demonstrated that high levels of serum IL-6, TNF-α and CRP were correlated with larger tumor size. The relation to tumor size could be related to the fact, that larger tumors may trigger a more potent immunological response manifested by the circulation of proinflammatory cytokines such as TNF-α[56].

PPAR-γ

PPAR-γ, a ligand-activated transcription factor, is a key regulator of adipogenic differentiation and glucose homeostasis. PPAR-γ ligands have recently been demonstrated to affect proliferation and differentiation in cancer cell lines. A gradually increasing number of studies demonstrated the association between PPAR-γ and colorectal cancer[57].

A recent study demonstrated a positive PPAR-γ immunostaining in 48 of 86 cases of colon cancer (56%). No association was found for PPAR-γ positivity with different Dukes' stages, histological grade of differentiation, tumor location, presence of lymph node and liver metastasis, venous invasion, or tumor cell proliferating capacity assessed as Ki-67 overexpression. On the contrary, PPAR-γ expression was statistically significant correlated with the expression of cell cycle-related molecules[58].

Another recent study demonstrated that PPAR-γ agonists have inhibitory effects on the proliferation of colon cancer cell lines associated with G1 cell cycle arrest and invasive activity. The latter effect is demonstrated in certain cell lines through the down-regulation of metalloproteinase-7 synthesis[59].

CONCLUSION

The association between metabolic syndrome and colorectal cancer is now supported by a large number of epidemiological studies[14,16,17,19,26]. The components of metabolic syndrome appear to have an additive effect on colon cancer development acting through different pathophysiological pathways. This evidence is based on studies of determinants of the metabolic syndrome (obesity, abdominal distribution of adiposity, physical inactivity), clinical consequences (type 2 diabetes, hypertension) of this syndrome, plasma or serum components of the definition of metabolic syndrome (hypertriglyceridemia, hyperglycemia, low HDL cholesterol), markers of hyperinsulinemia or insulin resistance (insulin, C-peptide), and serum inflammatory cytokines levels in relation to colon cancer or adenoma risk. High insulin and insulin resistance are common features of industrialized societies characterized by a large prevalence of overweight individuals and obesity, a diet rich in energy intake, and a lifestyle characterized by low calorie expenditure. Understanding the pathological mechanism that links metabolic syndrome and its components to carcinogenesis has a very important clinical significance. Controlling even one or two of the components of the metabolic syndrome may result in a longer, healthier and cancer-free life. Public health efforts aimed at reducing lifestyle patterns and dietary habits associated with this imbalance on insulin metabolism may have profound health benefits on a number of diseases including cancer, that represent major causes of mortality and morbidity in our societies.

REFERENCES

1 **Zimmet P**, Magliano D, Matsuzawa Y, Alberti G, Shaw J. The metabolic syndrome: a global public health problem and a new definition. *J Atheroscler Thromb* 2005; **12**: 295-300
2 **Vague J**. La differenciation sexuelle, facteur determinant des formes de l'obesite. *Presse Med* 1947; **55**: 339
3 **Grundy SM**, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA, Gordon DJ, Krauss RM, Savage PJ, Smith SC Jr, Spertus JA, Costa F. Diagnosis and management of the metabolic syndrome: an American Heart Association/ National Heart, Lung, and Blood Institute Scientific Statement. *Circulation* 2005; **112**: 2735-2752
4 **Kahn R**, Buse J, Ferrannini E, Stern M. The metabolic syndrome: time for a critical appraisal: joint statement from the American Diabetes Association and the European Association for the Study of Diabetes. *Diabetes Care* 2005; **28**: 2289-2304
5 **Flegal KM**, Carroll MD, Ogden CL, Johnson CL. Prevalence and trends in obesity among US adults, 1999-2000. *JAMA* 2002; **288**: 1723-1727
6 **Martínez MA**, Puig JG, Mora M, Aragón R, O'Dogherty P, Antón JL, Sánchez-Villares T, Rubio JM, Rosado J, Torres

R, Marcos J, Pallardo LF, Banegas JR. Metabolic syndrome: prevalence, associated factors, and C-reactive protein: the MADRIC (MADrid RIesgo Cardiovascular) Study. *Metabolism* 2008; **57**: 1232-1240

7 **Hildrum B**, Mykletun A, Hole T, Midthjell K, Dahl AA. Age-specific prevalence of the metabolic syndrome defined by the International Diabetes Federation and the National Cholesterol Education Program: the Norwegian HUNT 2 study. *BMC Public Health* 2007; **7**: 220

8 **Cameron AJ**, Shaw JE, Zimmet PZ. The metabolic syndrome: prevalence in worldwide populations. *Endocrinol Metab Clin North Am* 2004; **33**: 351-375, table of contents

9 **Ninomiya JK**, L'Italien G, Criqui MH, Whyte JL, Gamst A, Chen RS. Association of the metabolic syndrome with history of myocardial infarction and stroke in the Third National Health and Nutrition Examination Survey. *Circulation* 2004; **109**: 42-46

10 **Eckel RH**, Grundy SM, Zimmet PZ. The metabolic syndrome. *Lancet* 2005; **365**: 1415-1428

11 **Yusuf S**, Hawken S, Ounpuu S, Dans T, Avezum A, Lanas F, McQueen M, Budaj A, Pais P, Varigos J, Lisheng L. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. *Lancet* 2004; **364**: 937-952

12 **Kreger BE**, Splansky GL, Schatzkin A. The cancer experience in the Framingham Heart Study cohort. *Cancer* 1991; **67**: 1-6

13 **Boyle P**, Leon ME. Epidemiology of colorectal cancer. *Br Med Bull* 2002; **64**: 1-25

14 **Curado MP**, Edwards B, Shin HR, Storm H, Ferlay J, Heanue M, Boyle P, editors. Cancer Incidence in Five Continents, Vol. IX. IARC Scientific Publications No. 160, Lyon, IARC, 2007. Available from: URL: http://www-dep.iarc.fr/CI5_IX_frame.htm

15 **Garow D**. Metabolic syndrome is a risk factor for colorectal cancer in the United States. American College of Gastroenterology 2008 Annual Scientific Meeting. October 6, 2008

16 **Calle EE**, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N Engl J Med* 2003; **348**: 1625-1638

17 **Ahmed RL**, Schmitz KH, Anderson KE, Rosamond WD, Folsom AR. The metabolic syndrome and risk of incident colorectal cancer. *Cancer* 2006; **107**: 28-36

18 **Trevisan M**, Liu J, Muti P, Misciagna G, Menotti A, Fucci F. Markers of insulin resistance and colorectal cancer mortality. *Cancer Epidemiol Biomarkers Prev* 2001; **10**: 937-941

19 **Khaw KT**, Wareham N, Bingham S, Luben R, Welch A, Day N. Preliminary communication: glycated hemoglobin, diabetes, and incident colorectal cancer in men and women: a prospective analysis from the European prospective investigation into cancer-Norfolk study. *Cancer Epidemiol Biomarkers Prev* 2004; **13**: 915-919

20 **Saydah SH**, Platz EA, Rifai N, Pollak MN, Brancati FL, Helzlsouer KJ. Association of markers of insulin and glucose control with subsequent colorectal cancer risk. *Cancer Epidemiol Biomarkers Prev* 2003; **12**: 412-418

21 **Stocks T**, Lukanova A, Johansson M, Rinaldi S, Palmqvist R, Hallmans G, Kaaks R, Stattin P. Components of the metabolic syndrome and colorectal cancer risk; a prospective study. *Int J Obes* (Lond) 2008; **32**: 304-314

22 **Pischon T**, Lahmann PH, Boeing H, Friedenreich C, Norat T, Tjønneland A, Halkjaer J, Overvad K, Clavel-Chapelon F, Boutron-Ruault MC, Guernec G, Bergmann MM, Linseisen J, Becker N, Trichopoulou A, Trichopoulos D, Sieri S, Palli D, Tumino R, Vineis P, Panico S, Peeters PH, Bueno-de-Mesquita HB, Boshuizen HC, Van Guelpen B, Palmqvist R, Berglund G, Gonzalez CA, Dorronsoro M, Barricarte A, Navarro C, Martinez C, Quirós JR, Roddam A, Allen N, Bingham S, Khaw KT, Ferrari P, Kaaks R, Slimani N, Riboli E. Body size and risk of colon and rectal cancer in the European Prospective Investigation Into Cancer and Nutrition (EPIC). *J Natl Cancer Inst* 2006; **98**: 920-931

23 **Moore LL**, Bradlee ML, Singer MR, Splansky GL, Proctor MH, Ellison RC, Kreger BE. BMI and waist circumference as predictors of lifetime colon cancer risk in Framingham Study adults. *Int J Obes Relat Metab Disord* 2004; **28**: 559-567

24 **Otake S**, Takeda H, Suzuki Y, Fukui T, Watanabe S, Ishihama K, Saito T, Togashi H, Nakamura T, Matsuzawa Y, Kawata S. Association of visceral fat accumulation and plasma adiponectin with colorectal adenoma: evidence for participation of insulin resistance. *Clin Cancer Res* 2005; **11**: 3642-3646

25 **Hamoudi WTY**, Dumitrascu DL. Is there an association between coronary heart disease and colorectal carcinoma? Results from a pilot study. *Rom J Gastroenterol* 1997; **6**: 13-16

26 **Colangelo LA**, Gapstur SM, Gann PH, Dyer AR, Liu K. Colorectal cancer mortality and factors related to the insulin resistance syndrome. *Cancer Epidemiol Biomarkers Prev* 2002; **11**: 385-391

27 **Schoen RE**, Tangen CM, Kuller LH, Burke GL, Cushman M, Tracy RP, Dobs A, Savage PJ. Increased blood glucose and insulin, body size, and incident colorectal cancer. *J Natl Cancer Inst* 1999; **91**: 1147-1154

28 **Ma J**, Giovannucci E, Pollak M, Leavitt A, Tao Y, Gaziano JM, Stampfer MJ. A prospective study of plasma C-peptide and colorectal cancer risk in men. *J Natl Cancer Inst* 2004; **96**: 546-553

29 **Wei EK**, Ma J, Pollak MN, Rifai N, Fuchs CS, Hankinson SE, Giovannucci E. C-peptide, insulin-like growth factor binding protein-1, glycosylated hemoglobin, and the risk of distal colorectal adenoma in women. *Cancer Epidemiol Biomarkers Prev* 2006; **15**: 750-755

30 **Sandhu MS**, Dunger DB, Giovannucci EL. Insulin, insulin-like growth factor-I (IGF-I), IGF binding proteins, their biologic interactions, and colorectal cancer. *J Natl Cancer Inst* 2002; **94**: 972-980

31 **Grimberg A**, Cohen P. Role of insulin-like growth factors and their binding proteins in growth control and carcinogenesis. *J Cell Physiol* 2000; **183**: 1-9

32 **Warren RS**, Yuan H, Matli MR, Ferrara N, Donner DB. Induction of vascular endothelial growth factor by insulin-like growth factor 1 in colorectal carcinoma. *J Biol Chem* 1996; **271**: 29483-29488

33 **Jenkins PJ**. Acromegaly and cancer. *Horm Res* 2004; **62** Suppl 1: 108-115

34 **Giovannucci E**, Pollak MN, Platz EA, Willett WC, Stampfer MJ, Majeed N, Colditz GA, Speizer FE, Hankinson SE. A prospective study of plasma insulin-like growth factor-1 and binding protein-3 and risk of colorectal neoplasia in women. *Cancer Epidemiol Biomarkers Prev* 2000; **9**: 345-349

35 **Ma J**, Pollak MN, Giovannucci E, Chan JM, Tao Y, Hennekens CH, Stampfer MJ. Prospective study of colorectal cancer risk in men and plasma levels of insulin-like growth factor (IGF)-I and IGF-binding protein-3. *J Natl Cancer Inst* 1999; **91**: 620-625

36 **Williams AC**, Collard TJ, Perks CM, Newcomb P, Moorghen M, Holly JM, Paraskeva C. Increased p53-dependent apoptosis by the insulin-like growth factor binding protein IGFBP-3 in human colonic adenoma-derived cells. *Cancer Res* 2000; **60**: 22-27

37 **Barnard JA**, Beauchamp RD, Coffey RJ, Moses HL. Regulation of intestinal epithelial cell growth by transforming growth factor type beta. *Proc Natl Acad Sci USA* 1989; **86**: 1578-1582

38 **Giovannucci E**, Ascherio A, Rimm EB, Colditz GA, Stampfer MJ, Willett WC. Physical activity, obesity, and risk for colon cancer and adenoma in men. *Ann Intern Med* 1995; **122**: 327-334

39 **Kaaks R**, Toniolo P, Akhmedkhanov A, Lukanova A, Biessy C, Dechaud H, Rinaldi S, Zeleniuch-Jacquotte A, Shore RE, Riboli E. Serum C-peptide, insulin-like growth factor (IGF)-I, IGF-binding proteins, and colorectal cancer risk in women. *J Natl Cancer Inst* 2000; **92**: 1592-1600

40 **Cowey S**, Hardy RW. The metabolic syndrome: A high-risk

state for cancer? *Am J Pathol* 2006; **169**: 1505-1522
41 **Vona-Davis L**, Howard-McNatt M, Rose DP. Adiposity, type 2 diabetes and the metabolic syndrome in breast cancer. *Obes Rev* 2007; **8**: 395-408
42 **Barb D**, Williams CJ, Neuwirth AK, Mantzoros CS. Adiponectin in relation to malignancies: a review of existing basic research and clinical evidence. *Am J Clin Nutr* 2007; **86**: s858-s866
43 **Rose DP**, Komninou D, Stephenson GD. Obesity, adipocytokines, and insulin resistance in breast cancer. *Obes Rev* 2004; **5**: 153-165
44 **Wang Y**, Lam KS, Xu JY, Lu G, Xu LY, Cooper GJ, Xu A. Adiponectin inhibits cell proliferation by interacting with several growth factors in an oligomerization-dependent manner. *J Biol Chem* 2005; **280**: 18341-18347
45 **Goldstein BJ**, Scalia R. Adiponectin: A novel adipokine linking adipocytes and vascular function. *J Clin Endocrinol Metab* 2004; **89**: 2563-2568
46 **Bråkenhielm E**, Veitonmäki N, Cao R, Kihara S, Matsuzawa Y, Zhivotovsky B, Funahashi T, Cao Y. Adiponectin-induced antiangiogenesis and antitumor activity involve caspase-mediated endothelial cell apoptosis. *Proc Natl Acad Sci USA* 2004; **101**: 2476-2481
47 **Ferroni P**, Palmirotta R, Spila A, Martini F, Raparelli V, Fossile E, Mariotti S, Del Monte G, Buonomo O, Roselli M, Guadagni F. Prognostic significance of adiponectin levels in non-metastatic colorectal cancer. *Anticancer Res* 2007; **27**: 483-489
48 **Wei EK**, Giovannucci E, Fuchs CS, Willett WC, Mantzoros CS. Low plasma adiponectin levels and risk of colorectal cancer in men: a prospective study. *J Natl Cancer Inst* 2005; **97**: 1688-1694
49 **Slattery ML**, Wolff RK, Herrick J, Caan BJ, Potter JD. Leptin and leptin receptor genotypes and colon cancer: gene-gene and gene-lifestyle interactions. *Int J Cancer* 2008; **122**: 1611-1617
50 **Stattin P**, Lukanova A, Biessy C, Söderberg S, Palmqvist R, Kaaks R, Olsson T, Jellum E. Obesity and colon cancer: does leptin provide a link? *Int J Cancer* 2004; **109**: 149-152
51 **Chia VM**, Newcomb PA, Lampe JW, White E, Mandelson MT, McTiernan A, Potter JD. Leptin concentrations, leptin receptor polymorphisms, and colorectal adenoma risk. *Cancer Epidemiol Biomarkers Prev* 2007; **16**: 2697-2703
52 **Tamakoshi K**, Toyoshima H, Wakai K, Kojima M, Suzuki K, Watanabe Y, Hayakawa N, Yatsuya H, Kondo T, Tokudome S, Hashimoto S, Suzuki S, Kawado M, Ozasa K, Ito Y, Tamakoshi A. Leptin is associated with an increased female colorectal cancer risk: a nested case-control study in Japan. *Oncology* 2005; **68**: 454-461
53 **Chung YC**, Chang YF. Serum interleukin-6 levels reflect the disease status of colorectal cancer. *J Surg Oncol* 2003; **83**: 222-226
54 **Sonnenberg GE**, Krakower GR, Kissebah AH. A novel pathway to the manifestations of metabolic syndrome. *Obes Res* 2004; **12**: 180-186
55 **Valko M**, Izakovic M, Mazur M, Rhodes CJ, Telser J. Role of oxygen radicals in DNA damage and cancer incidence. *Mol Cell Biochem* 2004; **266**: 37-56
56 **Nikiteas NI**, Tzanakis N, Gazouli M, Rallis G, Daniilidis K, Theodoropoulos G, Kostakis A, Peros G. Serum IL-6, TNFalpha and CRP levels in Greek colorectal cancer patients: prognostic implications. *World J Gastroenterol* 2005; **11**: 1639-1643
57 **Fajas L**, Debril MB, Auwerx J. Peroxisome proliferator-activated receptor-gamma: from adipogenesis to carcinogenesis. *J Mol Endocrinol* 2001; **27**: 1-9
58 **Theocharis S**, Giaginis C, Parasi A, Margeli A, Kakisis J, Agapitos E, Kouraklis G. Expression of peroxisome proliferator-activated receptor-gamma in colon cancer: correlation with histopathological parameters, cell cycle-related molecules, and patients' survival. *Dig Dis Sci* 2007; **52**: 2305-2311
59 **Shen D**, Deng C, Zhang M. Peroxisome proliferator-activated receptor gamma agonists inhibit the proliferation and invasion of human colon cancer cells. *Postgrad Med J* 2007; **83**: 414-419

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ORIGINAL ARTICLE

EP4 agonist alleviates indomethacin-induced gastric lesions and promotes chronic gastric ulcer healing

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Abstract

AIM: To investigate EP4-selective agonist effect on indomethacin-induced gastric lesions and on the spontaneous healing of chronic gastric ulcers.

METHODS: In a mouse model of gastric bleeding with high dose of indomethacin (20 mg/kg), an EP4-selective agonist was administered orally. Stomach lesions and gastric mucous regeneration were monitored. In a mouse model of chronic gastric ulcer induced by acetic acid, EP4 agonist effect on the healing of chronic gastric ulcer was evaluated in the presence or absence of low dose indomethacin (3 mg/kg). In cultured human gastric mucous cells, EP4 agonist effect on indomethacin-induced apoptosis was assessed by flow cytometry.

RESULTS: The EP4-selective agonist reduced high dose indomethacin-induced acute hemorrhagic damage and promoted mucous epithelial regeneration. Low-dose indomethacin aggravated ulcer bleeding and inflammation, and delayed the healing of the established chronic gastric ulcer. The EP4 agonist, when applied locally, not only offset indomethacin-induced gastric bleeding and inflammation, but also accelerated ulcer healing. In the absence of indomethacin, the EP4 agonist even accelerated chronic gastric ulcer healing and suppressed inflammatory cell infiltration in the granulation tissue. *In vitro*, the EP4 agonist protected human gastric mucous cells from indomethacin-induced apoptosis.

CONCLUSION: EP4-selective agonist may prevent indomethacin-induced gastric lesions and promote healing of existing and indomethacin-aggravated gastric ulcers, *via* promoting proliferation and survival of mucous epithelial cells.

Key words: Prostaglandin E2; Non-steroidal anti-inflammatory drugs; Gastric bleeding; Gastric ulcer; EP4-subtype receptor

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INTRODUCTION

Over 300 million patients use non-steroidal anti-inflammatory drugs (NSAIDs) in the world to treat pain, arthritis, fever and other diseases. Nearly 30% of the users suffer from gastric lesions and bleeding. Mechanisms for such actions of NSAIDs seem to be complex and multifactorial, including the inhibition of prostaglandin (PG) synthesis, induction of apoptosis and necrosis of gastric mucosal cells[1-3], neutrophil penetration, dysfunction of microvessels, reduced secretion of bicarbonate and mucus, and increased gastric motility[4].

Proton pump inhibitors (PPIs) have been the mainstay for the treatment of gastric ulcers, primarily due to its abilities to reduce acid secretion[5]. An alternative approach is to administer misoprostol, a non-selective prostaglandin E1 (PGE1) analogue. PGE2/E1 has been shown to protect isolated gastric glands from indomethacin, independently of neural, vascular and hormonal factors[6]. Misoprostol has successfully prevented NSAID-induced bleeding, perforation or gastric outlet obstruction in patients[5,7], and reversed the negative effects of indomethacin on the maturation of granulation

tissue which PPIs were not able to do[8]. Misoprostol, however, produces severe side effects, such as diarrhea, gastrointestinal (GI) cramping pain, nausea, flatulence, dyspepsia, abortion, headache and poor tolerance[9]. Among the four PGE2 receptor subtypes (EP1-4), EP4 is constitutively expressed in gastric surface mucous cells, the first layer of lining cells of the stomach mucus[10-12], and its agonists have been shown to inhibit the production of chemokines and cytotoxic cytokines from immune cells[13,14], and to promote epithelial cell survival and growth by activating anti-apoptotic and proliferative cellular signaling pathways[1,15,16]. Previous studies, using a non-selective EP3/EP4 agonist or an EP4 antagonist, imply EP4's role in gastric ulcer healing[17]; however, direct evidence of EP4 agonists' role on gastric lesions is still missing. In the present study, we investigated therapeutic potentials of a highly-selective EP4 agonist for treatment of a mouse gastric ulcer model in the presence or absence of indomethacin at various levels.

MATERIALS AND METHODS

The EP4-selective agonist

Competition binding experiments were performed in a medium containing Hank's balanced salt solution, 20 mmol/L HEPES, pH 7.3, membranes (about 60 μg protein) or 2×10^5 cells from HEK 293 cells stably expressing the human EP4 receptor, [^{3}H] PGE_2 (10 nmol/L) and various concentrations of test compounds in a total volume of 300 μL, read with LS6500 multi-purpose scintillation counter (Beckman Coulter, CA). cAMP assay was carried out using AlphaScreen cAMP assay kits (PerkinElmer, Boston, MA) following manufacturer's instructions. Intracellular Ca^{2+} was monitored using a FLIPR Tetra system and assay kits from Molecular Devices following manufacturer's instructions. All assays were carried out in HEK-293 cells heterologously and stably expressing each of the eight human recombinant prostanoid receptors. For Ca^{2+} signals, hEP2, hEP4 and hDP were co-expressed with a chimeric G protein, Gqs, which converts the Gs signal to a Gq Ca^{2+} signal, and hEP3 with a chimeric G protein, Gqi. Subtype-selective compounds used here were PGE_2 for EP1, EP2, EP3 and EP4; BW245C for DP; 17-phenyl PGF2α for FP, carbacyclin for IP and U-46619 for TP.

The PGE2 analog used in this study bound hEP4 with a Ki of 6.7 ± 0.7 nmol/L, not other prostanoid receptors, and increased cAMP production with an EC_{50} of 0.25 ± 0.03 nmol/L. On the other hand, the drug at 10 μmol/L showed no detectable FLIPR signals in HEK 293 cells heterologously expressing hEP1, hFP, hIP and hTP, and also in hEP2 (Gqs), hEP4 (Gqs), hEP3 (Gqi), hDP (Gqs), respectively. This compound is unstable in liver microsomes and thus when locally applied, its systemic exposure was minimal.

Cell culture and apoptosis assay

Human gastric mucosal cells (AGS) were purchased from the American Type Culture Collection (Manassas, VA), and maintained on Ham's F-12 medium (GIBCO-BRL) with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin. The cells were seeded in 6-well plates at 1×10^5 cells/well. After overnight (37℃, 5% CO_2) culture, the cells were treated with indomethacin at 50, 100, 200 or 400 μmol/L for 24 h, under serum-free conditions, to induce apoptosis as reported elsewhere[18]. Cell apoptosis was quantitated with flow cytometry as below. To evaluate EP4 agonist effect on cell survival, 70% confluent cells were treated with the EP4 agonist at 0, 1, 3 or 10 nmol/L, respectively, followed by 400 μmol/L indomethacin 30 min later. Twenty-four hours later, cells were collected and washed in cold PBS, and fixed with cold 70% ethanol added drop by drop while vortex stirring. Following overnight fixation, the cells were stained with propidium iodide (10 μg/mL, Sigma) and RNase A (1 mg/mL, Sigma) for 30 min in the dark. The cells were sorted by flow cytometry using CellQuest software (Becton Dickson, San Diego, CA). The sub-2N population was quantified. The percentage of apoptotic cells was calculated by sub-2N population from each drug treatment minus vehicle treatment[19].

Indomethacin-induced gastric damage model

High dose indomethacin (20 mg/kg in 4% DMSO, corn oil) was orally gavaged to induce gastric damage in C57BL/6 mice (Charles River, Wilmington, MA) at 8 wk old[20,21]. Vehicle or the EP4 agonist at 0.1 mg/kg in 0.1 mL 4% DMSO-corn oil was orally gavaged, 24 h and 30 min prior to indomethacin. Gastric lesions were assessed 24 h after indomethacin administration. The stomachs were removed, inflated with 2% formalin, immersed in 2% formalin for 10 min and then opened along the great curvature. The area of hemorrhagic lesions was measured under a dissecting microscope (16 × magnification) with a square grid (× 10), summed per stomach, and used as a lesion score[20,21]. The stomachs were then fixed in 10% formalin and sectioned at 5 μm thickness. HE staining was performed as usual. To monitor cell proliferation, BrdU was injected intraperitoneally at 10 mg/mL in 0.1 mL of normal saline, 16 h prior to sacrifice. Paraffin-embedded sections were deparaffinized in xylene and rehydrated in ethanol. Antigen was retrieved with citrate buffer, pH 6.0, boiled for 5 min in a microwave and slowly cooled down at room temperature. Immunofluorescence staining of BrdU was then performed following manufacturer's instructions (Roche-Applied Science, Penzberg, Germany). Briefly, the sections were incubated with sufficient amount of anti-BrdU working solution at 4℃ in a humid atmosphere for 24 h; after 3 × washing, anti-mouse-Ig-fluorescein was applied for 30 min at 37℃ in a humid atmosphere. DAPI was used to co-stain the slides for 5 min. The slides were covered with antifade mount medium, and evaluated with a fluorescence microscope. Ten views from each section were randomly collected at original magnification, × 200. The percentages of BrdU-positive cells were counted in well-oriented glands of mucous layer.

Chronic gastric ulcer model and treatments

C57BL/6 mice at 3 mo old were shaved in the epigastric region and anesthetized with isofluorane. Following

sterilization with betadine and 70% ethanol, a midline incision was made to expose the stomach. Five microliters of 40% acetic acid was added through a 3 mm curette onto the serosal surface of the anterior wall of the stomach (just proximal to the antral gland area). The curette was placed tightly on the stomach surface to limit the spread of acetic acid. Thirty seconds later, acetic acid was wiped off and the surface was cleaned with normal saline. The abdomen wall was closed by 6-0 silk sutures, and the skin was closed by staples[22]. We first performed a study to monitor the dynamic changes of ulcers and animals. The mice lost some weight initially and recovered in 2 d. Their ulcer sizes were peaked at day 3 and then spontaneously healed within 2 wk. Vehicle or the EP4 agonist (0.1 mg/kg per day) and/or indomethacin (3 mg/kg per day) were orally gavaged from day 3 to day 6 in 0.1 mL 4% DMSO-corn oil. Then the animals were assessed on day 7. To study EP4 agonist effect on chronic gastric ulcer healing (without indomethacin), the EP4 agonist (0.1 mg/kg per day) or vehicle were given from day 3 to day 10, and evaluated on days 7 and 11, respectively.

On the day of sacrifice, blood was withdrawn, and hematology analysis was conducted by personnel who did not know the treatments (ADVIA120 Hematology System, Bayer, Tarrytown, NY). The stomachs were inflated with 2% formalin for 10 min and opened along the greater curvature. The stomachs were flattened on 3M paper. The ulcers were photographed under dissection microscopy (× 16) with a hooked camera, and images stored in the computer and analyzed by SPOT software. The stomachs were then fixed in 10% formalin and processed for sectioning. A slice cutting through the biggest diameter of each ulcer was sectioned and stained by HE.

All animal use protocols were approved and performed according to the guidelines of Allergan's animal care and use committee. Data shown are mean ± SE. Statistical analysis was conducted by student *t*-test.

RESULTS

EP4 agonist decreased indomethacin-induced apoptosis

Exposure of human gastric mucous epithelial cells (AGS) to indomethacin (0, 50, 100, 200, 400 μmol/L) for 24 h concentration-dependently induced cell apoptosis as determined using flow cytometry analysis (Figure 1A). Particularly, indomethacin at 400 μmol/L markedly increased apoptosis, nearly 10-fold greater than in the untreated cells, and its activity was significantly reduced upon treatment (30 min before) with a highly-selective and potent EP4 agonist (see materials and methods), in a dose-dependent manner (Figure 1B). The EP4 agonist at the highest dose, 10 nmol/L, significantly decreased indomethacin-induced apoptosis, by more than 50%.

EP4 agonist alleviated indomethacin-induced acute damage and promoted epithelial regeneration in mice

In rats, apoptosis of mucous epithelial cells contributes to indomethacin-induced lesions in stomachs[18], and

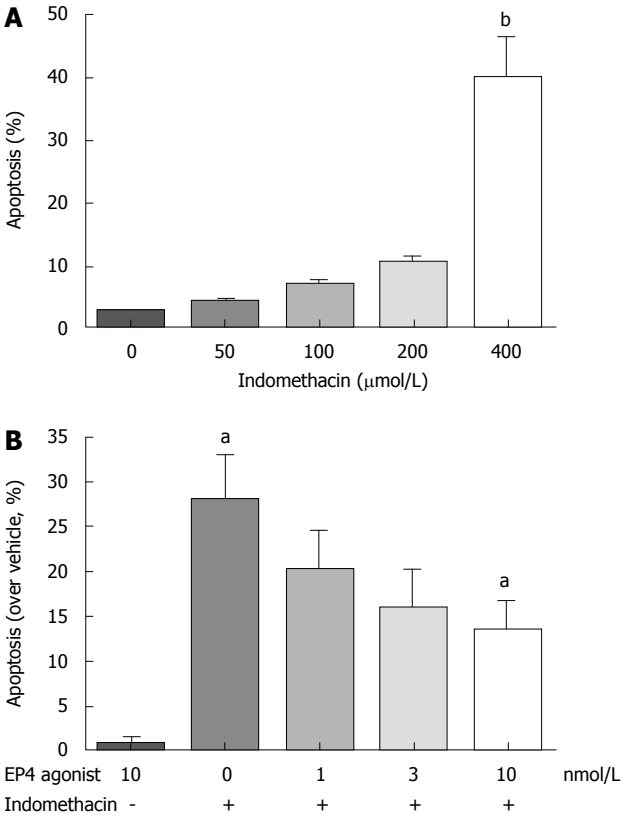

Figure 1 EP4 agonist effect on indomethacin-induced apoptosis in human gastric mucus epithelial cells (AGS). AGS cells were pre-treated with EP4 agonist for 30 min followed by addition of indomethacin for 24 h and sorted by flow cytometry after PI staining. Sub-2N population was quantified as apoptotic cells. A: indomethacin dose-dependently induced apoptosis, $^{b}P < 0.0001$ *vs* the rest, $n = 4$; B: shown are values minus vehicle controls, $^{a}P < 0.005$, $n = 5$-9; indomethacin dose was 400 μmol/L.

here we examined whether the EP4 agonist protects the gastric mucus layer from indomethacin. Indomethacin at high dose (20 mg/kg) produced band-shaped hemorrhagic lesions in the mucous layer mostly at the glandular part of the stomachs, occurring 7 h post indomethacin application. Histologically, there was edema and disorganization of the mucous layer, patchy mucous epithelial cell exfoliation, shallow ulcer formation and bleeding with infiltration of inflammatory cells in vehicle treated mice. The mucous layer of EP4-treated mice remained largely intact except for some sparse, focal superficial defects in mucous cells (Figure 2A). Treatment with the EP4 agonist (concentration 0.002%), 24 h and 30 min before indomethacin, significantly reduced gastric lesion scores, from an average of 16 to less than 6 (Figure 2B). BrdU staining-positive cells were largely limited to the isthmus and neck region in the tubular glands of the stomach mucosa layer in vehicle-treated mice (Figure 2A). BrdU-positive cells migrated much higher and lower along the tubular glands in the EP4 agonist-treated mice than vehicle-treated mice (Figure 2A). BrdU-positive cells in mucous layer were on average 29% in EP4 treated mice, and 21% in vehicle treated mice (Figure 2B). Taken together, the EP4 agonist may stimulate proliferation and migration of gastric epithelial progenitors, so as to accelerate mucous repair.

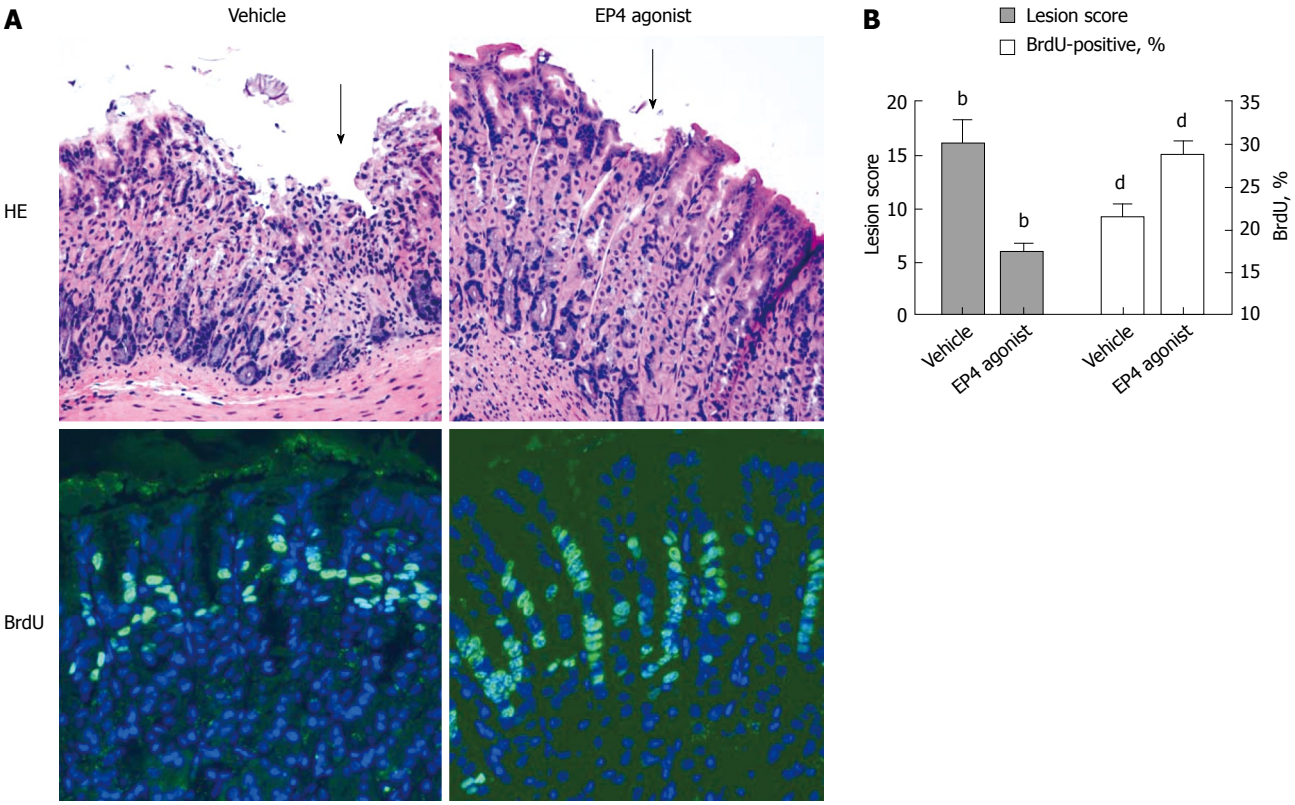

Figure 2 EP4 agonist effect on indomethacin-induced gastric lesion in mice. EP4 agonist was orally administered 24 h and 30 min prior to indomethacin dosing at 20 mg/kg. The stomachs were assessed for mucus lesions 24 h after indomethacin dosing. A: HE and BrdU immunohistochemistry staining of stomachs (× 200). Superficial mucosal cells had sloughed off gastric mucus with infiltration of inflammatory cells in vehicle-treated group (arrow points to one lesion site). The mucus of EP4 agonist-treated stomachs was almost normal, except for a sparse focal defect of superficial mucous cells without inflammatory cells (arrow points to one lesion site). BrdU labeling showed robust mucous epithelial regeneration and migration in EP4 agonist-treated rats compared with that of vehicle treatment; B: Quantification of gross lesion under dissection microscopy (× 16) and percentage of BrdU-positive cells among mucus cells. Shown are lesion scores, $^{b}P < 0.0001$, $n = 10$; and BrdU percentage, $^{d}P < 0.01$, $n = 10$, respectively.

Low dose indomethacin exacerbated chronic gastric ulcers in mice

A chronic gastric ulcer model was established by acetic acid application in mice. Low-dose indomethacin (3 mg/kg per day), which is sufficient to block *de novo* synthesis of PGE2, was applied 3 d post ulcer induction. On day 7, the indomethacin treatment increased gross ulcer areas by 76% as compared to vehicle-treated mice ($P < 0.01$, Figure 3A). Consistent with exacerbation of gastric ulcer sizes, hematology analysis revealed that indomethacin also worsened blood loss from the ulcers (Figure 3B), and higher lymphocyte surge as compared to untreated controls (Figure 3C). This supports the view that blocking of *de novo* synthesis of PGE2 delayed spontaneous repair of established gastric ulcer, and exacerbated inflammation and bleeding, which is similar to human gastric ulcer's responses to NSAIDs.

EP4 agonist ameliorated indomethacin exacerbation on chronic gastric ulcer in mice

We next investigated whether exogenous EP4 agonist is capable of promoting ulcer healing in the presence of indomethacin treatment. Indomethacin (3 mg/kg) with EP4 agonist (0.002% in 0.1 mL) or with vehicle was orally administered to mice with established gastric ulcers from day 3 to day 7. Mice treated with EP4 agonist had a smaller ulcer size than mice treated with vehicle, 75.04% ± 7.06% and 100.02% ± 9.44%, respectively, on day 7. Hematology analysis showed that EP4 agonist treatment significantly ameliorated loss of red blood cells, hemoglobin and hematocrit (Figure 4A). This may suggest that EP4 agonist-treated mice had either smaller ulcers or more mature granulation tissue than control mice. EP4 agonist leads to gastric mucous vasodilation, not vasoconstriction (mediated by EP3 receptor)[13] and mature granulation tissue is more resistant to noxious stimuli. The inflammation at ulcer sites was reflected by white blood cell counts in the peripheral circulation. EP4 agonist treatment decreased white blood cell counts from 6900/μL to 5600/μL, and lymphocyte counts from 4690/μL to 3330/μL ($P < 0.05$) (Figure 4B).

EP4 agonist accelerated the spontaneous healing of chronic gastric ulcer

We also examined the effects of EP4 agonist alone on gastric ulcer healing. By day 7, treatment with EP4 agonist reduced ulcer area by 40% as compared to that observed with vehicle treated mice (Figure 5A, $P < 0.005$). By day 11, the drug further reduced ulcer size by 70% (Figure 5A). There was much less inflammatory cell infiltration and necrosis tissue in the ulcers of EP4 agonist-treated animals, compared with untreated mice.

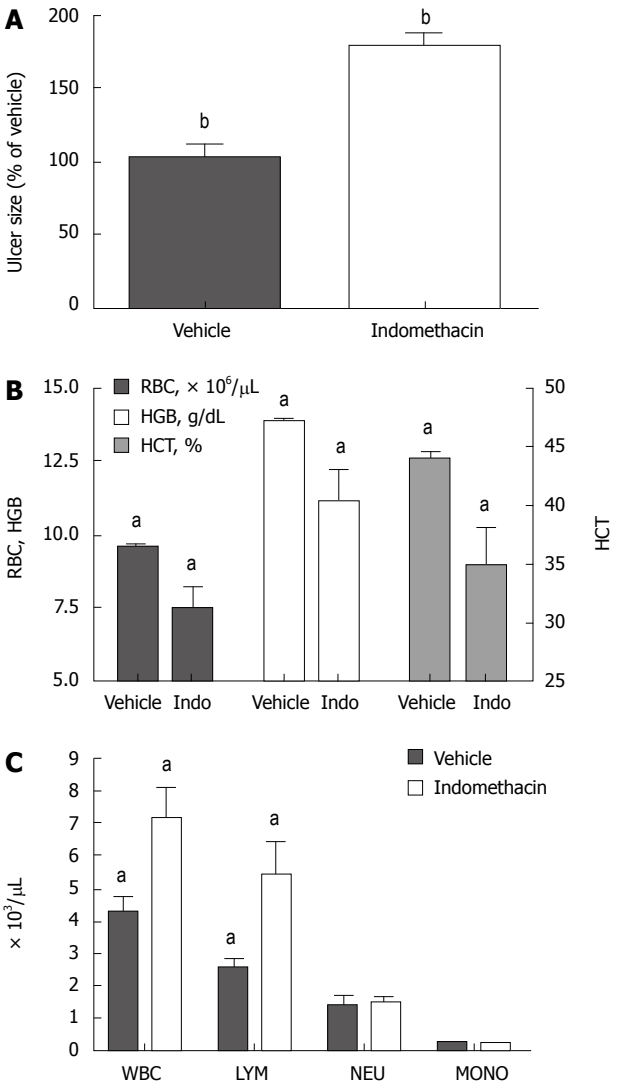

Figure 3 Low dose indomethacin effect on chronic gastric ulcer of mice. Chronic gastric ulcers were created by 40% acetic acid as detailed in "Methods". Then the animals were given indomethacin from day 3 to day 6 at 3 mg/kg per day and the ulcers were assessed after blood withdrawal on day 7 post-surgery. A: Ulcer sizes were measured under dissection microscopy with 16 × magnification; $^{b}P = 0.006$, $n = 18$; B: Hematology analysis of ulcerated animals on day 7, $^{a}P < 0.05$, $n = 6$; RBC: Red blood cell; HGB: Hemoglobin; HCT: Hematocrit; C: Hematology of ulcerated animals on day 7, $^{a}P < 0.05$, $n = 6$; WBC: White blood cells; LYM: Lymphocytes; NEU: Neutrophils; MONO: Monocytes.

On sectioning slides, inflammatory cell scores were 1.8 ± 0.2 for the EP4-agonist-treated and 2.7 ± 0.2 for the vehicle-treated mice ($P < 0.05$) (Figure 5B).

DISCUSSION

In the present study, we have shown that indomethacin, a prototypic cyclo-oxygenase (COX) inhibitor, at high dose, induced gastric epithelial apoptosis and produced gastric hemorrhagic lesions, and that the EP4-selective agonist we used here reduced such indomethacin-induced gastric injuries. Also, the EP4 agonist ameliorated ulcer bleeding and inflammation exacerbated by indomethacin at a low dose on existing ulcers, and promoted the spontaneous healing of chronic gastric ulcers in the absence of indomethacin.

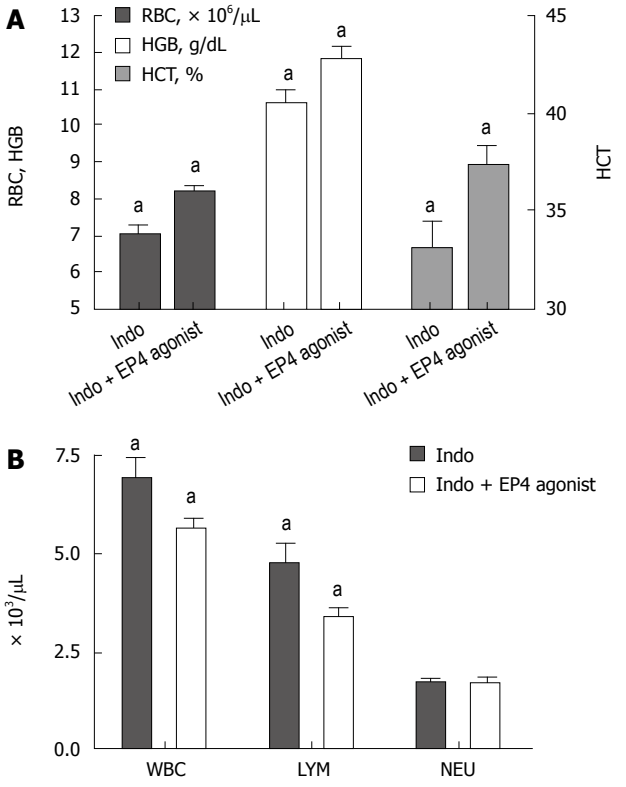

Figure 4 EP4 agonist alleviated indomethacin effect on gastric ulcer in mice. Mice with gastric ulcer were given low-dose indomethacin (3 mg/kg per day) with or without EP4 agonist from day 3 to day 6 post-surgery. The blood was withdrawn on day 7 for hematology analysis. A and B are results of hematology analysis, $^{a}P < 0.05$, $n = 20$.

Such indomethacin-induced gastric injuries and PGE2 analogue-induced gastric protection appear to be somewhat similar to their actions observed at cellular level: NSAIDs are known to bring about mitochondrial damage, caspase cleavage, and eventually cell apoptosis in human gastric mucus as well as animal primary gastric epithelial cells[3,18,23,24]. PGE2 and its analogs, on the other hand, inhibit indomethacin-induced mitochondrial damage and apoptosis in gastric epithelial cells[23,25], and a PGE1 analog, misoprostol, has been shown to reverse the inhibitory effect of NSAIDs on the regeneration of gastric mucous epithelial cells from human and animals[26]. 11-deoxy-PGE1 (EP3/EP4 agonist) reverses indomethacin-induced delay in the healing of chronic gastric ulcers[17]. One EP4-selective antagonist shows a deleterious effect on the spontaneous healing of chronic gastric ulcers with simultaneous suppression of vascular endothelial growth factor (VEGF) expression[17,27]. So gastric protective effects of PGE2 and its analogs may be partly caused by the activation of EP4, one of its 4 receptor subtypes, which is known to activate Gi- and Gs subtypes of G proteins, and to transduce PI3k/Akt/Erk1/2 and cAMP/PKA signaling[1,28,29]. Both pathways mediate pro-survival and proliferation signals in various epithelial cell lines, and are in line with our observation that the EP4 agonist inhibits indomethacin-induced apoptosis in human gastric mucosal cells (AGS), an established model for gastrointestinal effects of COX inhibitors[30], and expressing high levels of EP4

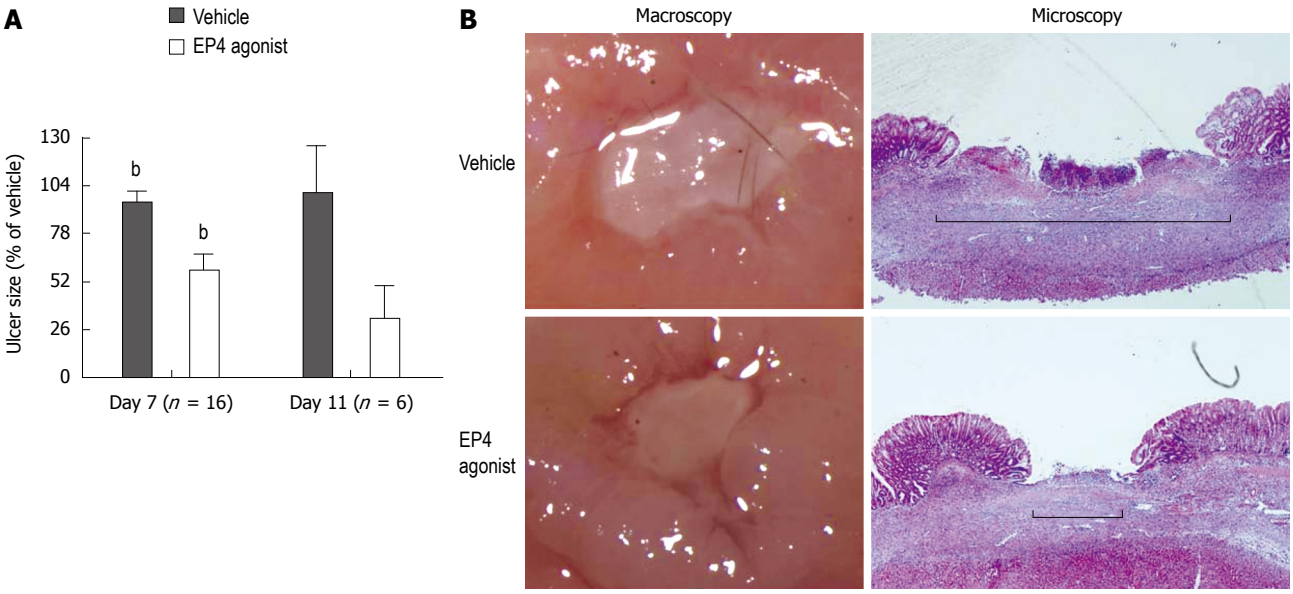

Figure 5 EP4 agonist promoted gastric ulcer healing in mice. Mice with gastric ulcers were treated with vehicle or EP4 agonist from day 3 to day 10 post-surgery. Gastric ulcers were assessed on day 7 or 11, respectively. A is quantification of ulcer sizes under dissection microscopy with 16 × magnification, $^{b}P = 0.003$, $n = 16$; B is representative images of gastric ulcers; the macroscopy images are at original magnification, × 16; the microscopy images are at original magnification, × 40 from slide sections.

transcripts (data not shown). Besides cell survival, the stimulation of intracellular cAMP from EP4 activation induces smooth muscle relaxation, and increases mucous blood supply and mucus secretion, which may additionally contribute to lessening indomethacin-induced injuries[25,31-33].

In addition to acute damages produced by high-dose indomethacin, we also observed the chronic deleterious gastric effects of indomethacin at low-dose, which is more relevant to common clinical situations. Consistent with earlier reports[17,34,35], low-dose indomethacin delayed healing of chronic gastric ulcers, exacerbated ulcer bleeding and inflammation, due to the inhibition of COX-2 expression and *de novo* synthesis of PGE2, and the inhibition of epithelial cell proliferation at the ulcer edge in both animals and humans. We have shown here that the EP4 agonist reversed such chronic effects induced by indomethacin at low dose.

Interestingly, we also observed here that the EP4 agonist accelerated the healing of chronic gastric ulcers under non-indomethacin challenged conditions. The major part of gastric ulcer healing is the restoration of gastric structure, which depends on the formation of the granulation tissue template made of gastric fibroblast cells and neovasculature. These fibroblasts express EP4 abundantly, and its activation is known to increase the synthesis of basic fibroblast growth factor, hepatocyte growth factor and VEGF[29,36-38]. All these factors may accelerate regeneration of fibroblasts and extracellular matrix, thus restoring ulcerated areas, and restituting epithelial cell layers[17,38]. Also the EP4 agonist showed anti-inflammatory activities as shown here and reported earlier[39]: fewer inflammatory cells in the blood and minimal infiltration in the ulcers from animals treated with the EP4 agonist. This should facilitate the healing process since the control of inflammation is a pre-requisite to rapid healing[35].

In summary, EP4 agonists may mimic the gastric protective effects of PGE2 in the presence or absence of NSAIDs, and may show advantages over non-selective analogs such as misoprostol by minimizing adverse effects arising from activating all 4 subtype receptors of PGE2.

ACKNOWLEDGMENTS

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COMMENTS

Background

Non-steroidal anti-inflammatory drugs (NSAIDs) including both cyclooxygenase (COX)-1/2 and COX-2-selective inhibitors, such as indomethacin, ibuprofen and celecoxib, have been prescribed in the world for treatment of pain, arthritis, menstrual symptoms and cancer, to name a few. However, nearly 30% of patients suffer from gastric lesions and bleeding. To mitigate NSAIDs' adverse effects on the stomach, misoprostol, a non-selective PGE1 analogue, has been prescribed as the first choice for prevention of NSAID-induced injuries in USA, but often induces severe adverse effects. There remain unmet medical needs for drugs with improved therapeutic profiles.

Research frontiers

PGE2/E1 interacts with 4 subtype receptors, EP1, 2, 3 and 4 in mammalian cells. Numerous studies have been performed to understand each subtype receptor's function and mechanism under various physiological and pathological conditions. High subtype receptor-selective ligands have been designed and tested to avoid adverse effects from non-selective drugs, such as misoprostol.

Innovations and breakthroughs

This study employed a novel, highly selective EP4 agonist, reported direct evidence for its protective activities against NSAIDs in the stomach, and further disclosed that the EP4 agonist may in part function through promoting mucous

epithelial cell survival and regeneration. This is the first study to show that an EP4 agonist may facilitate chronic gastric ulcer healing, although similar activity of EP4 in the stomach has been implicated in several concurrent studies using non-selective EP4 agonists or antagonists.

Applications

The concept from this paper would facilitate therapeutic developments of EP4-selective agonists for prevention of NSAIDs' adverse effects in the gastrointestinal (GI) tract as well as for monotherapy treatment of gastric ulcers. Further, EP4 agonists may provide gastric protection under conditions such as stress, radio/chemotherapy and other conditions compromising GI activities.

Terminology

Prostaglandin E2 (PGE2) is synthesized *via* key enzymes COX-1/2, under normal conditions primarily by COX-1 and under pathological conditions by inducible COX-2. PGE2 is a paracrine or autocrine hormone, and is involved in inflammation and pain, and also plays an important role in functional stability of the GI tract.

Peer review

In this manuscript, the authors investigated the effects of EP4-selective agonist on indomethacin-induced gastric lesions and spontaneous healing of chronic gastric ulcers in mice or cultured human gastric mucous cells. The study was well performed and very interesting.

REFERENCES

1 **Hoshino T**, Tsutsumi S, Tomisato W, Hwang HJ, Tsuchiya T, Mizushima T. Prostaglandin E2 protects gastric mucosal cells from apoptosis via EP2 and EP4 receptor activation. *J Biol Chem* 2003; **278**: 12752-12758

2 **Power JJ**, Dennis MS, Redlak MJ, Miller TA. Aspirin-induced mucosal cell death in human gastric cells: evidence supporting an apoptotic mechanism. *Dig Dis Sci* 2004; **49**: 1518-1525

3 **Redlak MJ**, Power JJ, Miller TA. Role of mitochondria in aspirin-induced apoptosis in human gastric epithelial cells. *Am J Physiol Gastrointest Liver Physiol* 2005; **289**: G731-G738

4 **Radi ZA**, Khan NK. Effects of cyclooxygenase inhibition on the gastrointestinal tract. *Exp Toxicol Pathol* 2006; **58**: 163-173

5 **Leontiadis GI**, Sreedharan A, Dorward S, Barton P, Delaney B, Howden CW, Orhewere M, Gisbert J, Sharma VK, Rostom A, Moayyedi P, Forman D. Systematic reviews of the clinical effectiveness and cost-effectiveness of proton pump inhibitors in acute upper gastrointestinal bleeding. *Health Technol Assess* 2007; **11**: 1-164

6 **Brzozowski T**, Tarnawski A, Hollander D, Sekhon S, Krause WJ, Gergely H. Comparison of prostaglandin and cimetidine in protection of isolated gastric glands against indomethacin injury. *J Physiol Pharmacol* 2005; **56** Suppl 5: 75-88

7 **Hooper L**, Brown TJ, Elliott R, Payne K, Roberts C, Symmons D. The effectiveness of five strategies for the prevention of gastrointestinal toxicity induced by non-steroidal anti-inflammatory drugs: systematic review. *BMJ* 2004; **329**: 948

8 **Arisawa T**, Harata M, Kamiya Y, Shibata T, Nagasaka M, Nakamura M, Fujita H, Hasegawa S, Nakamura M, Mizuno T, Tahara T, Ohta Y, Nakano H. Is omeprazole or misoprostol superior for improving indomethacin-induced delayed maturation of granulation tissue in rat gastric ulcers? *Digestion* 2006; **73**: 32-39

9 **Katzung BG**. Basic & clinical pharmacology. 9th ed. The McGraw-Hill Companies, 2004: 1040-1043

10 **Ding M**, Kinoshita Y, Kishi K, Nakata H, Hassan S, Kawanami C, Sugimoto Y, Katsuyama M, Negishi M, Narumiya S, Ichikawa A, Chiba T. Distribution of prostaglandin E receptors in the rat gastrointestinal tract. *Prostaglandins* 1997; **53**: 199-216

11 **Morimoto K**, Sugimoto Y, Katsuyama M, Oida H, Tsuboi K, Kishi K, Kinoshita Y, Negishi M, Chiba T, Narumiya S, Ichikawa A. Cellular localization of mRNAs for prostaglandin E receptor subtypes in mouse gastrointestinal tract. *Am J Physiol* 1997; **272**: G681-G687

12 **Northey A**, Denis D, Cirino M, Metters KM, Nantel F. Cellular distribution of prostanoid EP receptors mRNA in the rat gastrointestinal tract. *Prostaglandins Other Lipid Mediat* 2000; **62**: 145-156

13 **Hattori Y**, Ohno T, Ae T, Saeki T, Arai K, Mizuguchi S, Saigenji K, Majima M. Gastric mucosal protection against ethanol by EP2 and EP4 signaling through the inhibition of leukotriene C4 production. *Am J Physiol Gastrointest Liver Physiol* 2008; **294**: G80-G87

14 **Ratcliffe MJ**, Walding A, Shelton PA, Flaherty A, Dougall IG. Activation of E-prostanoid4 and E-prostanoid2 receptors inhibits TNF-alpha release from human alveolar macrophages. *Eur Respir J* 2007; **29**: 986-994

15 **Jiang GL**, Nieves A, Im WB, Old DW, Dinh DT, Wheeler L. The prevention of colitis by E Prostanoid receptor 4 agonist through enhancement of epithelium survival and regeneration. *J Pharmacol Exp Ther* 2007; **320**: 22-28

16 **Joseph RR**, Yazer E, Hanakawa Y, Stadnyk AW. Prostaglandins and activation of AC/cAMP prevents anoikis in IEC-18. *Apoptosis* 2005; **10**: 1221-1233

17 **Hatazawa R**, Tanaka A, Tanigami M, Amagase K, Kato S, Ashida Y, Takeuchi K. Cyclooxygenase-2/prostaglandin E2 accelerates the healing of gastric ulcers via EP4 receptors. *Am J Physiol Gastrointest Liver Physiol* 2007; **293**: G788-G797

18 **Aburaya M**, Tanaka K, Hoshino T, Tsutsumi S, Suzuki K, Makise M, Akagi R, Mizushima T. Heme oxygenase-1 protects gastric mucosal cells against non-steroidal anti-inflammatory drugs. *J Biol Chem* 2006; **281**: 33422-33432

19 **Jiang G**, Liu L, Buyse IM, Simon D, Huang S. Decreased RIZ1 expression but not RIZ2 in hepatoma and suppression of hepatoma tumorigenicity by RIZ1. *Int J Cancer* 1999; **83**: 541-546

20 **Morise Z**, Granger DN, Fuseler JW, Anderson DC, Grisham MB. Indomethacin induced gastropathy in CD18, intercellular adhesion molecule 1, or P-selectin deficient mice. *Gut* 1999; **45**: 523-528

21 **Swarnakar S**, Ganguly K, Kundu P, Banerjee A, Maity P, Sharma AV. Curcumin regulates expression and activity of matrix metalloproteinases 9 and 2 during prevention and healing of indomethacin-induced gastric ulcer. *J Biol Chem* 2005; **280**: 9409-9415

22 **Okabe S**, Amagase K. An overview of acetic acid ulcer models--the history and state of the art of peptic ulcer research. *Biol Pharm Bull* 2005; **28**: 1321-1341

23 **Nagano Y**, Matsui H, Muramatsu M, Shimokawa O, Shibahara T, Yanaka A, Nakahara A, Matsuzaki Y, Tanaka N, Nakamura Y. Rebamipide significantly inhibits indomethacin-induced mitochondrial damage, lipid peroxidation, and apoptosis in gastric epithelial RGM-1 cells. *Dig Dis Sci* 2005; **50** Suppl 1: S76-S83

24 **Tanaka K**, Tomisato W, Hoshino T, Ishihara T, Namba T, Aburaya M, Katsu T, Suzuki K, Tsutsumi S, Mizushima T. Involvement of intracellular Ca2+ levels in nonsteroidal anti-inflammatory drug-induced apoptosis. *J Biol Chem* 2005; **280**: 31059-31067

25 **Stenson WF**. Prostaglandins and epithelial response to injury. *Curr Opin Gastroenterol* 2007; **23**: 107-110

26 **Levi S**, Goodlad RA, Lee CY, Stamp G, Walport MJ, Wright NA, Hodgson HJ. Inhibitory effect of non-steroidal anti-inflammatory drugs on mucosal cell proliferation associated with gastric ulcer healing. *Lancet* 1990; **336**: 840-843

27 **Takeuchi K**, Tanaka A, Kato S, Aihara E, Amagase K. Effect of (S)-4-(1-(5-chloro-2-(4-fluorophenyoxy)benzamido)ethyl) benzoic acid (CJ-42794), a selective antagonist of prostaglandin E receptor subtype 4, on ulcerogenic and healing responses in rat gastrointestinal mucosa. *J Pharmacol Exp Ther* 2007; **322**: 903-912

28 **Nishihara H**, Kizaka-Kondoh S, Insel PA, Eckmann L. Inhibition of apoptosis in normal and transformed intestinal epithelial cells by cAMP through induction of inhibitor of apoptosis protein (IAP)-2. *Proc Natl Acad Sci USA* 2003; **100**: 8921-8926

29 **Rao R**, Redha R, Macias-Perez I, Su Y, Hao C, Zent R,

Breyer MD, Pozzi A. Prostaglandin E2-EP4 receptor promotes endothelial cell migration via ERK activation and angiogenesis in vivo. *J Biol Chem* 2007; **282**: 16959-16968

30 **Hall AJ**, Tripp M, Howell T, Darland G, Bland JS, Babish JG. Gastric mucosal cell model for estimating relative gastrointestinal toxicity of non-steroidal anti-inflammatory drugs. *Prostaglandins Leukot Essent Fatty Acids* 2006; **75**: 9-17

31 **Araki H**, Ukawa H, Sugawa Y, Yagi K, Suzuki K, Takeuchi K. The roles of prostaglandin E receptor subtypes in the cytoprotective action of prostaglandin E2 in rat stomach. *Aliment Pharmacol Ther* 2000; **14** Suppl 1: 116-124

32 **Takahashi S**, Takeuchi K, Okabe S. EP4 receptor mediation of prostaglandin E2-stimulated mucus secretion by rabbit gastric epithelial cells. *Biochem Pharmacol* 1999; **58**: 1997-2002

33 **Takeuchi K**, Aihara E, Hayashi M, Sasaki Y. Role of prostaglandin E receptor subtypes in gastroduodenal HCO3- secretion. *Med Chem* 2005; **1**: 395-403

34 **Motilva V**, Alarcon de la Lastra C, Bruseghini L, Manuel Herrerias J, Sanchez-Fidalgo S. COX expression and PGE(2) and PGD(2) production in experimental acute and chronic gastric lesions. *Int Immunopharmacol* 2005; **5**: 369-379

35 **Poonam D**, Vinay CS, Gautam P. Cyclo-oxygenase-2 expression and prostaglandin E2 production in experimental chronic gastric ulcer healing. *Eur J Pharmacol* 2005; **519**: 277-284

36 **Hatazawa R**, Tanigami M, Izumi N, Kamei K, Tanaka A, Takeuchi K. Prostaglandin E2 stimulates VEGF expression in primary rat gastric fibroblasts through EP4 receptors. *Inflammopharmacology* 2007; **15**: 214-217

37 **Sakai Y**, Fujita K, Sakai H, Mizuno K. Prostaglandin E2 regulates the expression of basic fibroblast growth factor messenger RNA in normal human fibroblasts. *Kobe J Med Sci* 2001; **47**: 35-45

38 **Takahashi M**, Ota S, Hata Y, Mikami Y, Azuma N, Nakamura T, Terano A, Omata M. Hepatocyte growth factor as a key to modulate anti-ulcer action of prostaglandins in stomach. *J Clin Invest* 1996; **98**: 2604-2611

39 **Kabashima K**, Saji T, Murata T, Nagamachi M, Matsuoka T, Segi E, Tsuboi K, Sugimoto Y, Kobayashi T, Miyachi Y, Ichikawa A, Narumiya S. The prostaglandin receptor EP4 suppresses colitis, mucosal damage and CD4 cell activation in the gut. *J Clin Invest* 2002; **109**: 883-893

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ORIGINAL ARTICLE

Voxel-based analyses of magnetization transfer imaging of the brain in hepatic encephalopathy

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Abstract

AIM: To evaluate the spatial distribution of cerebral abnormalities in cirrhotic subjects with and without hepatic encephalopathy (HE) found with magnetization transfer imaging (MTI).

METHODS: Nineteen cirrhotic patients graded from neurologically normal to HE grade 2 and 18 healthy control subjects underwent magnetic resonance imaging. They gave institutional-review-board-approved written consent. Magnetization transfer ratio (MTR) maps were generated from MTI. We tested for significant differences compared to the control group using statistical non-parametric mapping (SnPM) for a voxel-based evaluation.

RESULTS: The MTR of grey and white matter was lower in subjects with more severe HE. Changes were found in patients with cirrhosis without neurological deficits in the basal ganglia and bilateral white matter. The loss in magnetization transfer increased in severity and spatial extent in patients with overt HE. Patients with HE grade 2 showed an MTR decrease in white and grey matter: the maximum loss of magnetization transfer effect was located in the basal ganglia [SnPM (pseudo-)t = 17.98, P = 0.0001].

CONCLUSION: The distribution of MTR changes in HE points to an early involvement of basal ganglia and white matter in HE.

Key words: Brain; Hepatic encephalopathy; Magnetic resonance imaging; Liver cirrhosis; Magnetization transfer imaging

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INTRODUCTION

Hepatic encephalopathy (HE) is a frequent complication of liver cirrhosis, which is characterized by sleeping disorders, asterixis, and deficits in motor skills and reaction time. Twenty to eighty percent of patients with cirrhosis suffer from minimal HE (mHE)[1]. Five years after the diagnosis of mHE, 26% of patients with liver cirrhosis have episodes of overt HE. This condition is associated with a poor prognosis[2].

Studies using magnetization transfer contrast imaging (MTI) have demonstrated a decrease in the magnetization transfer ratio (MTR) in the brains of cirrhosis patients with mHE and overt HE[3-10]. MTR decrease in cirrhosis has been proposed to be an effect of astrocytic water retention[5-7,9,10], demyelination[5] and axonal loss[5], in addition to changes in blood flow and energy metabolism[9].

MTI has been used successfully to monitor normalization of cerebral abnormalities in cirrhosis patients following liver transplantation[8], and has been shown to detect increasing abnormalities following induced hyperammonemia[9].

The cited studies evaluated MTR maps using region-of-interest (ROI) based analyses. The MTR of normal appearing white matter[5-10] and anatomically defined areas of deep grey matter[3-5,10] have been the subject of previous studies on HE. To the best of our knowledge, no systematic evaluation of the spatial distribution of MTR abnormalities in HE has been published so far. The purpose of the present study is to evaluate the spatial distribution of MTI changes caused by central nervous system (CNS) abnormalities in HE.

MATERIALS AND METHODS

Subjects

Approval was obtained from the institution's review board. All patients and volunteers gave written informed consent after the neuropsychological tests and magnetic resonance imaging (MRI) had been explained fully. In this prospective study (for details see Table 1), 19 patients (14 male and five female) with non-alcoholic cirrhosis and 18 age-matched controls (eight male and 10 female) were included. Cirrhosis was caused by hepatitis C (n = 9), hemochromatosis (n = 2), primary chronic cholangitis (n = 2), hepatitis B (n = 1), Wilson's disease (n = 1) and cryptogenic cirrhosis (n = 4).

Subjects with a history of drug abuse, including alcohol, and those suffering from neurological or psychiatric diseases were excluded. Also excluded were patients who were taking CNS-relevant medications such as benzodiazepines, benzodiazepine antagonists and antidepressants. Further exclusion criteria were severe diseases such as spontaneous bacterial peritonitis, severe renal failure, uncontrolled diabetes mellitus or coronary heart disease. Since asterixis and hyperreflexia, as well as other more severe neurological conditions such as stupor or somnolence may interfere with safe and artefact-free MRI, patients with higher degrees of HE (grade 3 or 4) who also had these conditions were not considered for investigation. Also, patients needed to cooperate for neuropsychological examination and the patients' willingness represented a limitation in testing subjects with HE grade 3 or 4.

The severity of liver disease was determined according to the Child-Pugh-score[11]: patients were graded Child-Pugh A in nine cases, B in five and C in five.

Neuropsychological examination

Five computerized psychometric neurological tests were performed on each patient. The test battery included the visual pursuit test, motor performance series, Cognitrone, Vienna reaction test and Tachistoscopic Traffic Test Mannheim for Screen (TAVTMB) as part of the Vienna Test System (Schuhfried GmbH, Mödling, Austria, 1999). mHE was diagnosed if a patient showed no clinical overt symptoms of HE and performed at < 1 SD below the mean in at least two of the five computer psychometric tests of the test battery. Cirrhosis patients without clinical overt HE who performed at < -1 SD in only one of the tests were graded as having no HE[12]. Overt encephalopathy was graded according to the West-Haven criteria[13]. Cirrhosis patients were graded as no HE in five cases, mHE in three, HE 1 in six and HE 2 in five cases (Table 1).

Table 1 Nineteen patients with cirrhosis and 18 controls were enrolled (mean ± SD)

	Controls	Cirrhosis
n (male/female)	18 (8/10)	19 (14/5)
Age (yr)	55.7 ± 13.8	61.1 ± 12.4 NS
CFF (Hz)	41.3 ± 1.6	36.6 ± 4.9[b]
Child-Pugh grading		
A		9
B		5
C		5
HE grading		
HE 0		5
mHE		3
HE 1		6
HE 2		5

[b]P < 0.01 *vs* controls (Mann-Whitney U test). NS: No significant difference.

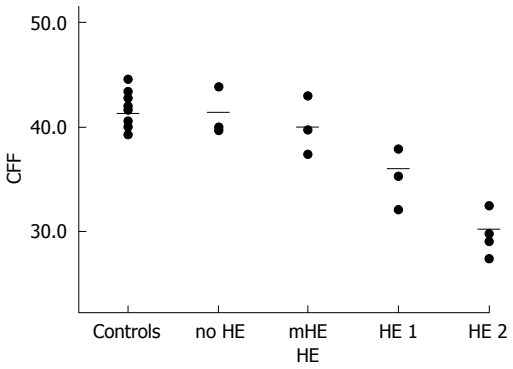

Figure 1 CFF of controls and patients. HE grading and CFF (Hz). Circles indicate individuals' CFF, bars represent means of groups (mean CFF of controls: 41.3 Hz; mean CFF of group with no HE: 41.4 Hz; mean CFF of group with mHE: 40.0 Hz; mean CFF of group with HE 1: 36.1 Hz; mean CFF of group with HE 2: 30.2 Hz).

In order to assess the severity of HE, the critical flicker frequency (CFF) was introduced in 2002. CFF is used in the evaluation of neurological deficits in low-grade HE and has been shown to respond readily to an improvement or deterioration of neurological symptoms[12].

CFF was determined in each patient using the Schuhfried Test System (Eberhard, G. Schuhfried GmbH, Mödling, Austria, 1994). A flickering light of 650 nm wavelength and 5.3 mcd was used for intrafoveal stimulation, while the flickering frequency decreased at 0.5-0.01 Hz/s. The CCF was defined as the frequency needed to perceive initially the stimulus as non-continuous. The means of eight repeat tests were determined (Figure 1).

MRI protocol

All scans were acquired on a 1.5-T clinical scanner (Magnetom Vision Plus; Siemens Medical Solutions, Erlangen, Germany) using the standard head coil. MTI was performed with two 2D gradient-echo sequences (TR 700 ms, TE 12 ms, flip angle α = 20°, one acquisition, 20 slices of 5 mm thickness, 0.5 mm gap), using a matrix

of 224 × 256 pixels with a field of view of 240 mm × 240 mm. The first set of images was obtained with no saturation pulse. The latter sequence used a saturation pulse 1.5 kHz below H_2O frequency, with a bandwidth of 250 Hz, 7.68 ms length and a flip angle of 500°. Additionally, routine examinations including T2- and T1-weighted imaging were performed to exclude patients with asymptomatic infarction or chronic ischemic changes.

Image processing

For each subject, the MTR was quantified as a percentage of signal loss between the two images according to the following equation: $MTR = (S_0 - S_S)/S_0 \times 100\%$. S_0 is the mean signal intensity of a pixel obtained from the sequence using no saturation pulse, and S_S is the mean signal intensity with the applied saturation pulse. Gray values in these secondary images represent MTR data, which yielded an MTR map.

Pixels that contained skull and soft tissue in the MTR maps were removed using the MRIcro-Software[14]. Images without skull and soft tissue pixels were used, since this has been reported to improve the validity of voxel-based evaluation of brain imaging data[14].

The MTR maps that contained only brain pixels were normalized into a standardized space using statistical parametric mapping (SPM5) software[15]. An MTR template was generated to ensure reliable normalization. For this, the images of all individuals were smoothed with a Gaussian kernel of 3 mm full width at half maximum, and normalized to the standard SPM EPI-template (Montreal Neurological Institute). The mean of the 37 normalized images was used as an MTR template for the normalization of each original MTR map. The default spatial normalization settings were applied. A Gaussian kernel of 3 mm full width at half maximum was used for the smoothing of the images.

It has been reported that brain atrophy is present in cirrhosis patients[16] and that comparison of atrophied and normal brains may lead to systematic errors[17]. To assess, whether atrophy interferes with voxel-based analysis in patients with liver cirrhosis, a test with binary images was performed. Binary images were generated from the normalized images of all patients and controls using MRIcro-Software. Pixels that contained brain were given the value 1. Pixels that contained cerebrospinal fluid (CSF) were set to 0.

Statistical analysis

Statistical mapping analysis has been applied in functional brain imaging [fMRI or positron emission tomography (PET)]. Recently, the method's successful use has been demonstrated in diffusion-weighted imaging[18], fluid-attenuated inversion-recovery imaging[19], perfusion-weighted imaging[20] and MTI[21].

We conducted a voxel-based analysis using the statistical non-parametric mapping (SnPM5b) software[22] based on SPM5[15]. A non-parametric approach was chosen because uniform variance across the volumes was not given and group size did not permit parametric tests. SnPM uses a permutation approach to account for the multiple comparison problem in voxel-by-voxel evaluation. It does not make the assumptions derived from random field theory underlying the multiple comparison corrections used in SPM[22].

For all statistical models employed, a threshold of $P < 0.001$ was used to determine significance. Non-parametric testing was conducted with 10000 random permutations when possible permutations exceeded this number. The anatomical localization of the maximum statistics was determined by co-examination of the SnPM (pseudo-)t map and the customized MTR template. The results are displayed as SnPM (pseudo-)t-map images superimposed on MTR maps of representative subjects.

Based on the hypothesis that cirrhosis patients (no HE, mHE, HE 1 and HE 2) had lower MTR values than controls, two-sample, one-sided permutation tests were conducted. Subject age was included as a confounder in the group comparison tests. Correlation between the MTR maps and CFF was tested. The binary images were tested on the hypothesis of a decreased brain volume in cirrhosis patients compared with controls.

RESULTS

MTR group comparisons

Compared with controls, cirrhosis patients without neurological deficits (no HE) displayed significantly decreased MTR values in the basal ganglia and in the hemispheric white matter (Table 2). The maximum statistics were located in the right putamen [(pseudo-)$t = 4.60$, $P = 0.0004$] (Figure 2). Statistics in the left putamen were (pseudo-)$t = 2.65$, $P = 0.0006$. In the group with mHE, a significant MTR decrease was found in hemispheric white matter, deep grey matter, brainstem and cerebellum. The cluster exhibiting the maximum statistics was in the right putamen (pseudo-)$t = 8.57$, $P = 0.0008$. Contralateral statistics were [(pseudo-)$t = 5.72$, $P = 0.0004$].

The groups with overt HE showed an MTR decrease in the entire brain. In HE 1, the maximum statistics were detected in the left posterior white matter [(pseudo-)$t = 8.82$, $P = 0.0001$]. The statistics of the right posterior white matter were (pseudo-)$t = 4.70$, $P = 0.0001$. In HE 2, the maximum statistics were found in the left globus pallidus [(pseudo-)$t = 17.98$, $P = 0.0001$]. The statistics of the right globus pallidus were (pseudo-)$t = 14.06$, $P = 0.0001$.

Binary data comparison

The SnPM results showed significant differences in external and internal CSF space between cirrhosis patients and controls (Figure 3). Spatial extent and maximum (pseudo-)t was higher in the patients with overt HE.

Correlation of CFF and MTR

In the cirrhosis patients, a positive correlation between CFF and MTR was found in basal ganglia and in supra- and infratentorial white matter (Figures 4 and 5, Table 3). Largest statistics detected in a brain parenchyma cluster

Table 2 Patients with no HE, mHE, HE 1 and HE 2 compared to controls (SnPM two-sample *t* test)

	Cluster level	Voxel level				SPM coordinates (mm)		
	k	*P* (FWE corrected)	*P* (FDR corrected)	(Pseudo-)*t*	*P* (uncorrected)	x	y	z
	209966	0.4753	0.5034	4.60	0.0004	-53	18	38
Controls *vs* no HE		0.7301	0.5034	4.27	0.0019	32	-101	-14
		0.8160	0.5034	4.14	0.0002	-27	17	55
	2652240	0.0045	0.0140	8.57	0.0008	-8	54	1
Controls *vs* mHE		0.0090	0.0140	8.27	0.0008	37	18	32
		0.0135	0.0140	7.72	0.0008	-19	-40	40
	288720	0.0003	0.0034	8.82	0.0001	-38	-74	1
Controls *vs* HE 1		0.0010	0.0034	8.31	0.0001	-38	-59	-10
		0.0017	0.0034	7.91	0.0001	-30	-26	1
	286240	0.0001	0.0006	17.98	0.0001	22	-6	-6
Controls *vs* HE 2		0.0001	0.0005	14.33	0.0001	-19	-38	36
		0.0001	0.0005	14.31	0.0001	18	2	-4

k: Number of voxels in significant clusters; FWE: Family-wise error; FDR: False discovery rate corrected and uncorrected; *P* and SnPM (pseudo-)*t* of most significant voxel clusters and their coordinates.

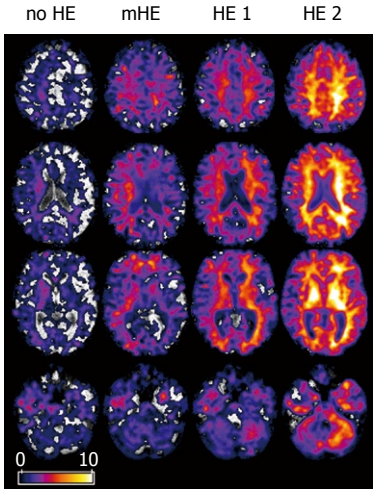

Figure 2 (Pseudo-)*t*-maps. MTR of patients with no HE, mHE, HE 1 and HE 2 compared to controls. Axial views superimposed on MTR maps of representative subjects. Colored areas represent voxels with significant decrease in MTR. Grey and white matter are involved. Local statistical maxima were found in basal ganglia and posterior white matter.

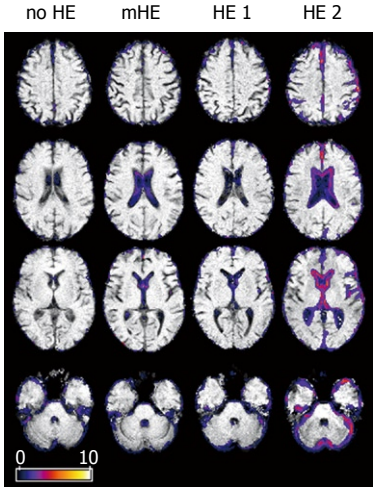

Figure 3 (Pseudo-)*t*-maps. Binary data of patients with no HE, mHE, HE 1 and HE 2 compared to controls. Axial views superimposed on the binary images of representative subjects showing differences in brain size. Colored areas represent voxels with significant differences. CSF space was increased in lateral cerebral fissure; parietal, frontal and cerebellar gyration were prominent in overt HE.

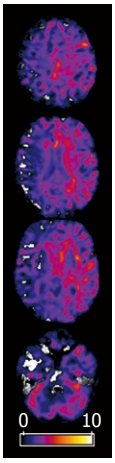

Figure 4 (Pseudo-)*t*-maps (*P* < 0.001). Areas with a correlation of subjects' CFF and MTR. Axial views superimposed on the MTR map of a representative subject (mHE). Colored areas represent voxels with significant positive correlation between CFF and MTR. The maximum statistics of a brain parenchyma cluster were found in left frontal white matter.

were in the left frontal white matter: (pseudo-)t = 7.06, P = 0.0001. Contralateral frontal white matter statistics were (pseudo-)t = 4.11, P = 0.0004.

DISCUSSION

MTI is an application of MRI that is used to assess non-water components in tissue. As opposed to conventional MRI, which is designed to represent different relaxation times in different tissues, MTI uses the exchange of magnetic properties between two brain tissue components: free water and macromolecules. Magnetization transfer takes place with and without physical exchange of protons between these two components. The extent of magnetization transfer is quantified using the ratio between two MR images. One image is acquired without and one with a high-frequency pulse that is designed to saturate the macromolecules. This results in a signal difference between the two images, as a result of the transfer of magnetization from free water protons towards macromolecules. The index derived from the two images can be calculated pixelwise and is rendered as an MTR map[23]. Changes in both components can lead to abnormal magnetization transfer. Depletion of macromolecules, a different macromolecule composition, as well as changes in brain water content may influence MTR.

Abnormal magnetization transfer is a known pathological feature in MRI of HE[24]. It has been reported to increase with the severity of HE. The present study provides a description of the distribution of these changes. In comparison to former studies that rely on simple ROI type analysis, a voxel-based approach was used to detect the localization of magnetization transfer changes in HE.

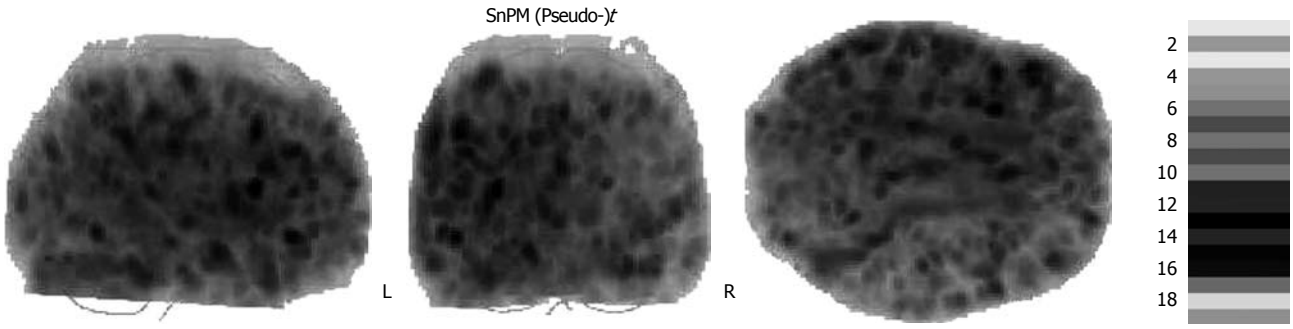

Figure 5 (Pseudo-)*t* results (*P* < 0.001). Voxel clusters with a correlation of subjects' CFF and MTR. SnPM glass brain representation of voxel clusters with a positive correlation between CFF and MTR (sagittal, coronal and axial view). A significant correlation was seen in all lobes.

Table 3 Correlation of MTR and CFF (SnPM *t* test)

	Cluster level	Voxel level				SPM coordinates (mm)		
	k	*P* (FWE corrected)	*P* (FDR corrected)	(Pseudo-)*t*	*P* (uncorrected)	x	y	z
Controls *vs* no HE	280567	0.0031	0.0137	7.06	0.0001	-34	18	8
		0.0167	0.0137	6.26	0.0001	-53	18	38
		0.0205	0.0137	6.19	0.0001	-54	35	-17

Spatial distribution of MTR abnormalities

MTR reduction in the brain of patients with cirrhosis involved white and grey matter, the brainstem and the cerebellum. The MTR decrease was more severe in patients with overt HE. The area of significantly reduced MTR was larger in patients with severe HE.

These results go beyond the findings of Iwasa *et al*[5], who have reported decreased MTR values in anatomically defined areas of the brain (globus pallidus, putamen, thalamus, corona radiata and subcortical white matter) in 37 cirrhosis patients with mHE, compared to controls. They also support and exceed the reports of Córdoba *et al*[6], Rovira *et al*[7] and Balata *et al*[9], who have described a loss of magnetization transfer effect in frontal and parietal white matter. In patients without overt HE, the maximum statistics were in the right basal ganglia. Patients with HE 2 had a more severe loss of MTR, which was localized bilaterally and symmetrically.

The data suggest that encephalopathy in patients with liver cirrhosis is unlikely to be related to structural damage of a specific area of the brain. In the dysmetabolic situation that accompanies hepatic insufficiency, our data show a diffuse pattern of brain involvement.

Pathological MRI of basal grey matter in HE has been described before the advent of MTI. Hyperintensity in T1-weighted imaging in the globus pallidus of patients with cirrhosis is a frequent finding[25]. Basal ganglia hyperintensity has been demonstrated to be reversible after successful liver transplantation[26]. Manganese deposition is a suggested condition to shorten T1 in the basal ganglia of cirrhosis patients[3,27], and to be an additive factor to the extrapyramidal symptoms in these patients[28]. In the mouse brain, significant shortening of T1 relaxation can be demonstrated after intravenous, intraperitoneal or subcutaneous administration of $MnCl_2$[29]. In a quantitative T1-mapping study of the brains of patients with liver cirrhosis and HE, Shah *et al*[30] have reported T1-shortening for the globus pallidus, caudate nucleus, and posterior limb of the internal capsule. They have discussed manganese deposition as a possible reason for their findings. No significant correlation between HE severity and T1 relaxation time could be found in the putamen, frontal white matter, white matter of the corona radiata, white matter in the occipital lobe, the anterior limb of the internal capsule, visual cortex, thalamus, or frontal cortex[30]. Patients with stage 1-2 primary biliary cirrhosis have been shown to exhibit a decrease in pallidal MTR (normalized to putamen), which correlates with blood manganese concentration[31].

In a study of diffusion-weighted imaging, Lodi *et al*[32] have reported increased diffusivity in white matter and basal ganglia, which suggests the presence of brain edema in chronic hepatic failure[32]. Increased diffusion in mHE and overt HE has been demonstrated to be reversible after successful treatment[33]. Both edema and manganese deposition may be explanations for the decrease in magnetization transfer effect.

White matter involvement in HE may be an effect of disturbed blood flow and energy metabolism[9], demyelination[5], axonal loss[5], as well as an effect of astrocytic water retention[5-7,9,10]. The loss in magnetization transfer reflected in a decrease in MTR has been proposed to be associated with brain edema in neuropsychiatric systemic lupus erythematosus[34], multiple sclerosis[35] and traumatic brain injury[36]. In non-cirrhotic patients with portal vein thrombosis, decreased MTR values of normal-appearing white matter have been found, which has been interpreted as an increase in free brain water[37].

The presence of white matter edema in chronic liver dysfunction is supported by the above findings of Lodi *et al*[32] and Kale *et al*[33], who reported increased diffusivity in white matter areas and in the basal ganglia.

MR spectroscopy has established the presence of a disturbed metabolic pattern in cirrhosis, which is considered

to represent a state of minimal brain swelling[26,38,39]. Reversibility after successful liver transplantation has been suggested to be an indicator of increased free cerebral water rather than structural damage[8].

The maximum statistics in the groups without overt HE were in the right putamen. In patients with HE 1 and HE 2, the highest (pseudo-)*t* was located in the left hemisphere. In each of the four groups, the corresponding contralateral brain area exhibited strongly significant, yet slightly lower statistics. The limited group size and the lack of information about the patients' handedness hinder interpretation of this finding. Unilateral brain involvement is unlikely under dysmetabolic conditions, with clinical symptoms of lack of awareness, constructional apraxia, dyscalculia, personality change and asterixis. A non-focal alteration of the dominant hemisphere might be expected to yield a stronger correlation with neuropsychological test results, than the same alteration of the non-dominant hemisphere. Nevertheless, a structurally higher susceptibility of the dominant hemisphere to metabolic toxins might be worthy of discussion. Further studies will possibly be dedicated to this question.

MTI and CFF

CFF assesses neurological deficits in cirrhosis as a continuous parameter, rather than offering the six-tier graduation used in the (revised) West-Haven criteria[40]. Since the test requires cooperation from the patients, its use in high-grade HE may be hindered. Patients suffering HE grade 3 and 4 were not enrolled in the present study. The CFF has been shown to be reliable in retest evaluation and responds rapidly to neurological deterioration or recovery[12]. Both parameters have been shown independently to decrease with increasing severity of HE[4-7,9,10,12].

In an fMRI study, Zafiris *et al*[41] have found impaired activation of visual cortex and abnormal neuronal coupling in cirrhosis patients with decreased CFF. Alzheimer-Ⅱ degeneration of glial Muller cells and mild visual impairment is another proposed mechanism of CFF decrease in HE[12].

A positive correlation was found between CFF and cerebral MTR. Our findings indicate that the MTR of large areas of normal-appearing white matter and deep grey matter correlates with a decrease in CFF in patients with HE.

Brain atrophy in HE

SnPM group comparison of the binary data sets showed prominent sulci, ventricles and lateral cerebral fissures as morphological correlations of brain atrophy.

In normal aging, a linear decrease in brain volume is known[42]. Although there was no significant difference between the mean age of patients (61.1 ± 12.4 years) and that of the control subjects (55.7 ± 13.8 years) in the present study, subject age was included in the analysis as a confounding variable. The findings from these images point towards an effect of cirrhosis on brain volume. Brain atrophy has been reported to be present in minimal and overt HE[27], which correlates with poor psychometric performance[16,43].

In voxel-based analysis following normalization, the comparison of normal and atrophied brains may lead to tests, in which voxels with mainly brain parenchyma in controls are tested against voxels that contain predominantly CSF in patients. This may result in non-valid differences. The topic of using the same spatial normalization algorithm for anatomically normal and atrophied brains has been addressed by Ishii *et al*[17]. Comparing SPM and NEUROSTAT normalization methods for the analysis of PET images in Alzheimer's disease, the authors have concluded that brains with atrophy tend to show artefacts caused by the anatomical standardization process.

In an MRI and PET study of schizophrenia, SPM results were deemed invalid because of incorrect spatial normalization of atrophied brains. This lead to an underestimation of metabolic activity in the caudate nucleus in patients with schizophrenia[44].

The present work shows a correlation between MTR and CFF in intraventricular and superficial voxels. This may be attributed to unreliable normalization of brains with atrophy in the patient group. Evaluating cortical or periventricular MTR values is limited by this effect.

In conclusion, in the brains of patients with non-alcoholic liver cirrhosis, MTR loss is seen. It involves basal ganglia and white matter, even in mild stages of HE. The changes are more severe and spatially more extended in patients with HE. Maximum statistics were found in the basal ganglia in overt HE, compared to healthy controls. The correlation of MTR and CFF is strongest in frontal white matter. Analyses of cerebral MTI of patients with liver cirrhosis might profit from inclusion of both basal ganglia and frontal white matter.

COMMENTS

Background

Hepatic encephalopathy (HE) is a frequent complication of liver cirrhosis and is associated with poor prognosis. Many studies have shown that magnetization transfer imaging (MTI) of the brain is sensitive to HE and subclinical forms of this condition [minimal HE (mHE)]. Treatment as well as induction of HE has been monitored successfully with MTI. Edema with water accumulation is a possible mechanism for MTI changes. Many brain areas have been investigated with MTI, but no systematic evaluation of the spatial pattern of MTI abnormalities in HE has been published.

Research frontiers

In the area of brain imaging in liver cirrhosis, the research hotspot is whether magnetic resonance imaging (MRI) can be used to detect HE early and to monitor its treatment. MTI is a MRI technique that is used to assess the interaction of free water protons and macromolecules. MTI is a promising research tool to assess minimal brain edema in HE.

Innovations and breakthroughs

In the previous application of MTI in HE, alterations have been reported for various brain areas such as the frontal and posterior white matter, basal ganglia and thalamus. More recent studies have demonstrated the sensitivity of MTI in mHE. These studies used simple region-of-interest (ROI) evaluations. The selection of ROIs was based on *a priori* assumptions from reports on white matter MRI anomalies and manganese deposits in the basal ganglia. To the best of the authors' knowledge, no systematic assessment of the distribution of brain MTI changes has been published on which to base the selection of ROIs in the imaging of HE. In the present study, they performed a voxel-based analysis (VBA) of MTI data of patients with HE and showed that abnormal signals were present throughout the brain. No one specific area was present where MTI changes in manifest HE were exclusive.

Applications

The study results suggest that MTI changes in HE are present throughout the brain. Test results in frontal and posterior white matter, thalamus and basal ganglia were statistically significant in cases with mHE, which might be areas that could be used as ROIs in monitoring HE by means of MTI.

Terminology

HE is a neurological condition with fatigue, sleeping disorders and motor deficits caused by liver cirrhosis. MTI is an MRI technique that is used to assess the interaction of free water protons and macromolecules, e.g. in mild brain edema. ROI is a means of evaluating MR images of the brain by measuring an image parameter in a defined area. VBA is a statistical evaluation of the entire brain MRI data of a study population.

Peer review

The manuscript entitled "Voxel-based analyses of magnetization transfer imaging of the brain in hepatic encephalopathy" is an interesting study that aimed to evaluate the spatial distribution of cerebral abnormalities in cirrhosis patients with and without HE found in MTI.

REFERENCES

1 **Gitlin N**, Lewis DC, Hinkley L. The diagnosis and prevalence of subclinical hepatic encephalopathy in apparently healthy, ambulant, non-shunted patients with cirrhosis. *J Hepatol* 1986; **3**: 75-82

2 **Rink C**, Haeting J, Nilius R. Prognosis assessment in patients with liver cirrhosis [Abstract]. *Hepatogastroenterology* 1990; **37** (suppl II): 5144

3 **Taylor-Robinson SD**, Oatridge A, Hajnal JV, Burroughs AK, McIntyre N, deSouza NM. MR imaging of the basal ganglia in chronic liver disease: correlation of T1-weighted and magnetisation transfer contrast measurements with liver dysfunction and neuropsychiatric status. *Metab Brain Dis* 1995; **10**: 175-188

4 **Iwasa M**, Kinosada Y, Watanabe S, Furuta M, Yuda H, Kawamura N, Nakayabu M, Esaki A, Sato T, Deguchi T, Nakatsuka A, Adachi Y. Hepatic cirrhosis: magnetisation transfer contrast in the globus pallidus. *Neuroradiology* 1998; **40**: 145-149

5 **Iwasa M**, Kinosada Y, Nakatsuka A, Watanabe S, Adachi Y. Magnetization transfer contrast of various regions of the brain in liver cirrhosis. *AJNR Am J Neuroradiol* 1999; **20**: 652-654

6 **Córdoba J**, Alonso J, Rovira A, Jacas C, Sanpedro F, Castells L, Vargas V, Margarit C, Kulisewsky J, Esteban R, Guardia J. The development of low-grade cerebral edema in cirrhosis is supported by the evolution of (1)H-magnetic resonance abnormalities after liver transplantation. *J Hepatol* 2001; **35**: 598-604

7 **Rovira A**, Grivé E, Pedraza S, Rovira A, Alonso J. Magnetization transfer ratio values and proton MR spectroscopy of normal-appearing cerebral white matter in patients with liver cirrhosis. *AJNR Am J Neuroradiol* 2001; **22**: 1137-1142

8 **Rovira A**, Córdoba J, Sanpedro F, Grivé E, Rovira-Gols A, Alonso J. Normalization of T2 signal abnormalities in hemispheric white matter with liver transplant. *Neurology* 2002; **59**: 335-341

9 **Balata S**, Olde Damink SW, Ferguson K, Marshall I, Hayes PC, Deutz NE, Williams R, Wardlaw J, Jalan R. Induced hyperammonemia alters neuropsychology, brain MR spectroscopy and magnetization transfer in cirrhosis. *Hepatology* 2003; **37**: 931-939

10 **Miese F**, Kircheis G, Wittsack HJ, Wenserski F, Hemker J, Mödder U, Häussinger D, Cohnen M. 1H-MR spectroscopy, magnetization transfer, and diffusion-weighted imaging in alcoholic and nonalcoholic patients with cirrhosis with hepatic encephalopathy. *AJNR Am J Neuroradiol* 2006; **27**: 1019-1026

11 **Pugh RN**, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R. Transection of the oesophagus for bleeding oesophageal varices. *Br J Surg* 1973; **60**: 646-649

12 **Kircheis G**, Wettstein M, Timmermann L, Schnitzler A, Häussinger D. Critical flicker frequency for quantification of low-grade hepatic encephalopathy. *Hepatology* 2002; **35**: 357-366

13 **Jalan R**, Hayes PC. Hepatic encephalopathy and ascites. *Lancet* 1997; **350**: 1309-1315

14 **Smith SM**. Fast robust automated brain extraction. *Hum Brain Mapp* 2002; **17**: 143-155

15 **Friston KJ**, Holmes AP, Worsley KJ, Poline JP, Frith CD, Frackowiak RSJ. Statistical parametric maps in functional imaging: A general linear approach. *Hum Brain Mapp* 1995; **2**: 189-210

16 **Amodio P**, Pellegrini A, Amistà P, Luise S, Del Piccolo F, Mapelli D, Montagnese S, Musto C, Valenti P, Gatta A. Neuropsychological-neurophysiological alterations and brain atrophy in cirrhotic patients. *Metab Brain Dis* 2003; **18**: 63-78

17 **Ishii K**, Willoch F, Minoshima S, Drzezga A, Ficaro EP, Cross DJ, Kuhl DE, Schwaiger M. Statistical brain mapping of 18F-FDG PET in Alzheimer's disease: validation of anatomic standardization for atrophied brains. *J Nucl Med* 2001; **42**: 548-557

18 **Chappell MH**, Uluğ AM, Zhang L, Heitger MH, Jordan BD, Zimmerman RD, Watts R. Distribution of microstructural damage in the brains of professional boxers: a diffusion MRI study. *J Magn Reson Imaging* 2006; **24**: 537-542

19 **Focke NK**, Symms MR, Burdett JL, Duncan JS. Voxel-based analysis of whole brain FLAIR at 3T detects focal cortical dysplasia. *Epilepsia* 2008; **49**: 786-793

20 **Rashid W**, Parkes LM, Ingle GT, Chard DT, Toosy AT, Altmann DR, Symms MR, Tofts PS, Thompson AJ, Miller DH. Abnormalities of cerebral perfusion in multiple sclerosis. *J Neurol Neurosurg Psychiatry* 2004; **75**: 1288-1293

21 **Audoin B**, Ranjeva JP, Au Duong MV, Ibarrola D, Malikova I, Confort-Gouny S, Soulier E, Viout P, Ali-Chérif A, Pelletier J, Cozzone PJ. Voxel-based analysis of MTR images: a method to locate gray matter abnormalities in patients at the earliest stage of multiple sclerosis. *J Magn Reson Imaging* 2004; **20**: 765-771

22 **Nichols TE**, Holmes AP. Nonparametric permutation tests for functional neuroimaging: a primer with examples. *Hum Brain Mapp* 2002; **15**: 1-25

23 **McGowan JC**. The physical basis of magnetization transfer imaging. *Neurology* 1999; **53**: S3-S7

24 **Grover VP**, Dresner MA, Forton DM, Counsell S, Larkman DJ, Patel N, Thomas HC, Taylor-Robinson SD. Current and future applications of magnetic resonance imaging and spectroscopy of the brain in hepatic encephalopathy. *World J Gastroenterol* 2006; **12**: 2969-2978

25 **Inoue E**, Hori S, Narumi Y, Fujita M, Kuriyama K, Kadota T, Kuroda C. Portal-systemic encephalopathy: presence of basal ganglia lesions with high signal intensity on MR images. *Radiology* 1991; **179**: 551-555

26 **Naegele T**, Grodd W, Viebahn R, Seeger U, Klose U, Seitz D, Kaiser S, Mader I, Mayer J, Lauchart W, Gregor M, Voigt K. MR imaging and (1)H spectroscopy of brain metabolites in hepatic encephalopathy: time-course of renormalization after liver transplantation. *Radiology* 2000; **216**: 683-691

27 **Zeneroli ML**, Cioni G, Crisi G, Vezzelli C, Ventura E. Globus pallidus alterations and brain atrophy in liver cirrhosis patients with encephalopathy: an MR imaging study. *Magn Reson Imaging* 1991; **9**: 295-302

28 **Butterworth RF**, Spahr L, Fontaine S, Layrargues GP. Manganese toxicity, dopaminergic dysfunction and hepatic encephalopathy. *Metab Brain Dis* 1995; **10**: 259-267

29 **Kuo YT**, Herlihy AH, So PW, Bhakoo KK, Bell JD. In vivo measurements of T1 relaxation times in mouse brain associated with different modes of systemic administration of manganese chloride. *J Magn Reson Imaging* 2005; **21**: 334-339

30 **Shah NJ**, Neeb H, Zaitsev M, Steinhoff S, Kircheis G, Amunts K, Häussinger D, Zilles K. Quantitative T1 mapping of hepatic encephalopathy using magnetic resonance imaging. *Hepatology* 2003; **38**: 1219-1226

31 **Forton DM**, Patel N, Prince M, Oatridge A, Hamilton G, Goldblatt J, Allsop JM, Hajnal JV, Thomas HC, Bassendine M,

Jones DE, Taylor-Robinson SD. Fatigue and primary biliary cirrhosis: association of globus pallidus magnetisation transfer ratio measurements with fatigue severity and blood manganese levels. *Gut* 2004; **53**: 587-592

32 **Lodi R**, Tonon C, Stracciari A, Weiger M, Camaggi V, Iotti S, Donati G, Guarino M, Bolondi L, Barbiroli B. Diffusion MRI shows increased water apparent diffusion coefficient in the brains of cirrhotics. *Neurology* 2004; **62**: 762-766

33 **Kale RA**, Gupta RK, Saraswat VA, Hasan KM, Trivedi R, Mishra AM, Ranjan P, Pandey CM, Narayana PA. Demonstration of interstitial cerebral edema with diffusion tensor MR imaging in type C hepatic encephalopathy. *Hepatology* 2006; **43**: 698-706

34 **Emmer BJ**, Steens SC, Steup-Beekman GM, van der Grond J, Admiraal-Behloul F, Olofsen H, Bosma GP, Ouwendijk WJ, Huizinga TW, van Buchem MA. Detection of change in CNS involvement in neuropsychiatric SLE: a magnetization transfer study. *J Magn Reson Imaging* 2006; **24**: 812-816

35 **Hähnel S**, Münkel K, Jansen O, Heiland S, Reidel M, Freund M, Aschoff A, Sartor K. Magnetization transfer measurements in normal-appearing cerebral white matter in patients with chronic obstructive hydrocephalus. *J Comput Assist Tomogr* 1999; **23**: 516-520

36 **Bagley LJ**, Grossman RI, Galetta SL, Sinson GP, Kotapka M, McGowan JC. Characterization of white matter lesions in multiple sclerosis and traumatic brain injury as revealed by magnetization transfer contour plots. *AJNR Am J Neuroradiol* 1999; **20**: 977-981

37 **Mínguez B**, García-Pagán JC, Bosch J, Turnes J, Alonso J, Rovira A, Córdoba J. Noncirrhotic portal vein thrombosis exhibits neuropsychological and MR changes consistent with minimal hepatic encephalopathy. *Hepatology* 2006; **43**: 707-714

38 **Kreis R**, Ross BD, Farrow NA, Ackerman Z. Metabolic disorders of the brain in chronic hepatic encephalopathy detected with H-1 MR spectroscopy. *Radiology* 1992; **182**: 19-27

39 **Ross BD**, Jacobson S, Villamil F, Korula J, Kreis R, Ernst T, Shonk T, Moats RA. Subclinical hepatic encephalopathy: proton MR spectroscopic abnormalities. *Radiology* 1994; **193**: 457-463

40 **Conn HO**. Subclinical hepatic encephalopathy. In: Bircher J, editor. Hepatic encephalopathy: syndromes and therapies. Bloomington: Medi-ed Press, 1994: 27-42

41 **Zafiris O**, Kircheis G, Rood HA, Boers F, Häussinger D, Zilles K. Neural mechanism underlying impaired visual judgement in the dysmetabolic brain: an fMRI study. *Neuroimage* 2004; **22**: 541-552

42 **Good CD**, Johnsrude IS, Ashburner J, Henson RN, Friston KJ, Frackowiak RS. A voxel-based morphometric study of ageing in 465 normal adult human brains. *Neuroimage* 2001; **14**: 21-36

43 **Moore JW**, Dunk AA, Crawford JR, Deans H, Besson JA, De Lacey G, Sinclair TS, Mowat NA, Brunt PW. Neuropsychological deficits and morphological MRI brain scan abnormalities in apparently healthy non-encephalopathic patients with cirrhosis. A controlled study. *J Hepatol* 1989; **9**: 319-325

44 **Reig S**, Penedo M, Gispert JD, Pascau J, Sánchez-González J, García-Barreno P, Desco M. Impact of ventricular enlargement on the measurement of metabolic activity in spatially normalized PET. *Neuroimage* 2007; **35**: 748-758

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ORIGINAL ARTICLE

Direct hepatic differentiation of mouse embryonic stem cells induced by valproic acid and cytokines

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Abstract

AIM: To develop a protocol for direct hepatic lineage differentiation from early developmental progenitors to a population of mature hepatocytes.

METHODS: Hepatic progenitor cells and then mature hepatocytes from mouse embryonic stem (ES) cells were obtained in a sequential manner, induced by valproic acid (VPA) and cytokines (hepatocyte growth factor, epidermal growth factor and insulin). Morphological changes of the differentiated cells were examined by phase-contrast microscopy and electron microscopy. Reverse transcription polymerase chain reaction and immunocytochemical analyses were used to evaluate the gene expression profiles of the VPA-induced hepatic progenitors and the hepatic progenitor-derived hepatocytes. Glycogen storage, cytochrome P450 activity, transplantation assay, differentiation of bile duct-like structures and tumorigenic analyses were performed for the functional identification of the differentiated cells. Furthermore, FACS and electron microscopy were used for the analyses of cell cycle profile and apoptosis in VPA-induced hepatic differentiated cells.

RESULTS: Based on the combination of VPA and cytokines, mouse ES cells differentiated into a uniform and homogeneous cell population of hepatic progenitor cells and then matured into functional hepatocytes. The progenitor population shared several characteristics with ES cells and hepatic stem/progenitor cells, and represented a novel progenitor cell between ES and hepatic oval cells in embryonic development. The differentiated hepatocytes from progenitor cells shared typical characteristics with mature hepatocytes, including the patterns of gene expression, immunological markers, *in vitro* hepatocyte functions and *in vivo* capacity to restore acute-damaged liver function. In addition, the differentiation of hepatic progenitor cells from ES cells was accompanied by significant cell cycle arrest and selective survival of differentiating cells towards hepatic lineages.

CONCLUSION: Hepatic cells of different developmental stages from early progenitors to matured hepatocytes can be acquired in the appropriate order based on sequential induction with VPA and cytokines.

Key words: Hepatic differentiation; Embryonic stem cells; Histone deacetylase inhibitor; Progenitor cell; Cell cycle arrest

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INTRODUCTION

Hepatocyte transplantation is an alternative therapy strategy for liver failure or end-stage liver diseases[1,2].

However, the shortage of donor hepatocytes and the difficulties for large scale hepatocyte amplification and function maintenance limit the clinical application of this cell-based therapy[3]. Embryonic stem (ES) cells, known for their capacity to proliferate indefinitely and differentiate into almost all types of cells including hepatocytes, have raised the hope of cellular replacement therapy for liver failure. The essential prerequisite for this purpose is to develop well-defined protocols for directing cellular differentiation into hepatic lineage, followed by selective isolation and proliferation *in vitro*. There have been several protocols available up till now for hepatic fate specification from ES cells, for example, the protocols based on spontaneous differentiation, combination of growth factors, co-culture with non-parenchymal liver cells and gene modifications[4-8]. However, most of the protocols currently used were devised to induce mature hepatocytes by using embryoid bodies that may result in low yield or purity of functional hepatocytes. Little documentation exists about a strategy for acquiring different developmental hepatic cells, for example, guiding differentiation of ES cells to hepatic progenitors and then to an entire population of mature hepatocytes to meet the requirements of precise study regarding the mechanisms of hepatic differentiation and potential clinical applications. Furthermore, more concise and reproducible methods to acquire abundant hepatic cells still remain to be developed, among which the direct hepatic differentiation from an ES monolayer without using embryoid bodies is most promising[9].

Valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, has been used as a new class of chemotherapeutic drug for cancer clinical purposes and as an inducer for stem cell differentiation[10-13]. It has exhibited profound therapeutic activity against hepatocellular carcinoma by inducing apoptosis and cell-cycle arrest[14-16], and dramatic effects on cellular differentiation from stem cells, including cardiomyocyte differentiation from ES cells, neuronal differentiation from neural stem cells, and osteogenic and hepatic differentiation from bone marrow-derived mesenchymal stem cells (MSC)[17-20]. In a previous study, we established a method for hepatic differentiation from MSC using VPA, which provided preliminary evidence that VPA could facilitate hepatic differentiation[21]. However, little is know about whether VPA can induce the hepatic differentiation of ES cells. Here, we report such research and the development of a protocol for direct hepatic lineage differentiation, from early developmental progenitors to a population of mature hepatocytes, based on sequential induction with VPA and cytokines. Results show that VPA can direct the hepatic specification of ES cells and largely participates in the differentiation of ES cells into hepatic progenitors. Further differentiation of hepatic progenitors into mature hepatocytes requires supplementation with cytokines. The present study may not only be helpful for the clinical application of hepatocyte transplantation, but also provide an *in vitro* research model for the better investigation and understanding of the entire developmental process of hepatocytes, from ES cells to hepatic progenitors, and then to mature hepatocytes. Furthermore, as VPA is an epigenetic modulator, so our results may also be of benefit to the research of mechanisms of epigenetic modifications during liver development.

MATERIALS AND METHODS

Reagents

VPA was purchased from Sigma (St Louis, MO); fetal bovine serum (FBS) was purchased from Hyclone (Rockville, MD); murine leukemia inhibitory factor (LIF) was purchased from Chemicon (Temecula, CA); mouse hepatocyte growth factor (mHGF), mouse epidermal growth factor (mEGF), oncostatin M (OSM), Insulin-Transferrin-Selenium (ITS), and collagen I were all from R&D systems (Minneapolis, MN); Matrigel was purchased from BD Biosciences (Palo Alto, CA); sheep anti-ALB antibodies were purchased from Biodesign (Saco, ME); rabbit anti-AFP, mouse anti-CK19 were from Dako (Copenhagen, Denmark); rat anti-OCT-4 was from R&D; mouse anti-SSEA-1 was from Developmental Hybridoma Bank of Iowa University; goat anti-mouse DLK was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rat anti-A6 was presented by Dr. Valentina Factor of NIH; FITC-conjugated bovine anti-sheep IgG, FITC-conjugated rabbit anti-goat IgG, FITC-conjugated goat anti-rabbit IgG, TRITC-conjugated goat anti-mouse IgG, TRITC-conjugated goat anti-rat IgG were all purchased from Dako; FITC-conjugated goat anti-mouse IgM was from Jackson Immunoresearch Laboratories Inc. All other reagents were from Sigma (St. Louis, MO).

Culture of mouse ES cells

Mouse ES D3 cells, provided by the Cell Biology Institute of the Chinese Academy of Sciences, were cultured on mitomycin C inactivated MEF feeder layers in high-glucose DMEM supplemented with 15% FBS, 2 mmol/L L-Glu, 0.1 mmol/L N-ME, 1% NEAA and 10 ng/mL murine LIF as described previously[22]. Briefly, MEF feeder cells were isolated from ICR mice at embryo day 13.5 and cultured at 37℃ and 5% CO_2. At approximately 80% confluence, the feeder cells were incubated with 10 μg/mL mitomycin C for 4 h and washed three times with PBS. Then the cells were replated at 8×10^4 cells/cm^2 on to tissue culture flasks. After allowed for attachment overnight, the ES cells were seeded.

Differentiation of hepatic cells from mouse ES cells

A protocol was designed to obtain the hepatic progenitor cells and then mature hepatocytes from mouse ES cells in a sequential manner (Figure 1). For differentiation of hepatic progenitor cells, ES cells were cultured in DME medium as described above with the exception of the withdrawal of feeder layer and LIF, and treated with 1 mmol/L VPA for 4-6 d, then the VPA was removed and recombinant mouse HGF 10 ng/mL was added for

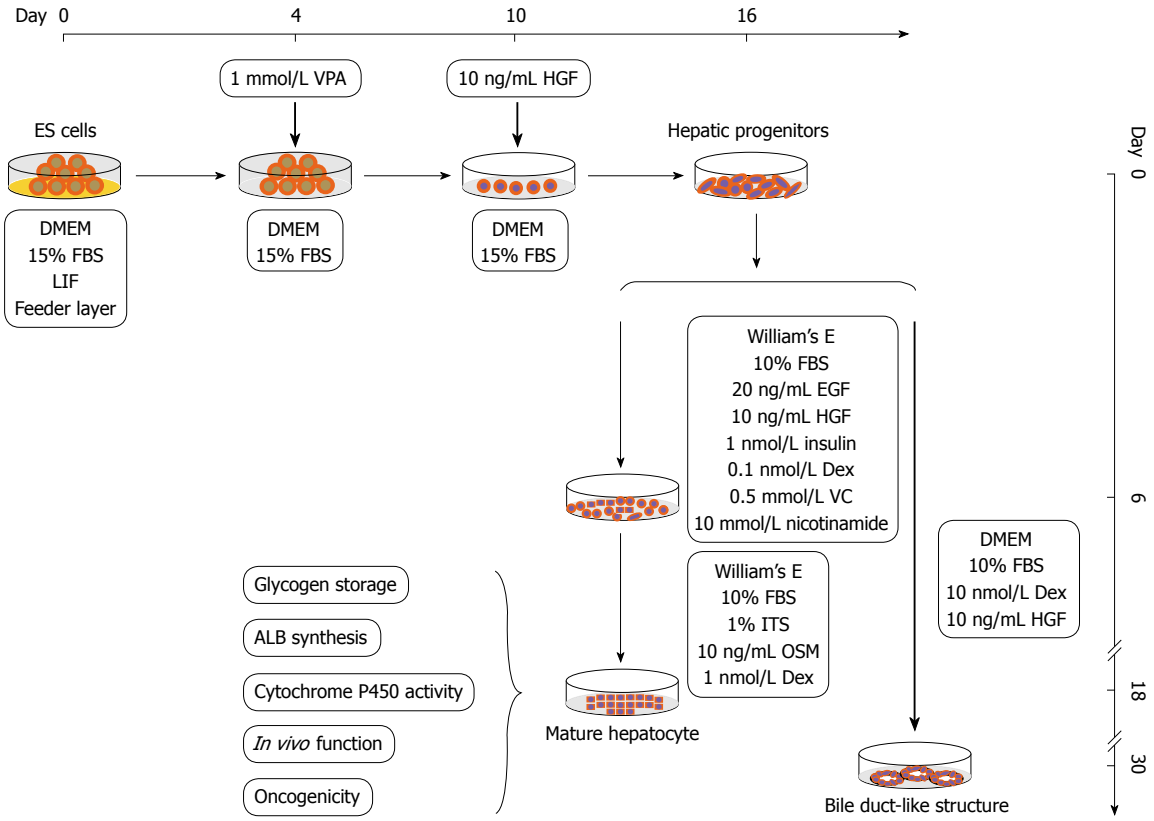

Figure 1 Schematic presentation of the protocol.

another 6-12 d until the hepatic progenitor cells became confluent. For differentiation of hepatocytes from hepatic progenitor cells, the progenitor cells were cultured in a William's E medium supplemented with 20 ng/mL mEGF, 10 ng/mL mHGF, 10^{-6} mol/L insulin, 10^{-7} mol/L Dex, 0.5 mmol/L ascorbic acid diphosphate, 10 mmol/L nicotinamide and 10% FBS (maturation medium Ⅰ) for 6 d, and then replaced with another William's E medium containing 1% ITS, 10 ng/mL OSM and 10^{-6} mol/L Dex (maturation medium Ⅱ) for another 6-12 d.

Molecular and structural identification of the differentiated cells

Gene expression analyses: Total RNA was extracted from the ES cells, hepatic progenitor cells, mature hepatocytes and adult mouse liver using Nucleospin RNA Kits (BD Biosciences, Palo Alto, CA). RNA samples were digested with DNase I for 15 min at room temperature. cDNAs were synthesized from 1 μg total RNA with a superscript Ⅲ first-strand synthesis kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and amplified with the following program: initial denaturation at 95℃ for 5 min followed by 30-40 cycles of 94℃ for 30 s, 50-56℃ for 30 s, 72℃ for 30 s and final extension at 72℃ for 10 min. The amplified products were analyzed by electrophoresis on 1.5% agarose gel and stained with ethidium bromide. All the primers used are listed in Table 1.

Immunocytochemical analyses: Cells were fixed using 4% paraformaldehyde (PFA) followed by ice-cooled methanol, and incubated with primary antibodies overnight at 4℃ after blocking with 5% normal goat serum. For the application of SSEA-1 antigen, the cells were fixed with PFA only. Following washing with PBS three times, the cells were incubated with fluorescence-conjugated secondary antibodies, and examined under a Carl Zeiss confocol laser-scanning microscope.

Electron microscopy: Cells were fixed in 2.5% glutaraldehyde in 0.1 mmol/L phosphate buffer saline (pH 7.4) at 4℃ for 24 h, post-fixed in 1% OsO_4 for 1 h, and embedded in Epon 812. They were cut into 30-40 nm sections for ultrastructural evaluation using a Philips TECNAL-10 transmission electron microscope.

Functional identification of the differentiated cells

Glycogen storage: At the final stage of differentiation, the cells were assessed by periodic acid-Schiff reaction for glycogen storage as previously described[23]. Briefly, cells were fixed in Carnoy's solution, oxidized in 1% periodic acid, treated with Schiff's reagent and examined under a Nikon microscope.

Cytochrome P450 activity: Cytochrome P450 activity was examined by ethoxyresorufin O-dealkylase assay. Briefly, cells were maintained under the same conditions in the presence or absence of 5 μmol/L phenobarbital for 24 h followed by treatment with 5 μmol/L ethoxyresorufin for 2-3 h, and then observed under Carl Zeiss confocol laser-scanning microscope at 355 nm excitation and 581 nm emission.

Table 1 Primers and annealing temperatures used for RT-PCR

Gene	Sequence (5'-3')	Product size (bp)	Annealing temperature (℃)
β-actin-F	TTCCTTCTTGGGTATGGAAT	200	55
β-actin-R	GAGCAATGATCTTGATCTTC		
AFP-F	CACTGCTGCAACTCTTCGTA	300	52
AFP-R	CTTTGGACCCTCTTCTGTGA		
HNF3β-F	GACCTCTTCCCTTTCTACCG	551	51
HNF3β-R	TTGAAGGCGTAATGGTGC		
TTR-F	TGCCTCGCTGGACTGGTAT	334	52
TTR-R	CAGAGTCGTTGGCTGTGAA		
DPPIV-F	GATTCCATACCCAAAGGC	587	55
DPPIV-R	GGTCACAACTAAGGCACT		
ALB-F	TCTTCGTCTCCGGCTCTG	475	55
ALB-R	CTGGCAACTTCATGCAAA		
OCT4-F	GGCGTTCTCTTTGGAAAGGTGTTC	313	57
OCT4-R	CTCGAACCACATCCTTCTCT		
AAT-F	AGAACCATTATCAGGCAGAA	675	55
AAT-R	AATAAGGAACGGCTAGTAAGA		
DLK-F	GGGGTGACTTCCGTTGC	510	52
DLK-R	GCTCCTCGCCGCTGTTAT		
HNF4-F	CTTCCAAGAGCTGCAGATTG	517	55
HNF4-R	CTTGTAGGATTCAGATCCCG		
G6P-F	TCAATCTCCTCTGGGTGGC	602	52
G6P-R	GGCAAAGGGTGTAGTGTCAAG		
TAT-F	CTTCAGTGCTGGATGTTCGC	619	55
TAT-R	CAGGGATTGGACGGGTTGTT		
TDO-F	TAAACAGAGCCAGCAAAG	868	56
TDO-R	ATGAGCGTGTCAATGTCC		
BG-F	CGTGAAGGATACGGGAGT	581	55
BG-R	CAGAGTTATTGACGAGGC		
GGT-F	TGTCCCTGGTGAAATCCG	577	55
GGT-R	GGCATAGGCAAACCGAAA		

AFP: α-fetoprotein; HNF: Hepatocyte nuclear factor; TTR: Transthyretin; DPPIV: Cytokeratin 18; ALB: Albumin; OCT4: Octamer-4; AAT: α-1-antitrypsin; Dlk: Δ-like (Pref-1, preadipocyte factor-1); G6p: Glucose-6-phosphatase; TAT: Tyrosine aminotransferase; TDO: Tryptophan 2,3-dioxygenase; BG: Biliary glycoprotein; GGT: γ-glutamyl transpeptidase.

Transplantation assay: To evaluate the *in vivo* function of differentiated hepatocytes, a transplantation assay was performed in CCl_4-intoxicated mice as previously described[24]. Five ICR mice were injected intraperitoneally with 10% CCl_4 in olive oil (1 mL/kg body weight). After 6 h, mice underwent intrasplenic transplantation of differentiated hepatocytes at 1×10^6 cells (0.1 mL of 1×10^7 cells/mL) per mouse. The injection site was ligated to prevent cell leakage and bleeding. Mice were then sacrificed 24 h after transplantation, and sera were collected separately. Liver function was assessed by measuring the total bilirubin (T-Bil), ALT, AST and urea levels. Ten CCl_4-intoxicated and untreated mice were used as controls.

Differentiation of bile duct-like structures: To evaluate the differentiation potential of progenitors into cholangiocytes, a spontaneous and directed bile duct-like structural differentiation assay was designed. For spontaneous differentiation, the progenitors were inoculated into the collagen I coated 96-well plate, and cultured with William's E medium supplemented with 10^{-6} mol/L Dex, 10 ng/mL mHGF for 20-40 d until the special bile duct-like structures appeared. For directed differentiation, the progenitors were seeded on a layer of Matrigel basement membrane matrix and cultured in the medium supplemented with 100 ng/mL mHGF and 50 ng/mL mEGF until the bile duct-like structures formed. Media were changed every 3 d.

Tumorigenic analyses: Undifferentiated ES cells and the hepatic progenitor cells were collected and suspended in DMEM medium (1×10^7 cells/mL). A 0.2 mL aliquot was injected subcutaneously into the backs of Balb/C nude mice. Each experimental group contained three animals. The animals were kept under a controlled lighting schedule with a 12-h dark period. Food and water were available *ad libitum*. All animals received humane care in compliance with institutional guidelines. Mice were sacrificed 6-8 wk after transplantation, the tumors and tumor-like tissues were fixed with formalin. After additional fixation in 4% paraformaldehyde for 2 h at 4℃, tissues were embedded in paraffin. Sections were stained with hematoxylin and eosin, and then microscopically observed.

Cell cycle and apoptosis analyses

Flow cytometry was performed to measure cell cycle distribution and apoptosis of VPA-treated cells. The measurements were made with a Becton Dickinson FACS Calibur machine, adapted for excitation with a 488 nm argon laser, and 582/42 nm band-pass filter for detecting propidium iodide emission.

Statistical analysis

The data are presented as mean ± SD. Statistical tests for the significance of differences between control and transplanted mice were performed by way of Student's *t* test. $P < 0.05$ was considered as statistically significant.

RESULTS

Differentiation of hepatic progenitors from mouse ES cells induced by VPA

To examine whether VPA has an effect on hepatic fate differentiation from mouse ES cells, the ES cultures were subjected to VPA (1 mmol/L) after withdrawing feeder layers and LIF. After treatment for 4-6 d, a small round-shaped progenitor-like population appeared. These cells were then treated with recombinant mouse HGF (10 ng/mL) which allowed the promotion of cellular proliferation. After another 6-12 d, this progenitor-like population underwent rapid growth, and finally proliferated into confluence. The acquired progenitor-like cells exhibited 8-10 μm in diameter, scant cytoplasm and a high nuclear to cytoplasmic ratio (Figure 2A), and resembled the blast-like oval cells proliferating during severe liver injury or the hepatoblasts found in fetal liver. Electron microscopic observation showed that the progenitor-like cells had abundant cell surface microvilli and shared scanty organelles except for a few mitochondria as in ES cells (Figure 2B and C). Noticeably, it was found that few mature functional hepatocytes

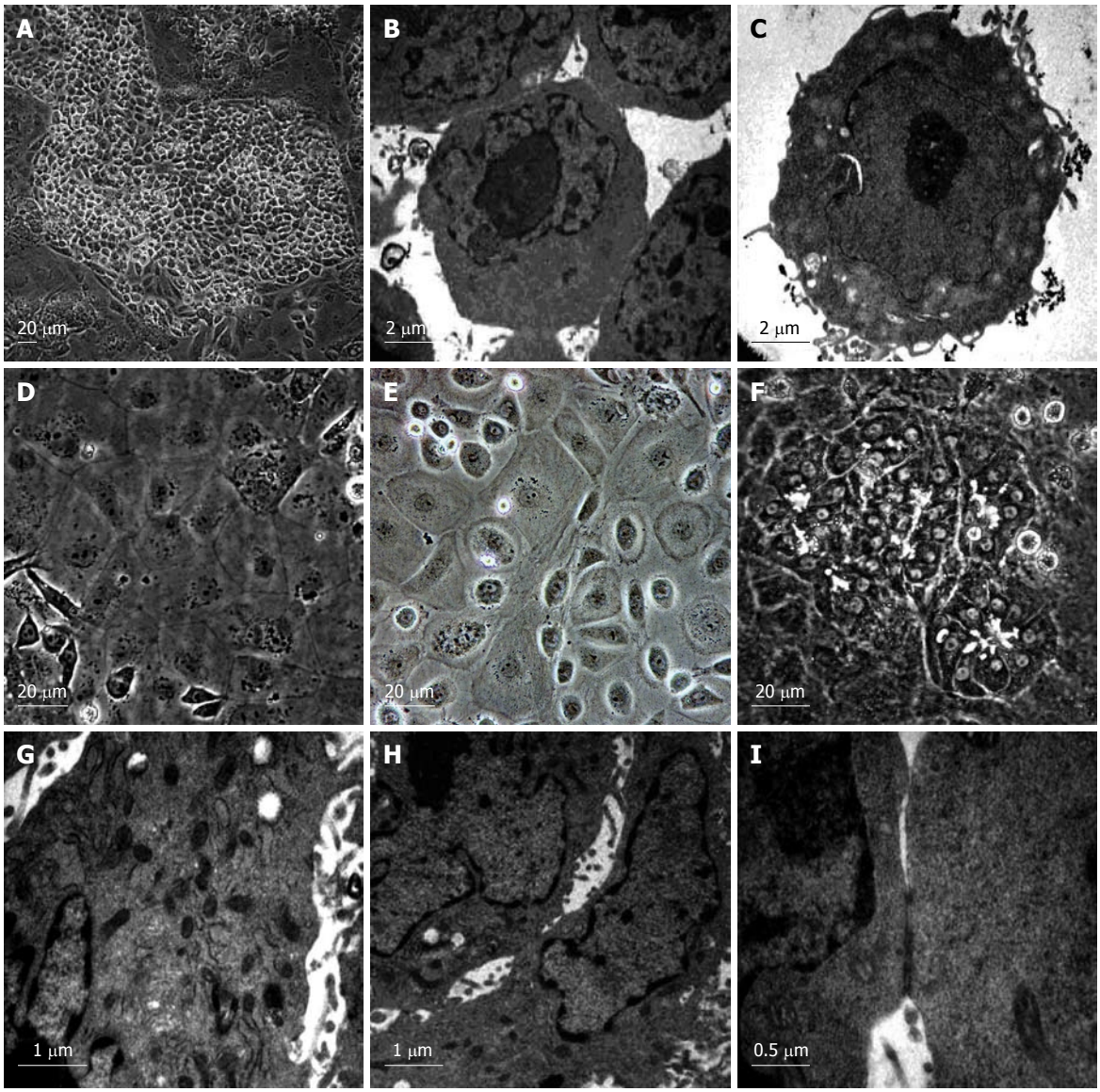

Figure 2 Morphological observation of the differentiated cells. A: Morphology of hepatic progenitor cells induced by VPA; B: Electron microscopic observation of the undifferentiated ES cells (control); C: Electron microscopic observation of the hepatic progenitor cells differentiated from ES cells; D, E: Morphological observation of typical hepatocytes differentiated from hepatic progenitor cells with flattened and cuboidal morphology, and acquired abundant granules in the cytoplasm; F: Morphological observation of another kind of hepatocyte-like cells differentiated from hepatic progenitor cells with rising and piled morphology, dark cytoplasm and light nuclei, and bile canaliculi-like structures found between these cells; G: Electron microscopic observation of the differentiated hepatocytes with abundant mitochondria in their cytoplasm; H: Electron microscopic observation of the bile canaliculi between adjacent cells as mentioned above (Figure 1F); I: The bile canaliculi between adjacent cells sealed with tight junctions.

could be acquired by treating the ES cells with VPA independently, and further differentiation into mature hepatocytes needed the combination of various cytokines, which indicated that VPA probably participates mainly in the regulation of early hepatic differentiation.

Differentiation of hepatocytes from progenitors

For maturation of functional hepatocytes from hepatic progenitors, the progenitor cells were further cultured in William's E medium (containing 10% FBS) supplemented with cytokines (EGF, HGF and insulin) and chemical inducers (Dex, ascorbic acid diphosphate and nicotinamide) for 6 d. Then, the cells were cultured in another medium containing ITS, OSM and Dex. After 6-12 d, two kinds of hepatocyte-like cells appeared. One exhibited typical hepatocyte features with a diameter of 20-40 μm, flattened and cuboidal morphology, and had acquired abundant granules in the cytoplasm (Figure 2D and E). The other displayed a rising/piled morphology with dark cytoplasm and light nuclei, and bile canaliculi-like structures were often found between these cells (Figure 2F). Ultrastructural analyses revealed that both kinds of hepatocyte-like cells contained numerous mitochondria and endoplasmic reticulum (Figure 2G). Bile canaliculi were also observed between adjacent cells and sealed with tight junctions (Figure 2H and I). These results showed that functional hepatocytes could be acquired from VPA-induced progenitors under sequential induction combinations.

Molecular identification of the hepatic lineage cells

To evaluate the characterization of the VPA-induced hepatic progenitors and the hepatic progenitor-derived

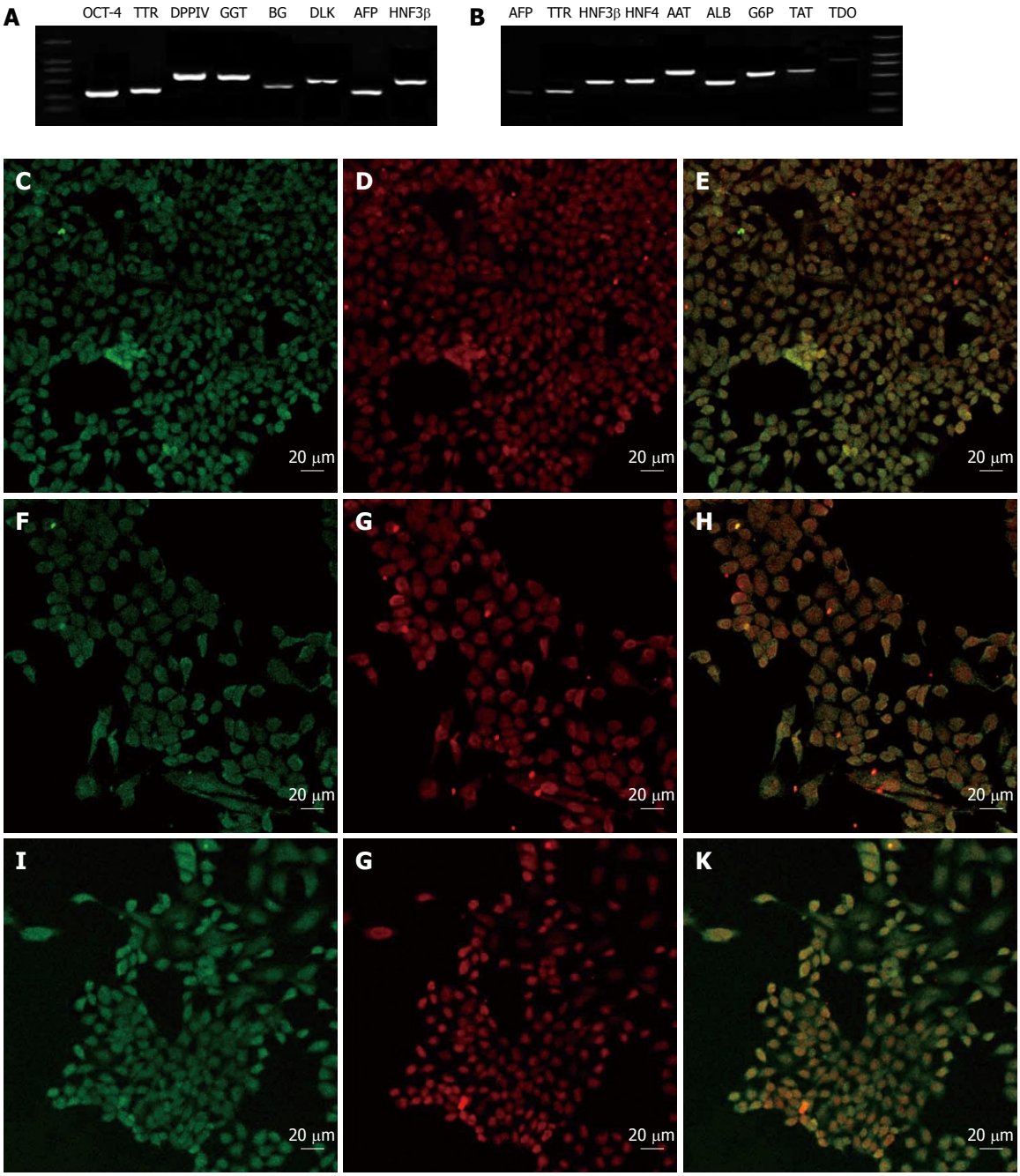

Figure 3 Gene expression analysis of VPA-induced hepatic lineage cells by RT-PCR and immunofluorescence staining. A: RT-PCR showed hepatic progenitor cells after the treatment with valproic acid expressing most typical markers of hepatic/hepatic stem cells; B: The hepatic progenitor-derived hepatocytes expressing typical markers of mature liver cells; C-E: Immunofluorescent images of AFP (C) and OCT-4 (D) staining in VPA-induced hepatic progenitor cells (E is the merged image of C and D); F-H: Immunofluorescent images of AFP (F) and CK19 (G) staining in VPA-induced hepatic progenitor cells (H is the merged image of F and G); I-K: Immunofluorescent images of AFP (I) and DLK (J) staining in VPA-induced hepatic progenitor cells (K is the merged image of I and J).

hepatocytes, a number of gene expression profiles were examined at mRNA and/or protein levels. The results showed that the undifferentiated ES cells expressed SSEA-1 and OCT-4, but no hepatic markers (data not shown). However, the progenitor cells expressed most typical markers of hepatic/hepatic stem cells, such as AFP, TTR, DPPIV, GGT, BG, HNF3β, CK19 and Dlk (Figure 3), but did not express the ES marker SSEA-1 or mature hepatocyte marker ALB (data not shown). Interestingly, the progenitor cells expressed the ES cell marker OCT-4, which suggested that the VPA-induced hepatic progenitor cells may share partial properties of ES cells (Figure 3A and D). Accordingly, the hepatic progenitor-derived hepatocytes expressed typical markers of mature liver cells, including ALB, AAT, HNF4, G6p, TAT, AFP, TTR, HNF3β and TDO (Figure 3B).

Functional characterization of the hepatic lineage cells

For functional evaluation of the progenitor cells, the *in vitro* bi-differentiation potential of the progenitors into bile duct-like structures and mature hepatocytes was determined. The results showed that bile duct-like structures

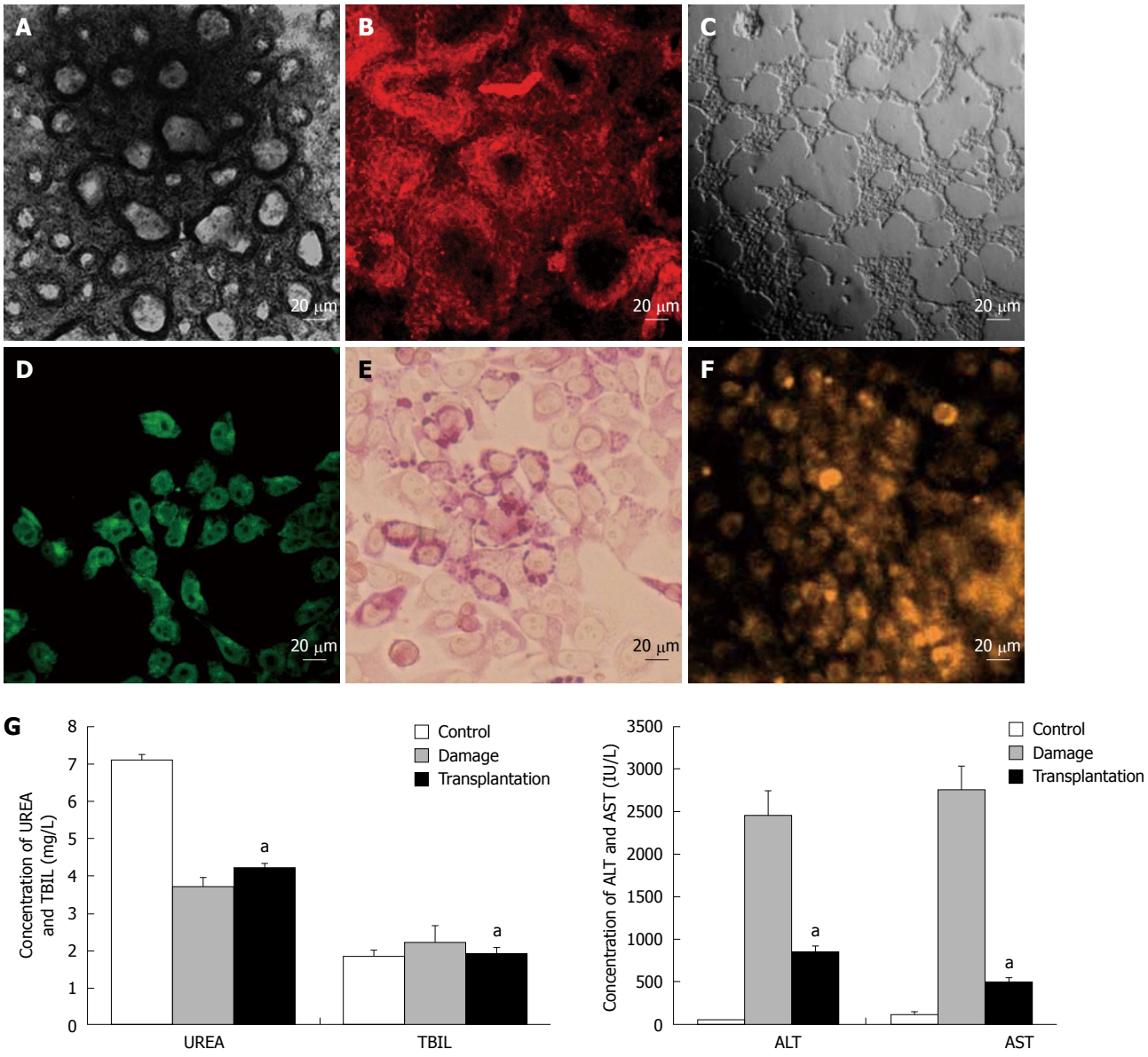

Figure 4 Functional characterization of the hepatic lineage cells. A: Bile duct-like structures formed from the VPA-induced hepatic progenitor cells cultured on collagen I coated dishes; B: Bile duct-like structures were stained CK19 positive; C: Bile duct-like structures formed from the VPA-induced hepatic progenitor cells cultured on Matrigel basement membrane matrix; D-F: Differentiated hepatocytes from hepatic progenitor cells could synthesize albumin (D), store glycogen (E) and possessed cytochrome P450 activity (F) after induction for 2-3 wk; G: When ES-HPCs derived hepatocytes were transplanted 6 h after liver intoxication, serum total bilirubin (TBIL), serum ammonia (UREA), AST and ALT levels were significantly improved towards normal at 1 d after the transplantation. $^{a}P < 0.05$.

could be formed from the progenitor cells in two ways. In the first way, the progenitor cells were cultured on collagen I coated dishes in the medium supplemented with 10^{-6} mol/L Dex and 10 ng/mL mHGF for about 30 d until the foci of small dark cells appeared, and these foci became organized and developed as doughnut-like structures identical to bile duct units (Figure 4A). In the second way, the progenitor cells were seeded on a layer of Matrigel basement membrane matrix and cultured in the medium supplemented with 100 ng/mL mHGF and 50 ng/mL mEGF as previously described. Many well-defined duct-like structures comprised of neatly aligned cells were found throughout the dish 1-2 d after inoculation, which developed into spherical 3-dimensional structures consisting of tightly packed columnar epithelium along with a central lumen when further maintained for another 15 d (Figure 4C). Immunofluorescence analysis demonstrated that the structures were CK19-positive (Figure 4B) and AFP-negative (data not shown), which are typical hallmarks seen in the bile duct.

Accordingly, typical hepatocytes could also be attained from the progenitors as mentioned above. Functional characterization analyses showed that the differentiated hepatocytes could synthesize albumin (Figure 4D), store glycogen (Figure 4E) and possessed cytochrome P450 activity (Figure 4F) after differentiation for 2-3 wk. Furthermore, an *in vivo* transplantation assay of hepatocytes in acute-injured liver was performed. The results showed that when the differentiated hepatocytes were transplanted 6 h after liver intoxication, serum T-Bil, serum ammonia, AST and ALT levels were significantly ($P < 0.05$) improved towards normal levels compared to con-

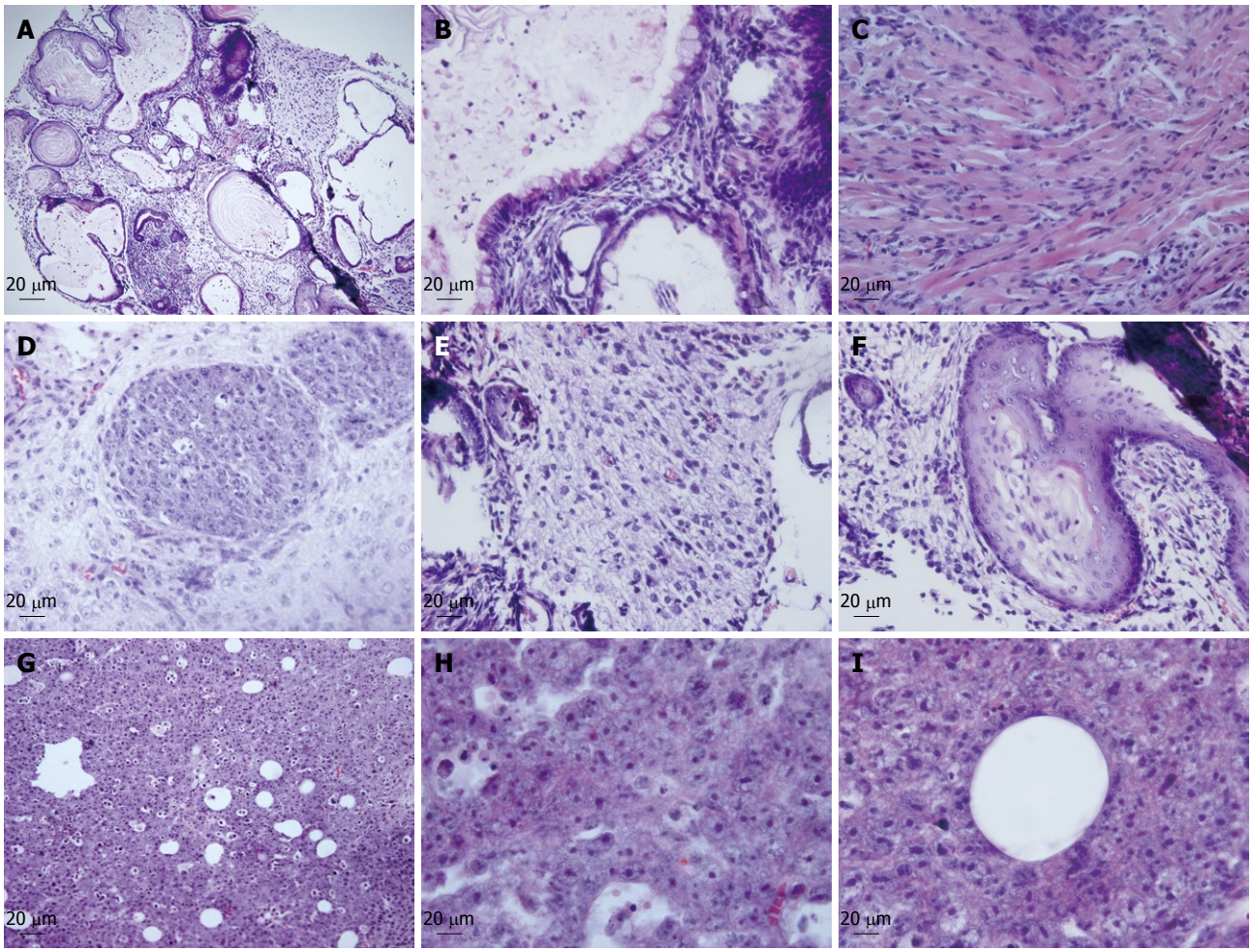

Figure 5 ***In vivo*** **multi-differentiation potential and tumorigenic analyses.** A-F: The teratomas derived from ES cells were composed of a variety of types of differentiated cells from all three primary germ layers including secretory glands (B), muscle (C), epithelium (D), neuroectodermal cells (E) and cartilage (F); G-I: The tumor-like bumps derived from ES-HPCs contained only epithelial (H) and mesenchymal-like cells, and duct-like structures (I) surrounded by several epithelial cells were also observed.

trol mice without hepatocyte transplantation (Figure 4G), suggesting that the hepatocytes functioned normally *in vivo* and their transplantation improved the liver function of CCl_4-treated mice.

Tumorigenic and in vivo differentiation potential analyses

To evaluate the *in vivo* oncogenicity and differentiation potential of the progenitor cells, we introduced these cells and undifferentiated mES cells (as control) into Balb/c nude mice. Two weeks post-inoculation, the mice implanted with ES cells developed apparent teratomas at the injection site, while the mice with progenitor cells developed tumor-like bumps about 3-4 wk later. After the development of 6-8 wk, the teratomas and tumor-like bumps were fixed and examined with HE staining. The teratomas derived from ES cells were composed of a variety of types of differentiated cells from all three primary germ layers including neuroectodermal cells, adipocytes, muscle and epithelium (Figure 5A-F). However, the tumor-like bumps derived from the progenitor cells contained only epithelial and mesenchymal-like cells, and duct-like structures surrounded by epithelial cells were also observed (Figure 5G-I). The results further confirmed that the progenitor cells have *in vivo* differentiation potential into hepatic lineages, and indicated the difference in *in vivo* potential between the undifferentiated ES cells and hepatic progenitor cells.

Cell cycle and apoptosis analyses of VPA-induced hepatic differentiation

To evaluate whether VPA had an effect on cell cycle profile, ES cells were exposed to 1 mmol/L VPA for 0, 3, 6 d and cell cycle analyses were performed with FACS. Results indicated that exposure to VPA decreased the proportion of cells in S phase and increased the G0/G1 phase proportion. Approximately 75% of the cells were arrested in the G0/G1 phase and only 10% of the cells were in the S phase after 6 d of treatment with VPA, whereas over 37% of control ES cells were in the S phase (Figure 6A). These data indicated that VPA could reduce the proliferation of ES cells and cause the inhibition of G1-S transition. In addition, DNA content analysis of the cells treated with VPA for 3 d showed that about 28% of the cells adopted apoptotic features (Figure 6B). Furthermore, the ultrastructural observations also showed that a considerable number of cells presented typical

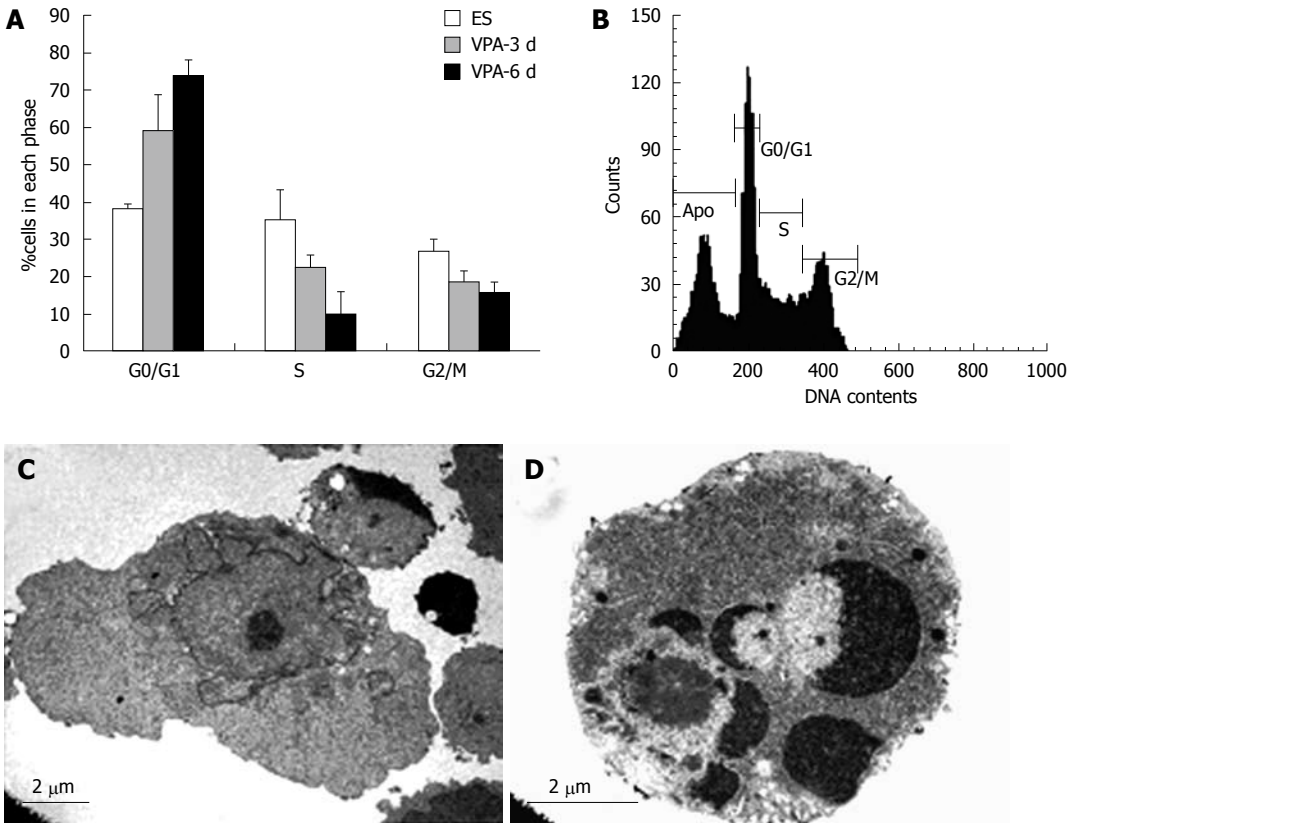

Figure 6 VPA-induced cell cycle arrest and apoptosis during hepatic differentiation. A: Cell cycle analysis revealed that exposure to VPA decreased the proportion of cells in S phase and increased the proportion of cells in the G0/G1 phase. Approximately 75% of the cells were arrested in the G0/G1 phase and only 10% of the cells in the S phase after 6 d of treatment with 1 mmol/L VPA, whereas greater than 37% of control ES cells were in the S phase; B: The analysis of apoptosis proportions during 3 d of treatment with VPA; C, D: Ultrastructural observations showed that some cells presented typical apoptotic morphology when treated with VPA for more than 5 d.

apoptotic morphology after being treated with VPA for more than 5 d (Figure 6C and D).

DISCUSSION

To develop well-defined *in vitro* protocols for directing cellular differentiation into hepatic lineage has become critical for better investigating the mechanisms of hepatocyte differentiation, and for providing seed cells for hepatic tissue engineering as well as for clinical purposes. Most of the protocols currently developed to induce the hepatic differentiation from ES cells were devised by using embryoid bodies. However, several disadvantages may exist in this method. For example, it is time-consuming to prepare the embryoid bodies, and hepatic differentiation through embryoid bodies may result in low yield and purity of functional hepatocytes. Therefore, direct hepatic fate differentiation from an ES monolayer without using embryoid bodies is a most promising concept. A previous study has demonstrated that hepatocyte-like cells could be directly induced from human ES cells by using a combination of cytokines, providing preliminary evidence of the possibilities for this method[25]. However, the direct hepatic differentiation of ES cells still remains a challenge and needs to be further developed. In the present study, we report a novel strategy for the direct hepatic fate differentiation of ES cells, which allows hepatic differentiation from progenitor cells to functional hepatocytes, based on a combination of VPA and cytokines. The results show this strategy has obvious advantages, such as being easy to operate, well reproducible and capable of acquirement of abundant and uniform progenitor cells and mature hepatocytes, the latter of which are suitable for the large-scale requirements of cell replacement therapy. Noticeably, following this strategy, we can harvest progenitor cells and mature hepatocytes in a sequential order. Thus, it may provide an *in vitro* research model which could meet the requirement of precise study regarding the mechanisms of hepatic differentiation at different developmental stages.

In addition, histone acetylation is considered to be one of the most important epigenetic regulation processes involved in gene expression, which is largely controlled by HDAC inhibitors including VPA. The observation that VPA could induce hepatic progenitor differentiation from ES cells suggested that epigenetic regulation mediated by histone acetylation might play an important role in early hepatic development. Therefore, the VPA-induced hepatic differentiation may also provide a model for the study of early hepatic developmental events, such as the relationship between the initiation of hepatic differentiation and epigenetic modification.

By FACS and ultrastructural analysis, it was found

that treatment of ES cells with VPA significantly reduced the proportion of the cells in the S phase and promoted the accumulation in the G0/G1 phase, which was accompanied by cellular apoptosis. This finding suggests that cell cycle arrest and apoptosis are involved in the VPA-induced hepatic specification. Further study is needed to elucidate the exact molecular and cellular mechanisms underlying the VPA-induced hepatic cell fate determination from ES cells.

Several lines demonstrated that the VPA-induced progenitor cells possessed some distinctive characteristics distinguished from ES cells or traditionally identified hepatoblasts or hepatic oval cells. For example, the progenitor cells exhibited typical epithelial morphology when cultured in a collagen coated dish, while ES cells formed multilayer compact colonies. There were many condensed-stained heterochromatin areas in the nucleus of ES cells, while the progenitor cells were almost all euchromatic. Also, the results of an *in vivo* differentiation assay revealed that ES cells formed typical teratomas containing the structures of three primary germ layers, while the progenitor cells formed tumor-like bumps containing only epithelial and mesenchymal cells. Moreover, the acquired progenitor cells expressed a number of typical markers of hepatoblasts or oval cells, such as AFP, TTR, Foxa2, DPPIV, GGT and BG. Importantly, Dlk, a marker of hepatic stem cells (hepatoblasts or hepatic oval cells) recently reported[26-28], was also expressed by the progenitor cells. However, investigation of the hepatic oval cell marker, A6, was negative[29,30]. In addition, the progenitor cells expressed OCT-4, a marker of multi-lineage stem/progenitor cells[31-33]. These data indicated that the acquired progenitor cells may represent a novel subset at a developmental stage between ES cells and the known hepatic stem/progenitor cells, suggesting that a novel progenitor population was involved in the VPA-induced hepatic differentiation. This finding will be of benefit for understanding more about the early differentiation of hepatocytes.

Importantly, mouse is an often-used model species for human disease, so the investigation of hepatic differentiation of mouse ES cells will be of benefit for the better understanding of mechanisms underlying human hepatic differentiation and the development involved. Our results also support the establishment of new strategies to acquire human hepatic progenitor cells as well as hepatocytes, which will be helpful for the solution of obtaining cell sources for clinical cytotherapy.

COMMENTS

Background

Embryonic stem (ES) cells, known for their capacity to proliferate indefinitely and differentiate into almost all types of cells including hepatocytes, have raised the hope of cellular replacement therapy for liver failure. There have been several protocols available for hepatic fate specification from ES cells, however, most of the protocols currently used result in low yield or purity of functional hepatocytes. Valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, has been demonstrated to facilitate the hepatic differentiation of mesenchymal stem cells. However, little is know about whether VPA could induce the hepatic differentiation of ES cells.

Research frontiers

The research hotspot is to develop well-defined protocols for directing cellular differentiation into hepatic lineage, followed by selective isolation and proliferation *in vitro*.

Innovations and breakthroughs

In the present study, the authors report a novel strategy for the direct hepatic fate differentiation of ES cells, which allows the hepatic differentiation from progenitor cells to functional hepatocytes, based on using a combination of VPA and cytokines. The results show that this strategy has obvious advantages, such as being easy to operate, well reproducible and capable of acquirement of abundant and uniform progenitor cells and mature hepatocytes, the latter of which are suitable for the large-scale requirements of cell replacement therapy. Noticeably, following this strategy, they can harvest progenitor cells and mature hepatocytes in a sequential order. Thus, it may provide an *in vitro* research model which could meet the requirements of precise study regarding the mechanisms of hepatic differentiation at different developmental stages. In addition, the observation that VPA could induce hepatic progenitor differentiation from ES cells suggested that epigenetic regulation mediated by histone acetylation might play an important role in early hepatic development. Therefore, the VPA-induced hepatic differentiation may also provide a model for the study of early hepatic developmental events, such as the relationship between the initiation of hepatic differentiation and epigenetic modification.

Applications

The present study may not only be helpful for the clinical application of hepatocyte transplantation, but also provide an *in vitro* research model for the better investigation and understanding of the entire developmental process of hepatocytes, from ES cells to hepatic progenitors, and then to a population of mature hepatocytes.

Terminology

VPA, a HDAC inhibitor, has been used as a new class of chemotherapeutic drug for cancer clinical purposes and as an inducer for stem cell differentiation.

Peer review

This is an important area for research to find new sources of hepatocytes for future clinical application. The authors have performed a good study.

REFERENCES

1 **Tanaka K**, Soto-Gutierrez A, Navarro-Alvarez N, Rivas-Carrillo JD, Jun HS, Kobayashi N. Functional hepatocyte culture and its application to cell therapies. *Cell Transplant* 2006; **15**: 855-864

2 **Strom SC**, Bruzzone P, Cai H, Ellis E, Lehmann T, Mitamura K, Miki T. Hepatocyte transplantation: clinical experience and potential for future use. *Cell Transplant* 2006; **15** Suppl 1: S105-S110

3 **Nussler A**, Konig S, Ott M, Sokal E, Christ B, Thasler W, Brulport M, Gabelein G, Schormann W, Schulze M, Ellis E, Kraemer M, Nocken F, Fleig W, Manns M, Strom SC, Hengstler JG. Present status and perspectives of cell-based therapies for liver diseases. *J Hepatol* 2006; **45**: 144-159

4 **Zhou QJ**, Huang YD, Xiang LX, Shao JZ, Zhou GS, Yao H, Dai LC, Lu YL. In vitro differentiation of embryonic stem cells into hepatocytes induced by fibroblast growth factors and bone morphological protein-4. *Int J Biochem Cell Biol* 2007; **39**: 1714-1721

5 **Yamamoto H**, Quinn G, Asari A, Yamanokuchi H, Teratani T, Terada M, Ochiya T. Differentiation of embryonic stem cells into hepatocytes: biological functions and therapeutic application. *Hepatology* 2003; **37**: 983-993

6 **Chinzei R**, Tanaka Y, Shimizu-Saito K, Hara Y, Kakinuma S, Watanabe M, Teramoto K, Arii S, Takase K, Sato C, Terada N, Teraoka H. Embryoid-body cells derived from a mouse embryonic stem cell line show differentiation into functional hepatocytes. *Hepatology* 2002; **36**: 22-29

7 **Yamada T**, Yoshikawa M, Kanda S, Kato Y, Nakajima Y, Ishizaka S, Tsunoda Y. In vitro differentiation of embryonic stem cells into hepatocyte-like cells identified by cellular uptake of indocyanine green. *Stem Cells* 2002; **20**: 146-154

8 **Cai J**, Zhao Y, Liu Y, Ye F, Song Z, Qin H, Meng S, Chen Y, Zhou R, Song X, Guo Y, Ding M, Deng H. Directed

differentiation of human embryonic stem cells into functional hepatic cells. *Hepatology* 2007; **45**: 1229-1239
9 **Teratani T**, Yamamoto H, Aoyagi K, Sasaki H, Asari A, Quinn G, Sasaki H, Terada M, Ochiya T. Direct hepatic fate specification from mouse embryonic stem cells. *Hepatology* 2005; **41**: 836-846
10 **Marks P**, Rifkind RA, Richon VM, Breslow R, Miller T, Kelly WK. Histone deacetylases and cancer: causes and therapies. *Nat Rev Cancer* 2001; **1**: 194-202
11 **Cress WD**, Seto E. Histone deacetylases, transcriptional control, and cancer. *J Cell Physiol* 2000; **184**: 1-16
12 **Glozak MA**, Seto E. Histone deacetylases and cancer. *Oncogene* 2007; **26**: 5420-5432
13 **Huang L**. Targeting histone deacetylases for the treatment of cancer and inflammatory diseases. *J Cell Physiol* 2006; **209**: 611-616
14 **Venturelli S**, Armeanu S, Pathil A, Hsieh CJ, Weiss TS, Vonthein R, Wehrmann M, Gregor M, Lauer UM, Bitzer M. Epigenetic combination therapy as a tumor-selective treatment approach for hepatocellular carcinoma. *Cancer* 2007; **109**: 2132-2141
15 **Pathil A**, Armeanu S, Venturelli S, Mascagni P, Weiss TS, Gregor M, Lauer UM, Bitzer M. HDAC inhibitor treatment of hepatoma cells induces both TRAIL-independent apoptosis and restoration of sensitivity to TRAIL. *Hepatology* 2006; **43**: 425-434
16 **Armeanu S**, Pathil A, Venturelli S, Mascagni P, Weiss TS, Göttlicher M, Gregor M, Lauer UM, Bitzer M. Apoptosis on hepatoma cells but not on primary hepatocytes by histone deacetylase inhibitors valproate and ITF2357. *J Hepatol* 2005; **42**: 210-217
17 **Hsieh J**, Nakashima K, Kuwabara T, Mejia E, Gage FH. Histone deacetylase inhibition-mediated neuronal differentiation of multipotent adult neural progenitor cells. *Proc Natl Acad Sci USA* 2004; **101**: 16659-16664
18 **Siebzehnrubl FA**, Buslei R, Eyupoglu IY, Seufert S, Hahnen E, Blumcke I. Histone deacetylase inhibitors increase neuronal differentiation in adult forebrain precursor cells. *Exp Brain Res* 2007; **176**: 672-678
19 **Rambhatla L**, Chiu CP, Kundu P, Peng Y, Carpenter MK. Generation of hepatocyte-like cells from human embryonic stem cells. *Cell Transplant* 2003; **12**: 1-11
20 **Cho HH**, Park HT, Kim YJ, Bae YC, Suh KT, Jung JS. Induction of osteogenic differentiation of human mesenchymal stem cells by histone deacetylase inhibitors. *J Cell Biochem* 2005; **96**: 533-542
21 **Chen Y**, Pan RL, Zhang XL, Shao JZ, Xiang LX, Dong XJ, Zhang GR. Induction of hepatic differentiation of mouse bone marrow stromal stem cells by the histone deacetylase inhibitor VPA. *J Cell Mol Med* 2008; [Epub ahead of print]
22 **Zhou QJ**, Shao JZ, Xiang LX, Hu RZ, Lu YL, Yao H, Dai LC. Generation of embryoid bodies from mouse embryonic stem cells cultured on STO feeder cells. *Cell Biol Int* 2005; **29**: 817-825
23 **Kania G**, Blyszczuk P, Jochheim A, Ott M, Wobus AM. Generation of glycogen- and albumin-producing hepatocyte-like cells from embryonic stem cells. *Biol Chem* 2004; **385**: 943-953
24 **Kumashiro Y**, Asahina K, Ozeki R, Shimizu-Saito K, Tanaka Y, Kida Y, Inoue K, Kaneko M, Sato T, Teramoto K, Arii S, Teraoka H. Enrichment of hepatocytes differentiated from mouse embryonic stem cells as a transplantable source. *Transplantation* 2005; **79**: 550-557
25 **Hay DC**, Zhao D, Ross A, Mandalam R, Lebkowski J, Cui W. Direct differentiation of human embryonic stem cells to hepatocyte-like cells exhibiting functional activities. *Cloning Stem Cells* 2007; **9**: 51-62
26 **Tanimizu N**, Tsujimura T, Takahide K, Kodama T, Nakamura K, Miyajima A. Expression of Dlk/Pref-1 defines a subpopulation in the oval cell compartment of rat liver. *Gene Expr Patterns* 2004; **5**: 209-218
27 **Tanimizu N**, Saito H, Mostov K, Miyajima A. Long-term culture of hepatic progenitors derived from mouse Dlk+ hepatoblasts. *J Cell Sci* 2004; **117**: 6425-6434
28 **Tanimizu N**, Nishikawa M, Saito H, Tsujimura T, Miyajima A. Isolation of hepatoblasts based on the expression of Dlk/Pref-1. *J Cell Sci* 2003; **116**: 1775-1786
29 **Engelhardt NV**, Factor VM, Medvinsky AL, Baranov VN, Lazareva MN, Poltoranina VS. Common antigen of oval and biliary epithelial cells (A6) is a differentiation marker of epithelial and erythroid cell lineages in early development of the mouse. *Differentiation* 1993; **55**: 19-26
30 **Petersen BE**, Grossbard B, Hatch H, Pi L, Deng J, Scott EW. Mouse A6-positive hepatic oval cells also express several hematopoietic stem cell markers. *Hepatology* 2003; **37**: 632-640
31 **Carlin R**, Davis D, Weiss M, Schultz B, Troyer D. Expression of early transcription factors Oct-4, Sox-2 and Nanog by porcine umbilical cord (PUC) matrix cells. *Reprod Biol Endocrinol* 2006; **4**: 8
32 **Mossman AK**, Sourris K, Ng E, Stanley EG, Elefanty AG. Mixl1 and oct4 proteins are transiently co-expressed in differentiating mouse and human embryonic stem cells. *Stem Cells Dev* 2005; **14**: 656-663
33 **Rathjen J**, Lake JA, Bettess MD, Washington JM, Chapman G, Rathjen PD. Formation of a primitive ectoderm like cell population, EPL cells, from ES cells in response to biologically derived factors. *J Cell Sci* 1999; **112** (Pt 5): 601-612

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BRIEF ARTICLE

Modification of sleep architecture in an animal model of experimental cirrhosis

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Abstract

AIM: To analyze the polygraphic sleep patterns during cirrhosis progression in a rat model by repeated CCl_4 administration.

METHODS: Male Wistar rats received three weekly injections of CCl_4 for 11 wk, and were analyzed before and during the induction of cirrhosis. Rats were implanted with electrodes to record their sleep patterns. Polygraph recordings were made weekly over 11 wk for 8 h, during the light period. After a basal recording, rats received three weekly injections of CCl_4. Histological confirmation of cirrhosis was performed after 11 wk.

RESULTS: The results showed a progressive decrease in total wake time that reached statistical significance from the second week of treatment. In addition, there was an increase in total time of slow wave sleep (SWS) II and rapid eye movement sleep (REM sleep) in most of the 11 wk. SWS I showed no significant variations. During the final weeks, a significant increase in REM sleep frequency was also observed. Histological analyses of the livers showed unequivocal signs of cirrhosis.

CONCLUSION: These data suggest that hepatic failure produced by CCl_4 administration is capable of modifying the sleep pattern even after only a few doses.

Key words: Experimental cirrhosis; Sleep; Rapid eye movement sleep; CCl_4; Wakefulness

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INTRODUCTION

The sleep-wakefulness cycle is an important circadian rhythm in mammals and is characterized by a reduction in the level of consciousness and by specific metabolic activities. Sleep is controlled by multiple areas of the brain and by several chemical factors, and can be readily modified by different activities, drugs, and pathological process, such as exercise, stress, alcoholism, depression, and metabolic diseases[1].

Cirrhosis, on the other hand, is an irreversible dysfunction of the liver characterized by damage of the parenchyma, alteration of the reticular structure and the connective tissue that sustains the lobules and sinusoids[2]. Cirrhosis can progress to hepatic encephalopathy

and to coma[3], but even before these advanced stages, functional alterations of several brain nuclei have been detected during early stages by magnetic transfer ratio, a magnetic resonance imaging technique[4]. Cirrhosis could also impact pulmonary function and might be involved in the development of obstructive sleep apnea syndrome (OSAS) in patients with ascitis; however, the early stages have not been associated with OSAS[5]. In the advanced stages, cirrhotic patients show an increased frequency of moderate obstructive sleep apnea[6]. In addition, cirrhotic patients with metabolic alterations frequently show abnormalities in electroencephalographic recordings[7]. Furthermore, patients with severe cirrhosis show a significant increase in power potency associated with the theta frequency and a decrease associated with the α frequency in the electroencephalogram (EEG)[8,9]. Recently, Mostacci *et al*[10] reported significant sleep alterations in 178 patients with cirrhosis when compared to normal control subjects using questionnaires: the basic Nordic sleep and the Epworth Sleepiness Scale. They reported that patients with cirrhosis complained of more daytime sleepiness, because they had fragmented nocturnal sleep caused by frequent nocturnal waking and had difficulties for falling asleep.

Through questionnaires of quality of life, sleep disturbances have been recognized as one of the early signs in patients with cirrhosis and hepatic encephalopathy[11]. Sleep disturbances in cirrhosis has not been correlated with clinical parameters or with cognitive impairment. Cirrhotic subjects with unsatisfactory sleep show higher scores for depression and anxiety, raising the possibility that the effects of these chronic emotional alterations might underlie the pathogenesis of sleep disturbances. Moreover, cirrhotic patients show reduced sleep time, increased latencies to sleep and frequent wakening. These alterations are not due to previously prescribed medications, but are related to abnormalities of the circadian system[12].

From this evidence we can conclude that hepatic cirrhosis causes a series of cerebral changes, which could modify several behaviors, such as sleep. Alterations of the sleep pattern as hepatic disease develops have not been reported. In addition, chronic administration of CCl_4 in rats induces reactive free radicals that attack membrane components, culminating in cell death[13] and promoting fibrosis[14]. This is a reliable procedure to induce experimental hepatic cirrhosis. The aim of this study was to analyze the sleep pattern in rats chronically treated with CCl_4 as hepatic damage progresses.

MATERIALS AND METHODS

Animals

Ten adult male Wistar rats (250-280 g) were used in this study. The animals were housed in a temperature-controlled room (22℃) and under a 12:12 normal light-dark cycle (light ON at 08:00 am). They were kept in individual clear polycarbonate cages with food and water available *ad libitum*. The experiments were performed following the guidelines of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

Sleep recording

Under deep general anesthesia induced with a cocktail (ketamine: 3.75 mg/100 g, xylazine: 0.19 mg/100 g, and acepromazine: 0.038 mg/100 g ip), rats were chronically implanted with a standard set of electrodes for sleep recording. Two stainless steel screw electrodes were implanted in the frontal and parietal cortex for the EEG and flexible wires were inserted in the neck muscles to record an electromyogram.

CCl_4 treatment

One week after surgery, and after 3 d of habituation to the recording conditions, rats (n = 10) polygraph recordings were made to obtain their basal sleep parameters. Thereafter, animals received an intraperitoneal (ip) injection, three times a week, containing different dilutions of CCl_4 and mineral oil, always in a total volume of 0.25 mL. Treatment lasted for 11 wk under the following pattern of administration: in the first week the animals received a solution with one part of CCl_4 and six parts of mineral oil (1:6). In the second week, the proportion of the solution changed to 1:5. In the third week the proportion of the solution was 1:4. From week four to week eleven the proportion of the solution was 1:3.

Polygraph recordings

Polygraph recordings of the rats were obtained for 8 h within the light period, once every week, with the Nihon-Kohden model polygraph. Thus, sleep recordings were done before treatment and after every three injections. In addition to the polygraph recordings, rat behavior was observed during the recording period. The polygraph recordings were scored visually according to standard criteria[15]. The frequency and the duration of wakefulness, slow wave sleep (SWS) Ⅰ, SWS Ⅱ, and rapid eye movement (REM) sleep were quantified.

Histological liver slices

Half of the animals died during the treatment (Initial n = 10; final n = 5). After sleep recordings, animals were killed by an overdose of pentobarbital and their livers were removed for histological examination. Liver slices were stained with hematoxylin-eosin and their morphological characteristics were determined.

Statistical analysis

All data were expressed as mean ± SD and analyzed by SPSS 11.0 software. Data were analyzed using an ANOVA for repeated measurements, followed by Fisher post hoc comparisons to detect significant differences between groups. $P < 0.05$ was considered statistically significant.

RESULTS

Sleep pattern

Treatment with CCl_4 induced behavioral changes in all

Table 1 CCl_4 effect on sleep architecture for 11 wk of treatment (min) (mean ± SE)

Treatment	Wake total time	SWS Ⅰ total time	SWS Ⅱ total time	REM sleep total time	Duration of REM sleep epochs	Frequency of REM sleep	Latency of REM sleep	Latency of SWS
Control (n = 10)	169.03 ± 14.94	89.67 ± 9.21	174.82 ± 9.516	42.32 ± 5.47	2.16 ± 0.21	17.3 ± 1.315	48.84 ± 11.54	37.06 ± 6.78
1 wk (n = 10)	142.93 ± 9.21	109.69 ± 12.89	158.40 ± 13.32	67.64 ± 4.89[b]	2.95 ± 0.27	23 ± 2.27	69.7 ± 26.61	25.60 ± 6.05
2 wk (n = 10)	128.85 ± 9.74[b]	87.45 ± 8.45	213.62 ± 6.07[a]	49.52 ± 7.39	2.56 ± 0.14	17.7 ± 1.93	80.27 ± 19.98	26.57 ± 5.58
3 wk (n = 9)	133.40 ± 9.26[a]	74.36 ± 14.90	214.38 ± 18.94[a]	57.30 ± 5.44	2.44 ± 0.13	22.22 ± 1.98	51.45 ± 16.44	18.99 ± 4.43
4 wk (n = 9)	107.77 ± 11.07[b]	80.69 ± 10.27	225.89 ± 10.20[b]	58.60 ± 7.94[a]	2.44 ± 0.160	22.22 ± 2.17	63.80 ± 17.81	17.84 ± 3.41
5 wk (n = 9)	107.52 ± 12.58[b]	74.27 ± 13.55	232.54 ± 14.85[b]	67.24 ± 5.45[b]	2.70 ± 0.27	23.55 ± 1.74	73.67 ± 19.52	25.04 ± 4.98
6 wk (n = 7)	121.19 ± 11.64[b]	86.08 ± 8.86	208.17 ± 16.42	63.64 ± 3.37[a]	2.72 ± 0.26	22.42 ± 2.01	79.99 ± 18.22	17.58 ± 2.30
7 wk (n = 7)	126.87 ± 7.28[b]	93.07 ± 10.69	191.5 ± 13.90	61.47 ± 8.03[a]	2.31 ± 0.21	24.85 ± 3.47	71.49 ± 23.91	19.81 ± 4.54
8 wk (n = 7)	99.64 ± 12.18[b]	82.11 ± 8.89	230.48 ± 14.96[b]	66.93 ± 9.00[b]	1.96 ± 0.23	31.42 ± 2.17[b]	75.81 ± 11.97	18.08 ± 4.95
9 wk (n = 6)	120.06 ± 17.93[b]	78.53 ± 7.85	215.29 ± 16.85[a]	65.41 ± 3.90[a]	2.56 ± 0.40	24.33 ± 2.78	85.26 ± 32.18	20.03 ± 6.91
10 wk (n = 5)	82.03 ± 8.25[b]	66.91 ± 13.22	256.29 ± 22.37[b]	68.89 ± 3.72[b]	2.60 ± 0.50	26.6 ± 2.56[b]	60.46 ± 36.58	17.15 ± 7.64
11 wk (n = 5)	72.79 ± 11.77[b]	77.34 ± 17.61	257.84 ± 18.77[b]	71.28 ± 7.67[b]	2.63 ± 0.41	26.4 ± 2.56[b]	39.64 ± 10.70	17.24 ± 5.22

[a]P < 0.05, [b]P < 0.01 *vs* control. Repeated measure ANOVA.

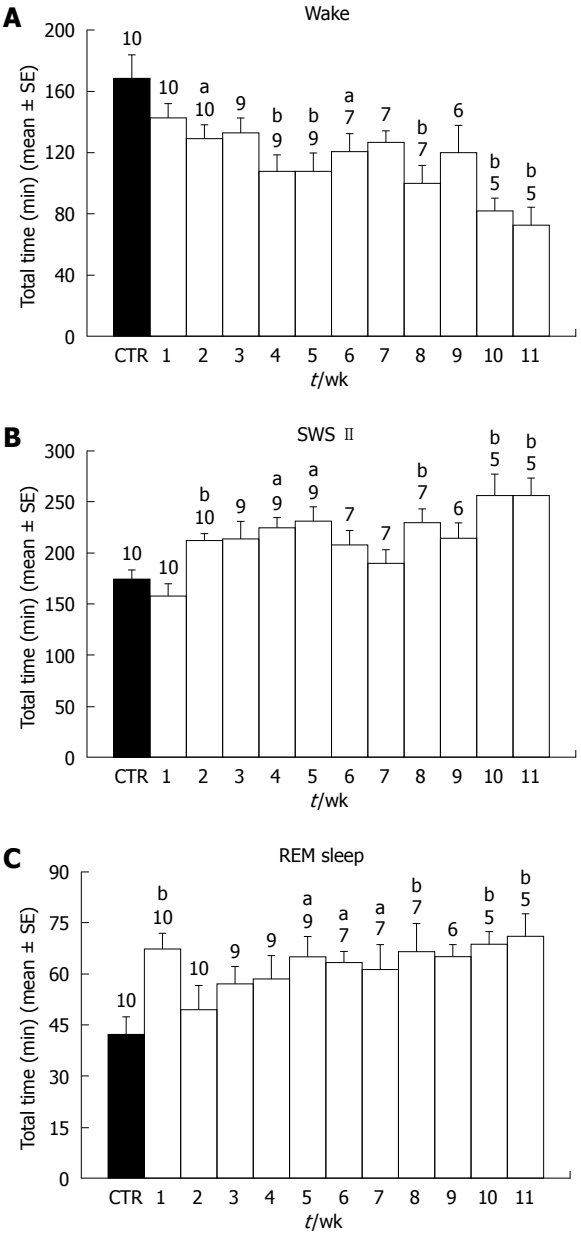

Figure 1 Percentage of Wakefulness (A), SWS Ⅱ (B), and REM sleep (C) in weekly 8 h sleep recordings during treatment with CCl_4. Numbers at the top of the bars express the number of subjects. [a]P < 0.05, [b]P < 0.01 *vs* control. Repeated measurements were analyzed by ANOVA.

rats, characterized mainly by a progressive decrease in locomotion. During the final 2 wk, four out of the five surviving animals showed ascitis. Five of the animals died before the end of the 11-wk period, so the size of the group decreased as the treatment progressed. Concerning sleep, CCl_4 administration elicited a decrease of wake time throughout the 11 wk of treatment. The decrease was observed from the first week of treatment but only reached statistical significance from the second week. This decrease in wake time grew larger as the treatment progressed, and during weeks 10 and 11, wake time was less than 50% of pretreatment values (Figure 1A). Concerning SWS Ⅰ, no significant modifications were observed. However, SWS Ⅱ time showed a significant increase from the second week of treatment. The increase remained constant with only small variations during the 11 wk. Only during weeks six and seven did the increase did not reach statistical significance (Figure 1B). REM sleep showed a significant increase in the first week of treatment (Figure 1C). However, REM sleep duration returned to control levels during the second and third weeks of treatment and thereafter, there was a significant increase that lasted until week eleven. The increase of REM sleep time was due mainly to the duration of each period, because no significant increases in REM sleep frequency were observed during most of the weeks. Only during weeks eight, ten, and eleven were there significant increases in REM sleep frequency (Figure 2).

Table 1 summarizes the data obtained concerning all the parameters recorded during pretreatment and during the 11 wk of recording.

Histological liver slices

Histological examination revealed the effects of chronic CCl_4 treatment on liver parenchyma. Figure 3 shows a sample of a liver treated with CCl_4. A normal liver can be observed in Figure 3A. In Figure 3B, the effects of CCl_4 treatment for 11 wk on liver histological features are shown. Clear indications of cirrhosis were observed (fibrosis, lipidic vacuoles, pycnotic nuclei, and necrosis).

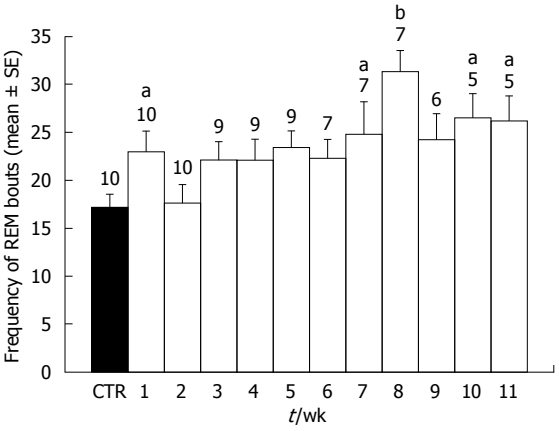

Figure 2 Frequency of REM sleep bouts observed in weekly eight-hour sleep recordings during treatment with CCl_4. Numbers at the top of the bars express the number of subjects. $^aP < 0.05$, $^bP < 0.01$ *vs* control. Repeated measurements were analyzed by ANOVA.

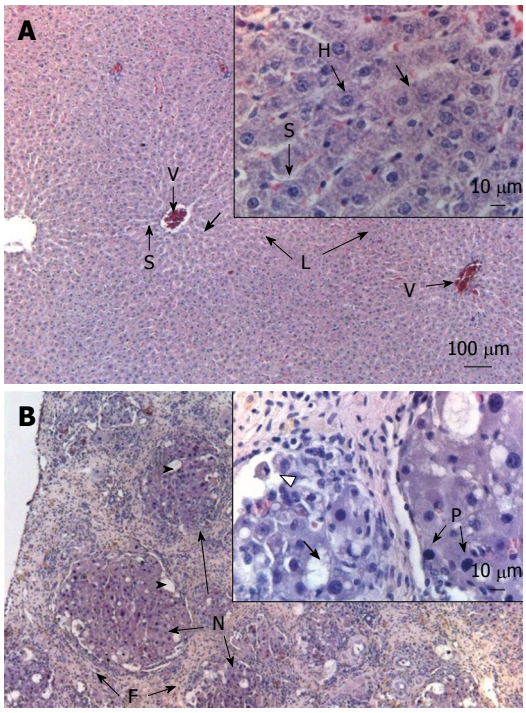

Figure 3 Effects of repeated administration of CCl_4 on liver morphology in rats after 11 wk of CCl_4 treatment. A: Normal aspect of an untreated liver. In a stain with HE, the typical sinusoidal (S) organization can be observed with lobules (L) surrounding the vessels (V). In a higher magnification, a normal hepatocyte (H) can be distinguished; B: The effects of CCl_4. A fibrotic process (F) is evident, with hepatocytes grouped in nodules (N) containing vacuoles. In a higher magnification, the bold arrow indicates hepatocytes with lipidic vacuoles can be observed as well as cells with pycnotic nucleus (P) or in necrosis (triangle).

DISCUSSION

The present results indicate that sleep-wake patterns changed as experimental cirrhosis progressed. REM sleep showed an acute response to the first administrations of CCl_4. Wake and SWS II time were significantly modified after 2 wk of CCl_4 treatment, and these effects showed a steady and slight increase during the following weeks. These results are consistent with previous reports showing that cirrhotic patients display a reduction in α activity and an increase in theta and delta activity[8].

Indirect tests, such as actigraphic recordings and questionnaires on sleep, have suggested that cirrhotic patients suffer from unsatisfactory sleep, mainly due to the reduction in the quality of sleep, produced by several awakenings throughout the night[12]. However, Steindl *et al*[16] did not find differences in polysomnographic recordings of cirrhotic patients compared to matched healthy controls. However, sleep diaries of these patients indicated more frequent nocturnal awakenings and daytime naps. Moreover, these researchers measured the levels of melatonin and found a significant increase during daytime, when melatonin is normally absent[16]. Recently, Velissaris *et al*[17] corroborated these results; they showed that melatonin circadian patterns were altered in cirrhosis patients without clinical encephalopathy. This disruption might reflect changes in the output of the circadian pacemaker located in the suprachiasmatic nucleus of the hypothalamus. It is possible that some of the metabolic disturbances generated by cirrhosis might also alter the function of this biological clock.

On the other hand, several diseases of sleep have been reported, such as the OSAS. OSAS is a frequent disease that has been extensively studied. In patients with advanced liver cirrhosis, OSAS has been reported to be associated with changes in autonomic nervous activities[18].

Recent molecular studies have shown an increased expression of genes associated with monoamine oxidase (MAO-A isoform) and nitric oxide synthase (nNOS isoform) in the brain of cirrhotic patients[3]. Moreover, oligodendroglial nodules have been observed in the white matter associated with CCl_4-induced liver failure[19,20]. In addition, in rats submitted to portacaval anastomosis and in patients with cirrhosis, alterations of the circadian system have been noticed, especially in the rhythm of circadian locomotor activity and in the rhythm of pineal melatonin release[1,21,22]. All of these data can account for sleep effects observed in the present study.

Likewise, it has been shown that the activity of the EEG can be modified by several substances, such as ammonium, a potent neurotoxic produced by cirrhotic patients[3,11,23,24]. This increase of ammonium affects the homeostasis levels of neurotransmitters and neuropeptides in plasma and in the brain. Thus, the levels of dopamine, acetylcholine, glutamate, nitric oxide, and GABA are modified[24-26]. It is possible that, in the present study, the animals treated with CCl_4 have several neurochemical alterations that could modify the levels of different neurotransmitters. These changes, in addition to the alterations in the biological clock, can produce disturbances in some nuclei involved in the regulation of sleep. Further research is needed to elucidate the precise mechanisms of the observed changes.

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COMMENTS

Background

Sleep disturbances have been described in patients with advanced cirrhosis. However, the development and mechanisms of these alterations are not known. In this study, the sleep pattern of rats submitted to experimental cirrhosis was analyzed as liver damage progressed. The results showed an early decrease of wake time and sustained increases of slow wave sleep (SWS) and later, of REM sleep. This data suggest that sleep alterations could be the warning signs of liver disease.

Research frontiers

This study adds information on the relationship between liver function and cerebral function. The mechanisms through which this reciprocal relationship works remain to be elucidated.

Innovations and breakthroughs

The animal model of liver cirrhosis induced by chronic administration of CCL_4 is a suitable model to analyze the relationship between liver failure and brain function disturbances.

Applications

This study highlights the need for gastroenterologists to pay attention to sleep disturbances as early signs of liver failure.

Terminology

SWS and rapid eye movement sleep and the two major sleep stages in most animal species.

Peer review

The content of the article will be interesting not only for the gastroenterologists, but also for other specialists. Further investigations of mechanisms of sleep alterations might yield potentially efficacious approaches to its clinical management.

REFERENCES

1 **Siegel J**. Sleep as a circadian rhythm. In: Siegel J, editor. The neural control of sleep & waking. New York: Springer-Verlag, 2002: 93-101
2 **Ham AW**, Cormack DH. Histology. Mexico: Interamericana, 1984: 783-812
3 **Butterworth RF**. Complications of cirrhosis III. Hepatic encephalopathy. *J Hepatol* 2000; **32**: 171-180
4 **Iwasa M**, Kinosada Y, Nakatsuka A, Watanabe S, Adachi Y. Magnetization transfer contrast of various regions of the brain in liver cirrhosis. *AJNR Am J Neuroradiol* 1999; **20**: 652-654
5 **Nikaina I**, Pastaka C, Zachou K, Dalekos GN, Gourgoulianis K. Sleep apnoea syndrome and early stage cirrhosis: a pilot study. *Eur J Gastroenterol Hepatol* 2006; **18**: 31-35
6 **Tanné F**, Gagnadoux F, Chazouillères O, Fleury B, Wendum D, Lasnier E, Lebeau B, Poupon R, Serfaty L. Chronic liver injury during obstructive sleep apnea. *Hepatology* 2005; **41**: 1290-1296
7 **Papenberg J**, Lanzinger G, Kommerell B, Hoyer S. Comparative studies of the electroencephalogram and the cerebral oxidative metabolism in patients with liver cirrhosis. *Klin Wochenschr* 1975; **53**: 1107-1113
8 **Amodio P**, Del Piccolo F, Pettenò E, Mapelli D, Angeli P, Iemmolo R, Muraca M, Musto C, Gerunda G, Rizzo C, Merkel C, Gatta A. Prevalence and prognostic value of quantified electroencephalogram (EEG) alterations in cirrhotic patients. *J Hepatol* 2001; **35**: 37-45
9 **Bahn E**, Nolte W, Kurth C, Ramadori G, Rüther E, Wiltfang J. Quantification of the electroencephalographic theta/alpha ratio for the assessment of portal-systemic encephalopathy following implantation of transjugular intrahepatic portosystemic stent shunt (TIPSS). *Metab Brain Dis* 2002; **17**: 19-28
10 **Mostacci B**, Ferlisi M, Baldi Antognini A, Sama C, Morelli C, Mondini S, Cirignotta F. Sleep disturbance and daytime sleepiness in patients with cirrhosis: a case control study. *Neurol Sci* 2008; **29**: 237-240
11 **Sherlock S**, Summerskill WH, White LP, Phear EA. Portal-systemic encephalopathy; neurological complications of liver disease. *Lancet* 1954; **267**: 454-457
12 **Córdoba J**, Cabrera J, Lataif L, Penev P, Zee P, Blei AT. High prevalence of sleep disturbance in cirrhosis. *Hepatology* 1998; **27**: 339-345
13 **Martínez M**, Mourelle M, Muriel P. Protective effect of colchicine on acute liver damage induced by CCl4. Role of cytochrome P-450. *J Appl Toxicol* 1995; **15**: 49-52
14 **Desmoulière A**, Xu G, Costa AM, Yousef IM, Gabbiani G, Tuchweber B. Effect of pentoxifylline on early proliferation and phenotypic modulation of fibrogenic cells in two rat models of liver fibrosis and on cultured hepatic stellate cells. *J Hepatol* 1999; **30**: 621-631
15 **Takeuchi E**. [Polygraphical study on the wakefulness-sleep cycle of the rat] *Shinrigaku Kenkyu* 1970; **41**: 248-256
16 **Steindl PE**, Finn B, Bendok B, Rothke S, Zee PC, Blei AT. [Changes in the 24-hour rhythm of plasma melatonin in patients with liver cirrhosis--relation to sleep architecture] *Wien Klin Wochenschr* 1997; **109**: 741-746
17 **Velissaris D**, Karanikolas M, Kalogeropoulos A, Solomou E, Polychronopoulos P, Thomopoulos K, Labropoulou-Karatza C. Pituitary hormone circadian rhythm alterations in cirrhosis patients with subclinical hepatic encephalopathy. *World J Gastroenterol* 2008; **14**: 4190-4195
18 **Ogata T**, Nomura M, Nakaya Y, Ito S. Evaluation of episodes of sleep apnea in patients with liver cirrhosis. *J Med Invest* 2006; **53**: 159-166
19 **Diemer NH**. Size and density of oligodendroglial nuclei in rats with CCl4-induced liver disease. *Neurobiology* 1975; **5**: 197-206
20 **Luse SA**, Wood WG. The brain in fatal carbon tetrachloride poisoning. *Arch Neurol* 1967; **17**: 304-312
21 **Steindl PE**, Finn B, Bendok B, Rothke S, Zee PC, Blei AT. Disruption of the diurnal rhythm of plasma melatonin in cirrhosis. *Ann Intern Med* 1995; **123**: 274-277
22 **Zee PC**, Mehta R, Turek FW, Blei AT. Portacaval anastomosis disrupts circadian locomotor activity and pineal melatonin rhythms in rats. *Brain Res* 1991; **560**: 17-22
23 **Del Piccolo F**, Sacerdoti D, Amodio P, Bombonato G, Bolognesi M, Mapelli D, Gatta A. Central nervous system alterations in liver cirrhosis: the role of portal-systemic shunt and portal hypoperfusion. *Metab Brain Dis* 2003; **18**: 51-62
24 **Hazell AS**, Butterworth RF. Hepatic encephalopathy: An update of pathophysiologic mechanisms. *Proc Soc Exp Biol Med* 1999; **222**: 99-112
25 **Bergeron M**, Reader TA, Layrargues GP, Butterworth RF. Monoamines and metabolites in autopsied brain tissue from cirrhotic patients with hepatic encephalopathy. *Neurochem Res* 1989; **14**: 853-859
26 **Rose C**, Felipo V. Limited capacity for ammonia removal by brain in chronic liver failure: potential role of nitric oxide. *Metab Brain Dis* 2005; **20**: 275-283

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BRIEF ARTICLE

Therapeutic effect of caffeic acid phenethyl ester on cerulein-induced acute pancreatitis

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Abstract

AIM: To evaluate the therapeutic role of caffeic acid phenethyl ester (CAPE) in a rat model of cerulean-induced acute pancreatitis (AP).

METHODS: Seventy male Wistar albino rats were divided into seven groups. Acute edematous pancreatitis was induced by subcutaneous cerulein injection (20 μg/kg) four times at 1-h intervals. CAPE (30 mg/kg) was given by subcutaneous injection at the beginning (CAPE 1 group) and 12 h after the last cerulein injection (CAPE 2 group). Serum amylase, lipase, white blood cell count, and tumor necrosis factor (TNF)-α levels were measured, and pancreatic histopathology was assessed.

RESULTS: In the AP group, amylase and lipase levels were found to be elevated and the histopathological evaluation showed massive edema and inflammation of the pancreas, with less fatty necrosis when compared with sham and control groups. Amylase and lipase levels and edema formation decreased significantly in the CAPE therapy groups ($P < 0001$); especially in the CAPE 2 group, edema was improved nearly completely ($P = 0001$). Inflammation and fatty necrosis were partially recovered by CAPE treatment. The pathological results and amylase level in the placebo groups were similar to those in the AP group. White blood cell count and TNF-α concentration was nearly the same in the CAPE and placebo groups.

CONCLUSION: CAPE may be useful agent in treatment of AP but more experimental and clinical studies are needed to support our observation of beneficial effects of CAPE before clinical usage of this agent.

Key words: Acute pancreatitis; Caffeic acid phenethyl ester; Cerulein

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INTRODUCTION

Acute pancreatitis (AP) is a process of acute inflammation in the pancreas, with variable involvement of regional tissues or organ systems. In most patients, acute necrotizing pancreatitis leads to remote organ failure, sepsis and a high death rate[1]. Pathophysiology of AP is poorly understood, but interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α as pro-inflammatory cytokines, oxidative stress and microvascular ischemia are important factors[2-4]. In recent years, pathogenesis-oriented treatments of AP have gained importance. Therefore, new experimental studies have focused on pathophysiological mechanisms such as oxidative stress and inflammatory cytokines[5,6]. Propolis is a natural substance that is produced by honeybees from the gum of

various plants. It contains several chemical compounds such as polyphenolic compounds like flavonoids, cinnamic acid derivates, various steroids, and amino acids[7,8]. Caffeic acid phenethyl ester (CAPE) is also a phenolic compound and an active substrate of propolis. Several investigators have shown that CAPE has anti-inflammatory activity by inhibiting the release of arachidonic acid from cell membranes, and suppressing cyclooxygenase (COX)-1 and COX-2 enzyme activity[9], antioxidant activity by lipoxygenase inhibition[10,11], and anti-proliferative, antimutagenic and antitumoral effects by inducing apoptosis in tumor cell lines[12]. In addition, CAPE is a potent and specific inhibitor of nuclear factor (NF)-κB and inhibits the activation of NF-κB that is induced by TNF-α and other inflammatory agents[13].

The aim of this study was to investigate the therapeutic efficacy of CAPE in the cerulean-induced acute edematous pancreatitis in rats.

MATERIALS AND METHODS

Animals

Seventy male Wistar albino rats, weighing 250-320 g were used from the Physiology Laboratory of Gaziantep University Medical School. The animals were housed under a 12-h light-dark cycle at a temperature of 24℃. Food was withdrawn 12 h before the experiment. All experiments were performed in accordance with the recommendations of the national guidelines for the care and handling of laboratory animals, and followed a protocol approved by the local animal ethics committee.

Experimental design

Acute edematous pancreatitis was induced by subcutaneous cerulein (Sigma, St Louis, MO, USA) injection (20 g/kg) four times at 1-h intervals[14]. Seventy male rats were divided into seven groups of 10.

Group 1 (sham): nothing was applied to the sham group. Group 2 (control): 1 mL saline was given by subcutaneous injecting four times at 1-h intervals, but no medication was applied. Animals were killed 12 h after the last injection. Group 3 (AP group): AP was induced by subcutaneous cerulein injection (20 g/kg dissolved in 1 mL saline) four times at 1-h intervals, but no medication was applied. Animals were killed 12 h after the last injection. Group 4 (CAPE 1) 30 mg/kg CAPE (Sigma) was given by subcutaneous injection at the beginning of the procedure, and at the same time, AP was induced by subcutaneous cerulein injection as described before. Group 5 (CAPE 2): AP was induced in the same way as described above, and CAPE (30 mg/kg) was given at 12 h after the last cerulein injection. Animals were killed 6 h after the CAPE injection. Group 6 (placebo 1): AP was induced by subcutaneous cerulein injection (20 μg/kg) four times at 1-h intervals, and 1 mL saline was given at the beginning of the studies. Animals were killed 12 h after the last injection. Group 7 (placebo 2): AP was induced in the same way as described above, and 1 mL saline was given at 12 h after the last cerulein injection. Animals were killed 6 h after the saline injection.

Table 1 Pathological grading system in experimental AP

Edema	0	No edema
	1	Interlobular edema
	2	Moderate interlobular edema + intra-acinar edema
	3	Severe interlobular and intra-acinar edema
Inflammatory infiltration	0	No infiltration
	1	Intravascular margination of granulocytes
	2	Granulocytes present in the perivascular tissue
	3	Diffuse infiltration of entire pancreatic gland
Fat necrosis	0	No necrosis
	1	1-4 necrotic cells (each microscopic area)
	2	5-10 necrotic cells
	3	11-16 necrotic cells

Assays of treatment efficacy

Under ketamine anesthesia, midline laparotomy was performed on all rats, except Groups 5 and 7, at 15 h (12 h after the last cerulein or saline injection). Groups 5 and 7 were killed 6 h after CAPE injection (Group 5) or saline injection (Group 7). Shortly after the blood specimens were taken from the inferior vena cava, the whole pancreas was extracted quickly and the animals were sacrificed. Blood samples were centrifuged at 3000 rpm for 10 min and the plasma was stored at -70℃ until assayed. White blood cell count, amylase, lipase and TNF-α concentrations were measured. Plasma TNF-α concentration was measured by immunoassay kit (Rat TNF-α immunoassay; R&D Systems Inc., Minneapolis, MN, USA), plasma amylase and lipase were measured by commercially available kits from Roche Diagnostics (Mannheim, Germany), using an enzymatic photometric method based on cleavage of the substrate ethylidene-4-nitrophenyl maltoheptaose. Results are expressed as U/L.

Histopathological scoring

Histopathological evaluation of the pancreas was made in order to understand the extent of the injury. Pancreatic tissue was fixed in formaldehyde solution and embedded in paraffin. Sections were stained with hematoxylin and eosin and were evaluated by light microscopy by two experienced pathologists who were blinded to the experimental treatment groups, according to the Schoenberg grading system[15] (Table 1). The tissues were scored using a scale for edema, neutrophil infiltration and fatty necrosis .

Statistical analysis

Results were given as mean ± SD. Comparisons between and among the groups were made using non-parametric test (Mann-Whitney *U* test) and one-way ANOVA. Data were evaluated statistically using SPSS for Windows version 10.0 (Chicago, IL, USA). $P < 0.05$ was taken as significant.

RESULTS

Serum amylase, lipase and TNF-α levels

Serum biochemical analysis of amylase, lipase and TNF-α levels and pathological examination results are shown in Table 2. Serum amylase and lipase levels were significantly increased in the cerulean-induced AP group

Table 2 Biochemical, values and pathological scores in cerulein-induced AP (mean ± SD)

Groups	TNF-α (pg/mL)	Amylase (U/L)	Lipase (U/L)	White blood cells	Edema	Leukocytic infiltration	Total pathological score	Fat necrosis
Sham	65.14 ± 1.7	665.14 ± 54	14.41 ± 1.7	9279 ± 1867	0.00	0.00	0.00	0.00
Control	63.29 ± 3.8	630.20 ± 64	14.92 ± 1.7	8755 ± 1098	0.00	0.00	0.00	0.00
AP	63.83 ± 3.8	4752 ± 1328[b]	112.3 ± 34.8[b]	8574 ± 1437	2.50 ± 0.5	2.80 ± 0.42	8.00	0.30 ± 0.48
CAPE 1	61.55 ± 8.0	1400 ± 680[d]	22.92 ± 6.9[d]	8407 ± 418	1.50 ± 0.7	2.50 ± 0.52	4.00[c]	0.00
CAPE 2	60.52 ± 6.5	1084 ± 533[d]	18.65 ± 3.7[d]	8940 ± 2746	0.50 ± 0.5[d]	2.40 ± 0.51	3.00[c]	0.00
Placebo 1	62.38 ± 9.5	4516 ± 749	49.2 ± 5.3	9267 ± 927	2.30 ± 0.6	2.70 ± 0.48	8.00	0.30 ± 0.48
Placebo 2	61.56 ± 2.5	4219 ± 235	52.54 ± 4.8	8544 ± 895	2.20 ± 0.6	2.70 ± 0.48	8.00	0.30 ± 0.48

[b]$P < 0.001$ *vs* group 1 and 2; [c]$P < 0.05$, [d]$P < 0.001$ *vs* group 3.

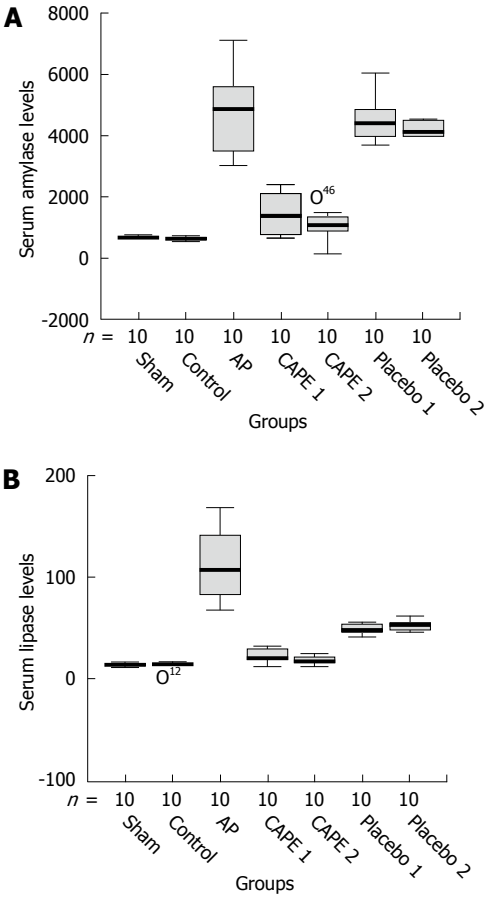

Figure 1 Serum amylase (A) and lipase (B) levels in the experimental groups.

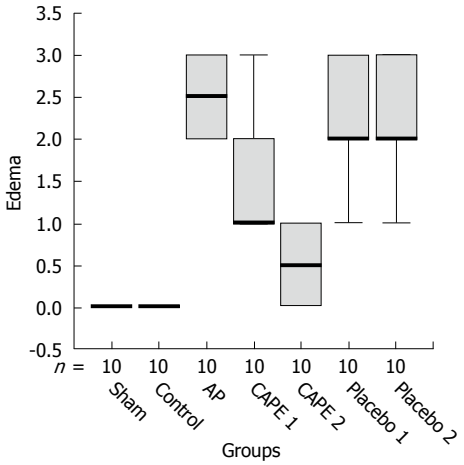

Figure 2 Edema scores in experimental AP groups.

when compared to the control and sham groups ($P < 0.001$). Amylase and lipase levels decreased significantly in the CAPE treatment groups ($P < 0001$) but the levels were higher than those of the control and sham groups. The levels of amylase and lipase in the placebo groups were similar to those in the AP group (Figure 1A and B). There were no statistically significant differences in serum TNF-α and white blood cell count between the study groups ($P > 0.05$, Table 2).

Pathological examination

In the AP group, histopathological evaluation showed massive edema and inflammation of the pancreas, with less fatty necrosis when compared with the control and sham groups. CAPE treatment significantly decreased edema formation, and the most striking finding was that edema was improved nearly completely in the CAPE 2 group ($P = 0001$, Figure 2). Polymorphonuclear leukocytic infiltration was increased in the AP groups ($P < 0.05$, Figure 3A). In the therapy groups, inflammation was partially recovered. In the AP groups, fatty necrosis score was 0.30 ± 0.48. We observed grade 1 fatty necrosis in only three rats in the AP groups. Fatty necrosis was ameliorated in the CAPE treatment groups but this improvement was not statistically significantly ($P > 0.05$). The pathological results of the placebo groups were similar to those in the AP groups. After CAPE treatment, the total pathological mean score was decreased significantly ($P < 005$) after CAPE treatment (Figure 3B).

DISCUSSION

Current therapeutic methods are usually insufficient for the treatment of severe AP, despite the development of new diagnostic and therapeutic procedures. Therefore, recently, several experimental studies have focused on the pathogenesis of AP. Several mechanisms, such as oxidative stress, COX-2 and inflammatory cytokines play an important role in the pathogenesis of the disease[2-4,16]. CAPE is a phenolic antioxidant, which is an active component of propolis. Previous investigators have demonstrated that CAPE has anti-inflammatory, antioxidant, anti-proliferative and antitumoral effects *in vitro* and *in vivo*[12]. In the light of previous findings,

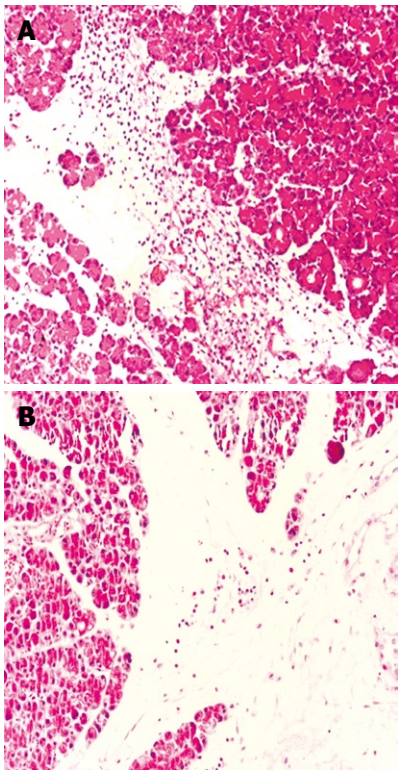

Figure 3 Histopathological features of cerulein induced AP group and after CAPE therapy. A: Severe edema and leukocytic infiltration of pancreas after cerulein-induced AP (HE, × 200); B: Decreased infiltration in pancreatic tissue after CAPE therapy (HE, × 200).

we investigated the therapeutic role of CAPE as a new agent for the treatment of AP.

TNF-α is a cytokine that plays a central role in the pathogenesis of the disease[2]. TNF receptor antagonist observed a reduction in the severity and mortality of experimental pancreatitis[17]. Plasma half-life of TNF-α is very short (14-18 min)[18], therefore, we studied TNF-α serum levels in rats, despite this kind of measurement being difficult. We obtained serum at 15 h, and that is probably why the results were low in all groups.

Norman *et al*[19] have shown marked amelioration of pancreatic tissue damage and decreased serum amylase and lipase levels after treatment with IL-1 antagonist. Oxidative stress plays an important role in the pathophysiology of AP. For this reason, several studies have reported the therapeutic effect of antioxidant agents. A previous study has disclosed that various antioxidant agents improve pancreatic edema in cerulean-induced pancreatitis, however antioxidants showed no improvement in a sodium-taurocholate model of pancreatitis in rats[20]. On the contrary, one recent study in a sodium-taurocholate model of pancreatitis in rats has shown that serum amylase and lipase, edema, leukocytic infiltration, parenchymal necrosis and hemorrhage were significantly decreased by N-acetylcysteine (NAC) treatment. In addition, in the NAC-treated rats, while serum nitrite/nitrate levels were significantly increased, serum concentration of the lipid peroxidation product was significantly decreased. The beneficial effect of NAC may result from its antioxidant activity and the production of and/or inhibition of degradation of nitric oxide[5]. In a similar study, Vaquero *et al*[21] have demonstrated that treatment with NAC reduces neutrophil infiltration and mRNA expression for IL-6, cytokines and inducible nitric oxide synthase in pancreatic tissue, by inhibition of NF-κB activity. In conclusion, NF-κB is a key regulator cytokine in induction and oxidative stress in AP. In experimental pancreatitis, the beneficial effect of antioxidants can be explained by inhibition of NF-κB activation. CAPE is a specific and potent inhibitor of NF-κB and causes inhibition of pro-inflammatory cytokine production[13]. Likewise, Fitzpatrick *et al*[22] have shown that CAPE (30 mg/kg) treatment significantly inhibits NF-κB, and colonic cytokines (TNF-α and IL-1β) are reduced in experimental colitis in rats.

AP is associated with induction of COX-2 expression. In cerulean-induced pancreatitis, Ethridge *et al*[16] have found that COX-2 gene expression is increased in pancreatic tissue. Although serum amylase and lipase are not reduced, the severity of pancreatic necrosis and leukocytic inflammation are significantly decreased by treatment with NS-398 (a COX-2 inhibitor). It has been demonstrated that COX-2 gene expression, activity of COX-1 and COX-2 enzymes, and release of arachidonic acid from cell membranes are inhibited by CAPE[9]. In light of this, we investigated the beneficial efficacy of CAPE (which is an antioxidant and anti-inflammatory agent) on the experimental model of cerulean-induced acute edematous pancreatitis in rats. As far as we know, there are no published data on the treatment effect of CAPE in experimental pancreatitis models. In the present study, we showed that CAPE ameliorated the harmful effects in a rat model of cerulein-induced pancreatitis. Serum amylase and lipase levels were decreased by CAPE treatment. In addition, CAPE treatment significantly reduced edema and total pathological mean score. Inflammation and fatty necrosis score were improved but the improvement was not statistically significantly.

There are several models of experimental pancreatitis, such as the cerulein-induced and sodium-taurocholate models. Pancreatic injury is evenly distributed throughout the pancreas in the cerulein-induced models. The reason why we chose the cerulein-induced AP model was that this form of pancreatitis is very similar to that in humans and it occurs within a short time[23]. This model is used widely to study potential agents for the treatment of AP[24]. In this model, pancreatic inflammation reaches the most severe stage at 12 h, which is why we ended the first part of the study at 12 h after cerulein injection[25]. Secondly, we formed a CAPE 2 group to study the effect of CAPE on the most severe stage of pancreatitis at 12 h. Here, our concern was to study the efficacy of the treatment in severe pancreatitis, especially in the full-blown situation. In fact, patients with AP often attend the hospital at an advanced stage, even sometimes with systemic complications.

In conclusion, in the cerulein-induced model of experimental AP, an improvement in the biochemical and histopathological findings were observed in the CAPE treatment groups. CAPE decreased pancreatic tissue injury and this supports the hypothesis that antioxidant

and anti-inflammatory treatment is effective in AP. It is important that CAPE was effective in the CAPE 2 group when AP had already occurred. This will enlighten the following phase 3 and phase 4 studies. Another important step will be to study the efficacy of CAPE in experimental necrotizing pancreatitis. Nevertheless, more experimental and clinical studies are needed to support our observation of the beneficial effects of CAPE before clinical usage of this agent.

COMMENTS

Background

Pathophysiology of acute pancreatitis (AP) is poorly understood. Therefore, new experimental therapeutic studies have focused on the pathophysiological mechanisms. The present experimental study investigated the therapeutic role of caffeic acid phenethyl ester (CAPE) as a new agent for the treatment of AP.

Research frontiers

CAPE is a specific and potent inhibitor of nuclear factor (NF)-κB and causes inhibition of pro-inflammatory cytokine production. CAPE (30 mg/kg) treatment significantly inhibited NF-κB, and colonic cytokines tumor necrosis factor-α and interleukin-1β were reduced in experimental colitis in rats.

Innovations and breakthroughs

There are no published data on the treatment effect of CAPE in experimental pancreatitis models. In the cerulein-induced model of experimental AP, improvement in biochemical and histopathological findings was observed in the CAPE treatment groups.

Applications

CAPE may be a useful agent in the treatment of AP but more experimental and clinical studies are needed to support our observation of its beneficial effects.

Terminology

CAPE is a phenolic compound and an active substrate of propolis. CAPE has anti-inflammatory, antioxidant, anti-proliferative and antitumoral effects *in vitro* and *in vivo*.

Peer review

This work provides experimental evidence for a protective function of CAPE in a rat model of acute pancreatitis. The study is generally well-designed and controlled. The results show that administration of CAPE reduces serum amylase and lipase levels in cerulein-treated rats and improves pathological scores in pancreatic tissue specimens.

REFERENCES

1 **Whitcomb DC**. Clinical practice. Acute pancreatitis. *N Engl J Med* 2006; **354**: 2142-2150
2 **Kusske AM**, Rongione AJ, Reber HA. Cytokines and acute pancreatitis. *Gastroenterology* 1996; **110**: 639-642
3 **Gukovskaya AS**, Gukovsky I, Zaninovic V, Song M, Sandoval D, Gukovsky S, Pandol SJ. Pancreatic acinar cells produce, release, and respond to tumor necrosis factor-alpha. Role in regulating cell death and pancreatitis. *J Clin Invest* 1997; **100**: 1853-1862
4 **Sweiry JH**, Mann GE. Role of oxidative stress in the pathogenesis of acute pancreatitis. *Scand J Gastroenterol Suppl* 1996; **219**: 10-15
5 **Yagci G**, Gul H, Simsek A, Buyukdogan V, Onguru O, Zeybek N, Aydin A, Balkan M, Yildiz O, Sen D. Beneficial effects of N-acetylcysteine on sodium taurocholate-induced pancreatitis in rats. *J Gastroenterol* 2004; **39**: 268-276
6 **Oruc N**, Ozutemiz AO, Yukselen V, Nart D, Celik HA, Yuce G, Batur Y. Infliximab: a new therapeutic agent in acute pancreatitis? *Pancreas* 2004; **28**: e1-e8
7 **Garcia-Viguera C**, Ferreres F, Tomas-Barberan FA. Study of Canadian propolis by GC-MS and HPLC. *Z Naturforsch* 1993; **48**: 731-735
8 **Gabrys J**, Konecki J, Krol W, Scheller S, Shani J. Free amino acids in bee hive product (propolis) as identified and quantified by gas-liquid chromatography. *Pharmacol Res Commun* 1986; **18**: 513-518
9 **Michaluart P**, Masferrer JL, Carothers AM, Subbaramaiah K, Zweifel BS, Koboldt C, Mestre JR, Grunberger D, Sacks PG, Tanabe T, Dannenberg AJ. Inhibitory effects of caffeic acid phenethyl ester on the activity and expression of cyclooxygenase-2 in human oral epithelial cells and in a rat model of inflammation. *Cancer Res* 1999; **59**: 2347-2352
10 **Sud'ina GF**, Mirzoeva OK, Pushkareva MA, Korshunova GA, Sumbatyan NV, Varfolomeev SD. Caffeic acid phenethyl ester as a lipoxygenase inhibitor with antioxidant properties. *FEBS Lett* 1993; **329**: 21-24
11 **Mirzoeva OK**, Sud'ina GF, Pushkareva MA, Korshunova GA, Sumbatian NV, Varfolomeev SD. [Lipophilic derivatives of caffeic acid as lipoxygenase inhibitors with antioxidant properties] *Bioorg Khim* 1995; **21**: 143-151
12 **Banskota AH**, Tezuka Y, Kadota S. Recent progress in pharmacological research of propolis. *Phytother Res* 2001; **15**: 561-571
13 **Natarajan K**, Singh S, Burke TR Jr, Grunberger D, Aggarwal BB. Caffeic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF-kappa B. *Proc Natl Acad Sci USA* 1996; **93**: 9090-9095
14 **Lampel M**, Kern HF. Acute interstitial pancreatitis in the rat induced by excessive doses of a pancreatic secretagogue. *Virchows Arch A Pathol Anat Histol* 1977; **373**: 97-117
15 **Schoenberg MH**, Büchler M, Gaspar M, Stinner A, Younes M, Melzner I, Bültmann B, Beger HG. Oxygen free radicals in acute pancreatitis of the rat. *Gut* 1990; **31**: 1138-1143
16 **Ethridge RT**, Chung DH, Slogoff M, Ehlers RA, Hellmich MR, Rajaraman S, Saito H, Uchida T, Evers BM. Cyclooxygenase-2 gene disruption attenuates the severity of acute pancreatitis and pancreatitis-associated lung injury. *Gastroenterology* 2002; **123**: 1311-1322
17 **Norman JG**, Fink GW, Franz MG. Acute pancreatitis induces intrapancreatic tumor necrosis factor gene expression. *Arch Surg* 1995; **130**: 966-970
18 **Makhija R**, Kingsnorth AN. Cytokine storm in acute pancreatitis. *J Hepatobiliary Pancreat Surg* 2002; **9**: 401-410
19 **Norman JG**, Franz MG, Fink GS, Messina J, Fabri PJ, Gower WR, Carey LC. Decreased mortality of severe acute pancreatitis after proximal cytokine blockade. *Ann Surg* 1995; **221**: 625-631; discussion 631-634
20 **Niederau C**, Niederau M, Borchard F, Ude K, Lüthen R, Strohmeyer G, Ferrell LD, Grendell JH. Effects of antioxidants and free radical scavengers in three different models of acute pancreatitis. *Pancreas* 1992; **7**: 486-496
21 **Vaquero** E, Gukovsky I, Zaninovic V, Gukovskaya AS, Pandol SJ. Localized pancreatic NF-kappaB activation and inflammatory response in taurocholate-induced pancreatitis. *Am J Physiol Gastrointest Liver Physiol* 2001; **280**: G1197-G1208
22 **Fitzpatrick LR**, Wang J, Le T. Caffeic acid phenethyl ester, an inhibitor of nuclear factor-kappaB, attenuates bacterial peptidoglycan polysaccharide-induced colitis in rats. *J Pharmacol Exp Ther* 2001; **299**: 915-920
23 **de Waal Malefyt R**, Haanen J, Spits H, Roncarolo MG, te Velde A, Figdor C, Johnson K, Kastelein R, Yssel H, de Vries JE. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J Exp Med* 1991; **174**: 915-924
24 **Adler G**, Rohr G, Kern HF. Alteration of membrane fusion as a cause of acute pancreatitis in the rat. *Dig Dis Sci* 1982; **27**: 993-1002
25 **Rongione AJ**, Kusske AM, Kwan K, Ashley SW, Reber HA, McFadden DW. Interleukin 10 reduces the severity of acute pancreatitis in rats. *Gastroenterology* 1997; **112**: 960-967

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BRIEF ARTICLE

CO_2 insufflation for potentially difficult colonoscopies: Efficacy when used by less experienced colonoscopists

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Abstract

AIM: To clarify the effectiveness of CO_2 insufflation in potentially difficult colonoscopy cases, particularly in relation to the experience level of colonoscopists.

METHODS: One hundred twenty potentially difficult cases were included in this study, which involved females with a low body mass index and patients with earlier abdominal and/or pelvic open surgery or previously diagnosed left-side colon diverticulosis. Patients receiving colonoscopy examinations without sedation using a pediatric variable-stiffness colonoscope were divided into two groups based on either CO_2 or standard air insufflation. Both insufflation procedures were also evaluated according to the experience level of the respective colonoscopists who were divided into an experienced colonoscopist (EC) group and a less experienced colonoscopist (LEC) group. Study measurements included a 100-mm visual analogue scale (VAS) for patient pain during and after colonoscopy examinations, in addition to insertion to the cecum and withdrawal times.

RESULTS: Examination times did not differ, however, VAS scores in the CO_2 group were significantly better than in the air group ($P < 0.001$, two-way ANOVA) from immediately after the procedure and up to 2 h later. There were no significant differences between either insufflation method in the EC group ($P = 0.29$), however, VAS scores for CO_2 insufflation were significantly better than air insufflation in the LEC group ($P = 0.023$) immediately after colonoscopies and up to 4 h afterwards.

CONCLUSION: CO_2 insufflation reduced patient pain after colonoscopy in potentially difficult cases when performed by LECs.

Key words: CO_2 insufflation; Colonoscopy; Difficult colonoscopy; Experienced colonoscopist; Training

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Uraoka T, Kato J, Kuriyama M, Hori K, Ishikawa S, Harada K, Takemoto K, Hiraoka S, Fujita H, Horii J, Saito Y, Yamamoto K. CO_2 insufflation for potentially difficult colonoscopies: Efficacy when used by less experienced colonoscopists. *World J Gastroenterol* 2009; 15(41): 5186-5192 Available from: URL: http://www.wjgnet.com/1007-9327/15/5186.asp DOI: http://dx.doi.org/10.3748/wjg.15.5186

INTRODUCTION

Colonoscopy has a high profile because of its increasingly important role in successfully preventing, detecting and treating colorectal cancer[1,2], however, some patients experience considerable abdominal pain and discomfort when the procedure is performed using air insufflation. In particular, the so-called "difficult colonoscopy" cases[3-6], which involve female patients with a relatively

low body mass index (BMI), patients with a history of abdominal and/or pelvic open surgery and male patients with diverticulosis, often require prolonged insertion to the cecum, thus this procedure can cause increased abdominal pain and discomfort for such patients.

Factors accounting for longer examination times and increased abdominal pain and discomfort can be derived from both a patient's condition and the examining colonoscopist's skill and experience[7-9]. Novice and even moderately skilled colonoscopists must improve their technical abilities by gaining experience in successfully handling difficult colonoscopies to become qualified experts, as a suitably high-level colonoscopy training environment has not been established as yet[10,11].

CO_2 insufflation has been reported to reduce patient abdominal pain and discomfort during and after colonoscopies[12-15]. Although the safety and efficacy of CO_2 insufflation during colonoscopies have been assessed in earlier studies, air insufflation is still the standard method due to a lack of suitable equipment and inadequate information as to when and on whom CO_2 insufflation should be used during colonoscopy examinations.

We decided to conduct a prospective randomized controlled trial to test the hypothesis that CO_2 insufflation reduces patient abdominal pain and discomfort during and after colonoscopy examinations in potentially difficult cases.

MATERIALS AND METHODS

Study protocol

Consecutive patients considered potentially difficult cases for colonoscopic intubation were included in this prospective randomized controlled trial which took place between September 2006 and October 2007. The aim of this study was to clarify the effectiveness of CO_2 insufflation during colonoscopy examinations, with the primary objectives of assessing both patient tolerance and the safety of CO_2 insufflation in these potentially difficult cases. A secondary objective was to clarify any differences between the two insufflation methods in relation to the experience level of the participating colonoscopists. This study was approved by the Ethics Committee at Okayama University Hospital.

Patients

Patients considered potentially difficult colonoscopy cases, based on published information and clinical experience, were selected, and included females with a relatively low BMI (BMI < 22), patients with a history of abdominal and/or pelvic open surgery, with the exception of low risk procedures for adhesions such as appendectomy or hernia repair, and male patients with previously diagnosed left-side diverticulosis[3-6].

The indications for colonoscopy examination were the standard clinical criteria: colorectal cancer screening, surveillance for polyps, a positive fecal occult blood test, abdominal symptoms or anemia. Exclusion factors included severe heart or lung disease, a prior colorectal resection, inflammatory bowel disease, severe hematochezia and repeat colonoscopy for therapeutic procedures including polypectomy.

Written informed consent was obtained from each patient and enrolled patients were randomly divided into two groups for colonoscopy examinations using either CO_2 or standard air insufflation. Group allocation for both patients and colonoscopists was performed by specially assigned nurses using standard randomization lists which contained consecutive patient numbers. Each number was linked to one of the two study groups for allocation purposes. These lists were not accessible by the participating colonoscopists.

Colonoscopy using CO_2 insufflation

Patients underwent bowel preparation with sodium picosulfate the day before their examinations and two liters of polyethylene glycol solution-containing lavage the morning of their colonoscopies. Scopolamine butylbromide (20 mg) was administered intramuscularly to suppress bowel movement, while patients with cardiac disease or benign prostatic hypertrophy received glucagon (1 IU) intramuscularly. Patients were not sedated, although midazolam (2-3 mg, iv) was administered based on the examining colonoscopist's judgment or when requested by the patient due to abdominal pain or distension. Examinations were performed using a pediatric variable-stiffness colonoscope (PVSC) with a distal tip diameter of 11.3 mm (PCF-Q260AI, Olympus Co, Tokyo, Japan).

Procedures were randomly performed by eight colonoscopists who had earlier been divided into two groups according to their colonoscopy experience: four highly experienced colonoscopists (EC) group each of whom had been in colonoscopy practice for over 10 years (TU, JK, KT and SH), and four less experienced colonoscopists (LEC) group with 5-7 years of colonoscopy practice during which each had performed 900-1500 colonoscopies (MK, SI, KH and HF).

If an examining colonoscopist from the LEC group failed to pass through the sigmoid-descending colon junction within 15 min or a patient complained of severe pain, a colonoscopist from the EC group replaced the initial examiner before midazolam was administered and continued insertion to the cecum. When such a case involved a colonoscopist from the EC group as the initial examiner, a more experienced member of the EC group would continue the procedure. After reaching the cecum, the initial examiner proceeded with withdrawal of the colonoscope.

A "complete colonoscopy" was defined as successful insertion to the cecum bottom or terminal ileum. Insertion to the cecum and withdrawal time was recorded for every colonoscopy.

CO_2 insufflation and monitoring system

CO_2 was administered using a commercial CO_2 regulator (Gas Regulator, Crown, Model FR-IIS-P; Yutaka Engineering, Tokyo, Japan) connected to a CO_2 bottle.

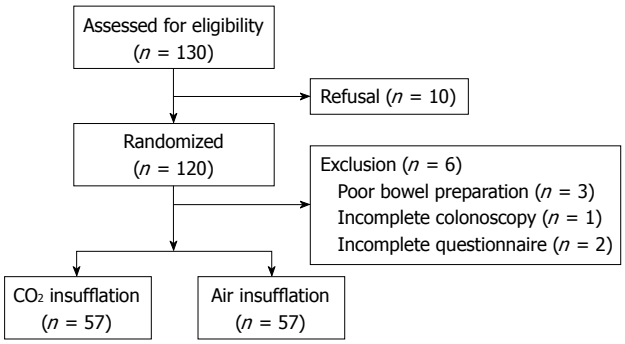

Figure 1 Patient flow chart.

The regulator delivered CO_2 at the rate of 2 L/min. CO_2 and air insufflations were used in a blind fashion both to patients and colonoscopists with full-day colonoscopy sessions randomly assigned CO_2 or air insufflation to avoid unblinding caused by set-up changes between patients.

CO_2 partial pressure was continuously measured using a transcutaneous CO_2 monitoring system (TOSCA 500; Radiometer Basel AG, Switzerland). Processed transcutaneous CO_2 readings ($PtcCO_2$) correlate closely with directly obtained arterial blood gas results[16,17]. Sensors were attached to a patient's ear lobe with a monitor-specific clip. A colonoscopy assistant recorded readings and an independent observer monitored gas readings to avoid potential serious side effects. CO_2 insufflation was stopped immediately if $PtcCO_2$ registered > 60 mmHg during any colonoscopy examination.

Pain and discomfort measurement

A 100-mm visual analogue scale (VAS) consisting of a horizontal line 100 mm in length was used for measuring patient abdominal pain and discomfort (0 mm = painless, 100 mm = extremely painful)[18]. Patients recorded the pain level experienced upon reaching the cecum bottom, immediately following their examinations and 30 min, 1, 2, 4 and 6 h afterwards. The VAS score was the distance measured to the nearest millimeter from the left end of the line to the point of the patient's mark.

Another member of the medical staff, who did not know how the procedures were performed, interviewed the patients 30 min after completion of their colonoscopies. A questionnaire was then given to the patients to take home to complete as instructed at intervals of 1, 2, 4 and 6 h and the completed forms were then mailed to the hospital the following day. The completed questionnaires were subsequently mailed to our medical office. No follow-up phone calls were made as 98% of all questionnaires were promptly returned.

Statistical analysis

A preliminary pilot study was conducted to estimate the SD in pain measurements. With an assumed SD of 19 mm, the study sample size was calculated at 110 patients in order to have an 80% power with two-sided α levels of 0.05 to detect any differences in VAS scores between the two insufflation groups ($\geqslant$ 10 mm was considered clinically important).

The outcomes for our secondary objective to clarify any differences between the two insufflation methods in relation to the experience level of participating colonoscopists were analyzed on an intention-to-treat basis, given the fact that a number of the initial examining colonoscopists were replaced during the insertion phase of the procedure. Statistical comparisons were made using chi-square and Fisher's exact tests. ANOVA was used for repeated measures statistical analysis of pain. Some variables were not distributed normally, thus the Wilcoxon rank sum test was applied for supplementary analysis to compare groups at each measurement point. Statistical analyses were performed using Prism version 5.0 (GraphPad Software, San Diego, CA, USA) and JMP version 6.3 (SAS Institute, Cary, NC, USA). A P value < 0.05 was considered significant.

RESULTS

Baseline characteristics

A total of 130 patients were asked to participate and 120 consenting patients were randomized into two groups prior to their colonoscopy examinations (Figure 1). Three poor bowel preparation patients were not included and one (0.85%, 1/117) incomplete intubation patient in the air insufflation group with a history of abdominal and pelvic open surgery, whose examination was performed by an EC, was not submitted for consideration. Completed questionnaires were received from 98% of the 116 remaining patients, thus a final total of 114 patients (68% female/32% male) were analyzed in this study. Exactly half or 57 patients were examined using CO_2 insufflation and the other 57 patients were examined with air insufflation. There were no significant differences in baseline patient characteristics including eligibility criteria for potentially difficult cases between the two groups (Table 1).

Outcome measures comparing CO_2 and air insufflation groups

There were no significant differences in procedure times including intubation, withdrawal and total time between the two groups (Table 2). Midazolam was administered to two patients (4%) in each group. There were no instances of $PtcCO_2$ > 60 mmHg in the CO_2 insufflation patients or any procedure-related complications in either group.

Figure 2 shows the mean VAS scores during and after colonoscopy examinations. VAS scores in the CO_2 insufflation group were significantly better than those in the air insufflation group (P < 0.001, ANOVA for repeated measures). The overall mean difference was 5.3 mm (95% CI: 3.5-7.1, P < 0.001). Comparison by nonparametric analysis at each measurement point produced results favoring CO_2 insufflation immediately following the examinations and up to 2 h afterwards. The maximum mean difference of 9.2 mm (95% CI: 0.4-18.0, P = 0.0049) was recorded 30 min after the examinations.

Table 1 Patient characteristics (n = 114) n (%)

	CO_2 group (n = 57)	Air group (n = 57)	P value
Median age, yr (IQR)	65 (59-73)	62 (47-71)	0.107
Females	39 (68)	38 (67)	1.00
Eligibility criteria for difficult cases[1]			
Females with relatively low BMI (< 22)	35 (61)	36 (63)	0.133
Previous abdominal and/or pelvic open surgery	41 (72)	37 (65)	0.546
Males with previously diagnosed left-side diverticulosis	6 (11)	2 (4)	0.271
One or more previous colonoscopies	16 (28)	15 (26)	1.00

[1]Some patients had more than one difficult case factor. IQR: Interquartile range; BMI: Body mass index.

Table 2 Use of antispasmodic drugs & median procedure times for CO_2 & air insufflation groups

	CO_2 group (n = 57)	Air group (n = 57)	P value
Patients receiving antispasmodic drug (%)	54 (95)	56 (98)	0.616
Median total procedure time, min (IQR)	22.5 (17.9-29.6)	22.3 (16.3-43.9)	0.734
Insertion to cecum	10.3 (6.5-16.6)	9.6 (5.8-16.2)	0.601
Withdrawal	11.9 (10.1-13.6)	12.0 (9.8-14.2)	0.986

Table 3 Median procedure times for colonoscopist groups

	EC group (n = 53)	LEC group (n = 61)	P value
Median total procedure time, min (IQR)	19.5 (15.3-25.8)	23.8 (19.2-34.5)	0.005
Insertion to cecum	7.7 (5.1-13.2)	12.5 (7.0-18.9)	0.036
Withdrawal	10.9 (10.0-13.0)	12.5 (10.2-15.1)	0.003
Examiner replaced during intubation	1	5	0.213

EC: Experienced colonoscopist; LEC: Less experienced colonoscopist.

Subgroup analysis

Based on the subgroup analysis relative to experience level of the participating colonoscopists, we evaluated 53 patients (46%) in the EC group and 61 patients (54%) in the LEC group. There were no significant differences in eligibility criteria for potentially difficult cases between the two groups, however, the EC group achieved insertion to the cecum significantly faster, while withdrawal and total procedure times were also significantly shorter than those in the LEC group (Table 3). The number of replacements by another colonoscopist was larger in the LEC group (5) than in the EC group (1), however, there was no significant difference between the two groups.

Figure 3 shows the mean VAS scores for 27 CO_2 insufflation patients and 26 air insufflation patients during and following colonoscopy examinations performed by the EC group. There were no significant differences in the mean VAS scores between the two patient groups

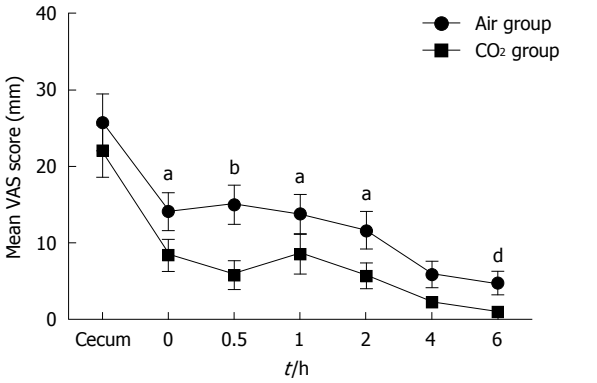

Figure 2 Mean VAS scores at corresponding measurement points during and after colonoscopy examinations in CO_2 and air insufflation groups. VAS scores for CO_2 insufflation were significantly better than those for air insufflation ($^dP < 0.001$, ANOVA for repeated measures). $^aP < 0.05$, $^bP < 0.01$ *vs* the CO_2 group at each measurement point by Wilcoxon rank sum test. VAS: Visual analogue scale.

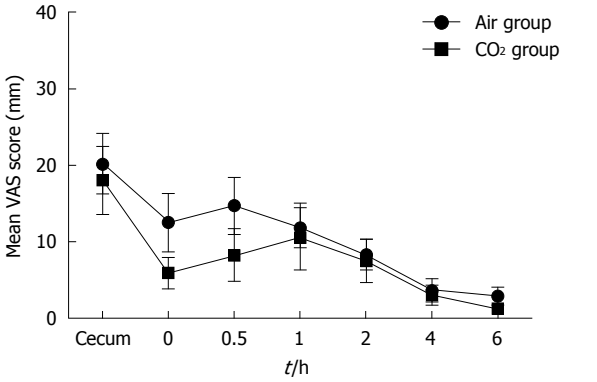

Figure 3 Mean VAS scores at corresponding measurement points during and after colonoscopy examinations for experienced colonoscopists (EC group) in CO_2 and air insufflation groups. There were no significant differences in VAS scores between the two insufflation groups for EC group (P = 0.29, ANOVA for repeated measures).

(P = 0.29, ANOVA for repeated measures). A comparison of the two patient groups at each measurement point also revealed no significant differences. The maximum mean difference of 6.5 mm (95% CI: -3.7-16.6, P = 0.207) occurred 30 min after the examinations.

In the LEC group, 30 CO_2 insufflation patients were evaluated along with 31 air insufflation patients. The mean VAS scores in the CO_2 insufflation group were significantly better than those in the air insufflation group (P = 0.023, ANOVA for repeated measures) (Figure 4). The overall mean difference was 7.5 mm (95% CI: 4.9-10.0, $P < 0.001$). A comparison of the two groups by nonparametric analysis at each measurement point produced results favoring CO_2 insufflation from immediately after the examinations up to 4 h later with the maximum mean difference of 11.6 mm (95% CI: 3.4-19.8, P = 0.006) occurring 30 min after the examinations.

DISCUSSION

The increase in patient abdominal pain and discomfort

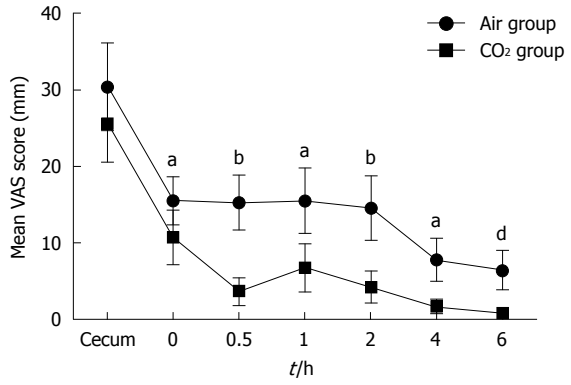

Figure 4 Mean VAS scores at corresponding measurement points during and after colonoscopy examinations for less experienced colonoscopists (LEC group) in CO_2 and air insufflation groups. VAS scores for CO_2 insufflation were significantly better compared to air insufflation for LEC group ($^dP = 0.023$, ANOVA for repeated measures). $^aP < 0.05$, $^bP < 0.01$ *vs* the CO_2 group at each measurement point by Wilcoxon rank sum test.

often encountered in difficult colonoscopy examination cases is a concern that needs to be satisfactorily resolved by colonoscopists. In this study, we successfully demonstrated the clinical effectiveness of CO_2 insufflation in potentially difficult colonoscopy examination cases. We also clarified the efficacy of CO_2 insufflation for LECs compared to highly ECs.

CO_2 with its characteristic rapid rate of absorption into surrounding tissue has been reported to be more suitable than atmospheric air in various clinical settings[12-15]. In fact, several randomized trials have shown that CO_2 insufflation reduced post-colonoscopy abdominal pain and discomfort compared to conventional air insufflation in ambulatory settings. Bretthauer *et al*[15] demonstrated that CO_2 insufflation was not only effective, but also safe during colonoscopies in patients receiving conscious sedation. Saito *et al*[19] introduced the use of CO_2 insufflation during lengthier colorectal endoscopic submucosal dissections in patients receiving conscious sedation. Their results demonstrated the effectiveness and safety of CO_2 insufflation as well as a resultant reduction in total dosage of midazolam. CO_2 insufflation has also been applied in endoscopic retrograde cholangiopancreatography (ERCP)[20] and endoscopic dilatation therapy using a double balloon endoscope[21]. There have been few detailed investigative reports on the use of CO_2 insufflation during difficult colonoscopy cases. In addition, the effect of the relative experience of colonoscopists using CO_2 insufflation has not been previously analyzed.

This study validated our theory that CO_2 insufflation is more effective than air insufflation in potentially difficult colonoscopy cases with the comparative difference for the two procedures being particularly discernable between LECs and ECs. Colonoscopy is a technically demanding procedure requiring considerable instruction and on-the-job experience for optimal performance. A suitable training program and sufficient opportunities to improve practical skills in a clinical setting are essential for beginners as well as colonoscopists with a moderate degree of experience[10,11,22].

Difficult colonoscopy examinations performed by LECs require additional time as do ERCP and therapeutic endoscopic procedures, and can cause patient abdominal pain and discomfort both during the procedure and afterwards. The results of our study demonstrated a difference not only in intubation times, but also in withdrawal and overall examination times according to the experience of the participating colonoscopists. Avoiding prolonged insufflation especially during insertion, however, might have led to similar results in the LEC group concerning the clinical effectiveness of CO_2 in reducing patient pain and discomfort.

Lee *et al*[8] recommended that trainees perform over 150 examinations in a colonoscopy training program to be technically competent for diagnostic colonoscopy. Our results revealed significant differences in examination times and patient abdominal pain and discomfort after colonoscopy between the EC and LEC groups. The four colonoscopists in the LEC group had each performed a minimum of 900 colonoscopies, thus the question arises as to whether a minimum of 150 cases referred to in the report by Lee above, is sufficient for conducting examinations in potentially difficult colonoscopy cases.

A recent study in Ontario, Canada analyzed factors associated with incomplete colonoscopies based on the following settings: an academic hospital, a community hospital and private medical offices. The incomplete colonoscopy rate was highest in private offices with an odds ratio increase of more than three-fold[3], thus introducing CO_2 insufflation may be particularly useful in reducing patient complaints in non-hospital environments. We refrained from using novice colonoscopists in this study because of the formidable nature of potentially difficult colonoscopy cases. Such novices should only conduct difficult colonoscopies after gaining the necessary experience performing routine colonoscopy examinations.

A number of techniques and devices have reportedly been effective in reducing patient abdominal pain and discomfort during difficult colonoscopies, improving the rate of successful insertion to the cecum, shortening insertion time to the cecum and reducing the dosage of sedatives[23] including the use of a pediatric colonoscope[24], variable stiffness colonoscope[25], gastroscope[26], double balloon endoscope[27] and hood attached to the top of the colonoscope[28]. A PVSC featuring both variable stiffness on demand and a thin diameter was used in our trial. Previously, this instrument was shown not to be superior to adult or standard pediatric colonoscopes[29-32]. However, there have been reports that use of the PVSC made it possible to complete colonoscopies that would have been much more difficult or impossible to perform using an adult colonoscope, including patients who had undergone hysterectomies[31] and patients with diverticular disease and severe stenosis[32].

There was only one case (0.85%) of incomplete insertion to the cecum in our study and just four (3.5%) patients required sedation. Complete screening colonoscopy without sedation or with on-demand sedation in

academic medical centers has been reported to be in the 88%-99% range[33-36], with the optimum intubation rate obtained using a PVSC. In this study, the PVSC more than likely contributed to the impressive successful intubation rates and reduction in pain during insertion to the cecum achieved in both groups, as well as the favorable intubation times for each group. In several studies performed by ECs at academic medical centers, insertion to the cecum times varied between 7-13 min for colonoscopies performed without sedation or with on-demand sedation[33-36]. Our median intubation times of 7.7 and 12.5 min for ECs and LECs, respectively, were in line with these earlier reports.

In conclusion, we clearly demonstrated the clinical effectiveness of CO_2 insufflation in potentially difficult colonoscopy examination cases performed without sedation. We also successfully clarified the efficacy of CO_2 insufflation for LECs.

COMMENTS

Background

Colonoscopy is the preferred method for preventing, detecting and treating colorectal cancer, however, prolonged cecal intubation can cause increased patient abdominal pain and discomfort especially in difficult cases, such as female patients with a relatively low body mass index, patients with a history of abdominal and/or pelvic open surgery and male patients with diverticulosis. CO_2 with its rapid rate of absorption has been reported to be more suitable than atmospheric air as an insufflation agent in various clinical settings, although air insufflation is still the standard method due to a lack of suitable equipment and inadequate information as to when and on whom CO_2 insufflation should be used during colonoscopy examinations.

Research frontiers

This prospective randomized controlled study was conducted to clarify the effectiveness of CO_2 insufflation in potentially difficult cases, particularly in relation to colonoscopist experience level.

Innovations and breakthroughs

The clinical effectiveness of CO_2 insufflation was clearly demonstrated in potentially difficult colonoscopy examination cases performed without sedation. The procedure that was followed also clarified the efficacy of CO_2 insufflation for less experienced colonoscopists (LEC) particularly in comparison to more experienced colonoscopists.

Applications

The use of CO_2 insufflation can be incorporated into existing and future colonoscopy training programs in order to further improve the technical skills of colonoscopists.

Peer review

The authors successfully demonstrated that CO_2 insufflation with its rapid rate of CO_2 absorption and improved efficacy reduced patient pain in potentially difficult cases particularly when colonoscopy examinations were performed by LECs.

REFERENCES

1 **Winawer SJ**, Zauber AG, Ho MN, O'Brien MJ, Gottlieb LS, Sternberg SS, Waye JD, Schapiro M, Bond JH, Panish JF. Prevention of colorectal cancer by colonoscopic polypectomy. The National Polyp Study Workgroup. *N Engl J Med* 1993; **329**: 1977-1981

2 **Winawer S**, Fletcher R, Rex D, Bond J, Burt R, Ferrucci J, Ganiats T, Levin T, Woolf S, Johnson D, Kirk L, Litin S, Simmang C. Colorectal cancer screening and surveillance: clinical guidelines and rationale-Update based on new evidence. *Gastroenterology* 2003; **124**: 544-560

3 **Shah HA**, Paszat LF, Saskin R, Stukel TA, Rabeneck L. Factors associated with incomplete colonoscopy: a population-based study. *Gastroenterology* 2007; **132**: 2297-2303

4 **Kim WH**, Cho YJ, Park JY, Min PK, Kang JK, Park IS. Factors affecting insertion time and patient discomfort during colonoscopy. *Gastrointest Endosc* 2000; **52**: 600-605

5 **Anderson JC**, Gonzalez JD, Messina CR, Pollack BJ. Factors that predict incomplete colonoscopy: thinner is not always better. *Am J Gastroenterol* 2000; **95**: 2784-2787

6 **Nelson DB**, McQuaid KR, Bond JH, Lieberman DA, Weiss DG, Johnston TK. Procedural success and complications of large-scale screening colonoscopy. *Gastrointest Endosc* 2002; **55**: 307-314

7 **Bernstein C**, Thorn M, Monsees K, Spell R, O'Connor JB. A prospective study of factors that determine cecal intubation time at colonoscopy. *Gastrointest Endosc* 2005; **61**: 72-75

8 **Lee SH**, Chung IK, Kim SJ, Kim JO, Ko BM, Hwangbo Y, Kim WH, Park DH, Lee SK, Park CH, Baek IH, Park DI, Park SJ, Ji JS, Jang BI, Jeen YT, Shin JE, Byeon JS, Eun CS, Han DS. An adequate level of training for technical competence in screening and diagnostic colonoscopy: a prospective multicenter evaluation of the learning curve. *Gastrointest Endosc* 2008; **67**: 683-689

9 **Eckardt AJ**, Swales C, Bhattacharya K, Wassef WY, Phelan NP, Zubair S, Martins N, Patel S, Moquin B, Anwar N, Leung K, Levey JM. Open access colonoscopy in the training setting: which factors affect patient satisfaction and pain? *Endoscopy* 2008; **40**: 98-105

10 **Bowles CJ**, Leicester R, Romaya C, Swarbrick E, Williams CB, Epstein O. A prospective study of colonoscopy practice in the UK today: are we adequately prepared for national colorectal cancer screening tomorrow? *Gut* 2004; **53**: 277-283

11 **Bisschops R**, Wilmer A, Tack J. A survey on gastroenterology training in Europe. *Gut* 2002; **50**: 724-729

12 **Bretthauer M**, Thiis-Evensen E, Huppertz-Hauss G, Gisselsson L, Grotmol T, Skovlund E, Hoff G. NORCCAP (Norwegian colorectal cancer prevention): a randomised trial to assess the safety and efficacy of carbon dioxide versus air insufflation in colonoscopy. *Gut* 2002; **50**: 604-607

13 **Sumanac K**, Zealley I, Fox BM, Rawlinson J, Salena B, Marshall JK, Stevenson GW, Hunt RH. Minimizing postcolonoscopy abdominal pain by using CO(2) insufflation: a prospective, randomized, double blind, controlled trial evaluating a new commercially available CO(2) delivery system. *Gastrointest Endosc* 2002; **56**: 190-194

14 **Church J**, Delaney C. Randomized, controlled trial of carbon dioxide insufflation during colonoscopy. *Dis Colon Rectum* 2003; **46**: 322-326

15 **Bretthauer M**, Lynge AB, Thiis-Evensen E, Hoff G, Fausa O, Aabakken L. Carbon dioxide insufflation in colonoscopy: safe and effective in sedated patients. *Endoscopy* 2005; **37**: 706-709

16 **Gisiger PA**, Palma JP, Eberhard P. OxiCarbo®, a single sensor for the non-invasive measurement of arterial oxygen saturation and CO2 partial pressure at the ear lobe. *Sens Actuators B Chem* 2001; **76**: 527-530

17 **Heuss LT**, Chhajed PN, Schnieper P, Hirt T, Beglinger C. Combined pulse oximetry/cutaneous carbon dioxide tension monitoring during colonoscopies: pilot study with a smart ear clip. *Digestion* 2004; **70**: 152-158

18 **Wewers ME**, Lowe NK. A critical review of visual analogue scales in the measurement of clinical phenomena. *Res Nurs Health* 1990; **13**: 227-236

19 **Saito Y**, Uraoka T, Matsuda T, Emura F, Ikehara H, Mashimo Y, Kikuchi T, Kozu T, Saito D. A pilot study to assess the safety and efficacy of carbon dioxide insufflation during colorectal endoscopic submucosal dissection with the patient under conscious sedation. *Gastrointest Endosc* 2007; **65**: 537-542

20 **Bretthauer M**, Seip B, Aasen S, Kordal M, Hoff G, Aabakken L. Carbon dioxide insufflation for more comfortable endoscopic retrograde cholangiopancreatography: a randomized, controlled, double-blind trial. *Endoscopy* 2007; **39**: 58-64

21 **Hirai F**, Matsui T, Yao K, Sou S, Seki T. Efficacy of carbon

dioxide insufflation in endoscopic balloon dilation therapy by using double balloon endoscopy. *Gastrointest Endosc* 2007; **66**: S26-S29

22 **Grassini M**, Verna C, Battaglia E, Niola P, Navino M, Bassotti G. Education improves colonoscopy appropriateness. *Gastrointest Endosc* 2008; **67**: 88-93

23 **Rex DK**, Chen SC, Overhiser AJ. Colonoscopy technique in consecutive patients referred for prior incomplete colonoscopy. *Clin Gastroenterol Hepatol* 2007; **5**: 879-883

24 **Marshall JB**, Perez RA, Madsen RW. Usefulness of a pediatric colonoscope for routine colonoscopy in women who have undergone hysterectomy. *Gastrointest Endosc* 2002; **55**: 838-841

25 **Brooker JC**, Saunders BP, Shah SG, Williams CB. A new variable stiffness colonoscope makes colonoscopy easier: a randomised controlled trial. *Gut* 2000; **46**: 801-805

26 **Kozarek RA**, Botoman VA, Patterson DJ. Prospective evaluation of a small caliber upper endoscope for colonoscopy after unsuccessful standard examination. *Gastrointest Endosc* 1989; **35**: 333-335

27 **Kaltenbach T**, Soetikno R, Friedland S. Use of a double balloon enteroscope facilitates caecal intubation after incomplete colonoscopy with a standard colonoscope. *Dig Liver Dis* 2006; **38**: 921-925

28 **Kondo S**, Yamaji Y, Watabe H, Yamada A, Sugimoto T, Ohta M, Ogura K, Okamoto M, Yoshida H, Kawabe T, Omata M. A randomized controlled trial evaluating the usefulness of a transparent hood attached to the tip of the colonoscope. *Am J Gastroenterol* 2007; **102**: 75-81

29 **Shumaker DA**, Zaman A, Katon RM. Use of a variable-stiffness colonoscope allows completion of colonoscopy after failure with the standard adult colonoscope. *Endoscopy* 2002; **34**: 711-714

30 **Rex DK**. Effect of variable stiffness colonoscopes on cecal intubation times for routine colonoscopy by an experienced examiner in sedated patients. *Endoscopy* 2001; **33**: 60-64

31 **Shumaker DA**, Zaman A, Katon RM. A randomized controlled trial in a training institution comparing a pediatric variable stiffness colonoscope, a pediatric colonoscope, and an adult colonoscope. *Gastrointest Endosc* 2002; **55**: 172-179

32 **Kaffes AJ**, Mishra A, Ding SL, Hope R, Williams SJ, Gillespie PE, Bourke MJ. A prospective trial of variable stiffness pediatric vs. standard instrument colonoscopy. *Gastrointest Endosc* 2003; **58**: 685-689

33 **Yörük G**, Aksöz K, Unsal B, Buyraç Z, Buran T, Yazicioğlu N, Yildiz C, Yalçin HC. Colonoscopy without sedation. *Turk J Gastroenterol* 2003; **14**: 59-63

34 **Ladas SD**. Factors predicting the possibility of conducting colonoscopy without sedation. *Endoscopy* 2000; **32**: 688-692

35 **Konishi K**, Kaneko K, Kurahashi T, Yamamoto T, Kushima M, Kanda A, Tajiri H, Mitamura K. A comparison of magnifying and nonmagnifying colonoscopy for diagnosis of colorectal polyps: A prospective study. *Gastrointest Endosc* 2003; **57**: 48-53

36 **Takahashi Y**, Tanaka H, Kinjo M, Sakumoto K. Sedation-free colonoscopy. *Dis Colon Rectum* 2005; **48**: 855-859

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BRIEF ARTICLE

Crosstalk between angiogenesis, cytokeratin-18, and insulin resistance in the progression of non-alcoholic steatohepatitis

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Abstract

AIM: To elucidate the possible crosstalk between angiogenesis, cytokeratin-18 (CK-18), and insulin resistance (IR) especially in patients with non-alcoholic steatohepatitis (NASH).

METHODS: Twenty-eight patients with NASH and 11 with simple fatty liver disease (FL) were enrolled in this study and underwent clinicopathological examination. The measures of angiogenesis, CK-18, and IR employed were CD34-immunopositive vessels, CK-18-immunopositive cells, and homeostasis model assessment of IR (HOMA-IR), respectively. The correlations of these factors with NASH were elucidated.

RESULTS: Significant development of hepatic neovascularization was observed only in NASH, whereas almost no neovascularization could be observed in FL and healthy liver. The degree of angiogenesis was almost parallel to liver fibrosis development, and both parameters were positively correlated. Similarly, CK-18 expression and HOMA-R were significantly increased in NASH as compared with FL and healthy liver. Furthermore, CK-18 and HOMA-IR were also positively correlated with the degree of neovascularization.

CONCLUSION: These results indicate that the crosstalk between angiogenesis, CK-18, and IR may play an important role in the onset and progression of NASH.

Key words: Angiogenesis; Cytokeratin-18; Fatty liver; Insulin resistance; Non-alcoholic steatohepatitis; Liver fibrosis

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) ranges from simple steatosis to cirrhosis. Fatty liver (FL) has been recognized as a benign and non-progressive condition[1,2]. However, non-alcoholic steatohepatitis (NASH) is now widely known as a liver disease which may progress to liver cirrhosis and finally hepatocellular carcinoma (HCC). Fibrosis development is only seen in NASH but not in simple FL. The pathogenesis of NASH is not well understood, but it is unlikely that one of the recognized mechanisms explains all pathogenic processes of NASH. Thereby, NASH may develop as a consequence of a multifactorial process[2].

Angiogenesis i.e. formation of new vessels by sprouting from the pre-existing vasculature, is of central importance for embryonic development and organogenesis. Abnormally pathological angiogenesis is observed in rheumatoid arthritis, psoriasis, diabetic retinopathy, tumor growth, and even in fibrogenesis[3]. Although previous studies conducted to determine the molecular process associated with fibrosis and angiogenesis were performed independently, recent studies have revealed that both biological phenomena developed synergistically[4]. It was shown that neovascularization significantly increased during the development of liver fibrosis both in human and animal experimental studies[5,6]. In the NASH experimental model, we previously demonstrated that angiogenesis plays an important role in the progression of NASH[7]. We also reported that neovascularization developed in patients with NASH whereas no marked augmentation was observed in FL or the healthy liver[8].

Recent studies have suggested that increased hepatocyte apoptosis has an important role in progression from simple FL to NASH, and correlates with disease severity and hepatic fibrosis[9,10]. During apoptosis, some intracellular proteins are cleaved by caspase. A neoepitope in cytokeratin-18 (CK-18), the major intermediate filament protein in the liver, becomes available at an early caspase cleavage event during apoptosis and is not detectable in the viable or necrotic cells[11]. It has been reported that CK-18 fragments were markedly elevated in NAFLD patients as compared with healthy controls, and that the plasma levels of CK-18 correlated with the expression levels in the liver. A significant increase in CK-18 expression could be observed in patients with NASH as compared with the simple FL patients, and the expression level of CK-18 correlated with the presence of fibrosis[10]. The same group recently conducted a multicenter validation study which yielded similar results, and CK-18 was identified as an independent predictor of NASH[12].

NASH is well known as a liver disease that frequently complicates insulin resistance (IR) status[13]. Current evidence points to IR and subsequent hyperinsulinemia as the key pathogenic factors in NAFLD and progression from simple FL to NASH[13]. It has been reported that IR accelerated the progression in a NASH animal model, and that an insulin-sensitizing agent could reverse the underlying pathogenesis involved through improvement in IR[14]. We also have demonstrated that IR itself significantly promoted liver fibrosis development in diabetic rats[15]. Several studies have shown that insulin-sensitizing agents improved the metabolic and histologic parameters, most notably liver injury and fibrosis, not only in experimental models, but also in patients with NASH[16,17]. Collectively, these findings indicate that neovascularization, CK-18 expression, and IR status play pivotal roles in the progression of NASH. We previously demonstrated that angiogenesis and IR play important roles in animal NASH models[7,15]. However, no study has been conducted as yet to examine the interaction among these parameters in NASH using human cases.

In the current study, we elucidated the possible correlation between angiogenesis, CK-18, and IR especially in fibrosis development, which is one of the specific characteristic features of NASH compared with simple FL.

MATERIALS AND METHODS

Patients and methods

We recruited a total of 39 patients with NAFLD between 2001 and 2007 at Nara Medical University, and three healthy volunteers were enrolled as a control group. Twenty-eight patients with NASH (17 males and 11 females) and 11 patients with simple FL (seven males and four females), diagnosed by histological examination, were enrolled in this study. First, all patients were re-evaluated clinically for evidence of diseases including diabetes mellitus and hypertension. Alcohol-induced hepatitis was excluded according to each patient's self-report and was confirmed by the family. The height and weight were measured, and the body mass index (BMI) was calculated. Hepatitis B virus surface antigen and hepatitis C virus antibody were negative in all patients. The standard liver function tests were performed for all patients. The fasting blood levels of glucose and insulin were assessed, and the homeostasis model assessment parameter of IR (HOMA-IR) was calculated. The serum fibrosis markers, namely, hyaluronic acid, type IV collagen 7S (7S-collagen), and amino-terminal peptide of type-Ⅲ pro-collagen, were measured by latex agglutination, enzyme immunoassay, and radioimmunoassay, respectively, using routine laboratory methods. The serum levels of leptin and adiponectin were measured by ELISA kit (R&D systems, Tokyo, Japan) according to the manufacturer's instructions as described previously[18,19].

Histology and immunohistochemistry

Liver biopsies obtained for diagnostic purposes were histologically examined. The liver biopsy specimens exceeded 1.5-2 cm in length and 1.4 cm in width, and contained more than five portal areas in each sample that was sufficient to perform the immunohistochemical analysis. In all samples, serial sections were used for analysis. The first section was routinely stained with hematoxylin and eosin for histological examination. Another section was stained with Sirius Red to detect fibrosis development. The fibrosis stages was scored in an F0 to F4 scale, where F0 means absence of fibrosis; F1, portal fibrosis with few septa; F2, few septa; F3, abundant septa with cirrhosis; F4, cirrhosis, as described elsewhere[20]. Regarding the 28 patients with NASH, 10 had low-grade fibrosis (F0 and F1), and 18 had high-grade fibrosis (F2 to F4). For determination of neovascularization, we employed immunohistochemical detection of CD34, which is widely used as a marker of neovascularization, using paraffin-embedded sections as described previously[21]. We also performed immunohistochemical staining of CK-18 in the liver. Liver tissue samples were fixed in 4% formaldehyde solution and embedded in paraffin. Then, 5 μm tissue sections were incubated in 0.1% hydrogen peroxide in 70%

Table 1 Characteristic features of patients with FL and NASH (mean ± SD)

	NASH (*n* = 28)	FL (*n* = 11)	*P*-value
Age (yr)	45.4 ± 14.7	43.6 ± 14.1	0.587
Sex (M/F)	17/11	7/4	0.660
BMI (kg/m^2)	28.5 ± 6.91	25.8 ± 1.16	0.042
Total cholesterol (mg/dL)	218.6 ± 43.3	203.4 ± 48.3	0.350
Triglycerides (mg/dL)	186.1 ± 69.3	237.1 ± 98.2	0.075
FFA (mmol/L)	0.532 ± 0.207	0.897 ± 0.804	0.079
Fasting plasma glucose (mg/dL)	113.3 ± 30.0	98.8 ± 19.2	0.116
Fasting plasma insulin (μU/mL)	20.8 ± 11.5	10.0 ± 3.91	0.018
HOMA-IR	5.79 ± 4.15	2.26 ± 0.96	0.038
HbA1c (%)	6.09 ± 1.29	5.25 ± 0.53	0.130
Serum albumin (g/dL)	4.57 ± 0.33	4.54 ± 0.39	0.774
AST (IU/L)	63.4 ± 27.0	37.5 ± 10.0	0.012
ALT (IU/L)	105.5 ± 65.7	69.8 ± 31.2	0.035
γ-GTP (IU/L)	68.4 ± 37.1	64.2 ± 47.8	0.770
ZTT (kU)	7.87 ± 4.60	5.89 ± 2.39	0.224
Total bilirubin (mg/dL)	0.80 ± 0.25	1.00 ± 0.98	0.305
ICG R15 (%)	10.9 ± 4.28	9.00 ± 2.61	0.264
Procollagen-Ⅲ-peptide (U/mL)	0.83 ± 0.31	0.96 ± 0.23	0.454
Collagen Ⅳ-7S (ng/mL)	4.46 ± 1.83	4.40 ± 1.52	0.762
Hyaluronic acid (ng/mL)	60.4 ± 57.6	15.0 ± 9.06	0.036
Platelets (10^4/μL)	21.2 ± 8.83	23.2 ± 4.85	0.470
Leptin (ng/mL)	7.23 ± 3.98	2.58 ± 0.43	0.040
Adiponectin (ng/mL)	5.06 ± 1.90	8.25 ± 0.95	0.016

FL: Fatty liver; NASH: Non-alcoholic steatohepatitis; M: Male; F: Female.

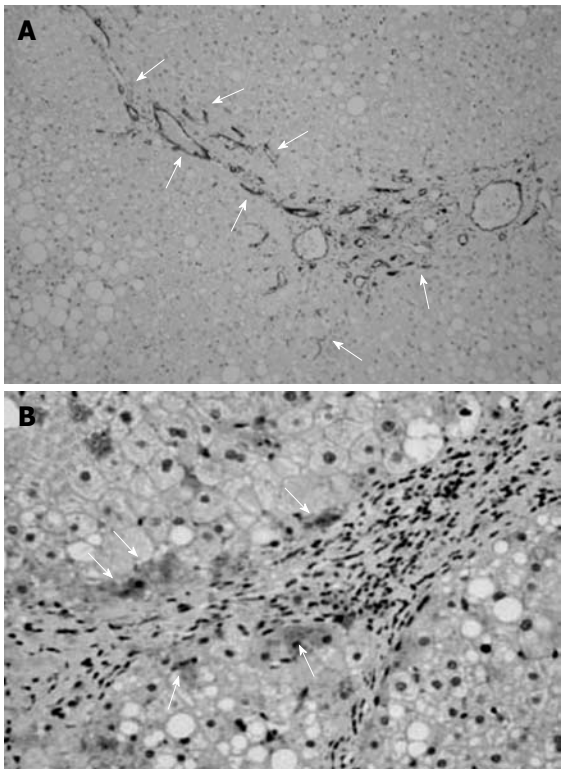

Figure 1 Representative microphotographs of CD34-positive neovessels and cytokeratin-18 (CK-18)-positive cells in the liver of patients. A: Marked CD34-positive immunopositive vessels could be found in NASH along with liver fibrosis development; B: The immunopositivity of CK-18 was mainly observed in the hepatocytes. The arrows indicate CD34 and CK-18-immunopositivity in A and B, respectively. The original magnification was × 100.

methanol for 30 min to inhibit the endogenous peroxidase activity. Microwave antigen retrieval was performed at 500 W for 15 min with pH 9 antigen retrieval solution (Nichirei Bioscience Inc., Tokyo, Japan). Then, 10% fetal bovine serum with 0.3% Triton X was used to prevent nonspecific staining. The slides were subsequently incubated overnight at 4℃ in humidified chambers with primary rabbit polyclonal anti-CD34 antibody at a dilution of 1:100 and primary rabbit polyclonal anti-CK-18 antibody (DAKO, Kyoto, Japan) at a dilution of 1:100. The sections were rinsed three times in a phosphate-buffered solution and further incubated with a biotinylated secondary antibody for 30 min at room temperature. Antigen-antibody complexes were detected by the avidin-biotin-peroxide method, using diaminobenzidine as a chromogenic substrate (DAKO, Carpentaria, CA, USA). Finally, the slides were counter-stained with hematoxylin and then examined microscopically. Immunopositive quantitation of CD34 and Sirius Red-positive liver fibrosis areas were evaluated with Adobe Photoshop software and NIH image software as described previously[22]. The intensity of CK-18 staining was scored from 0 to 4 as previously described[8] with minor modification; 0: no staining; 1: mild (punctured labeling); 2: mild to moderate (dense labeling in a few lesions); 3: moderate; 4: strong (dense and homogeneous labeling in large areas).

Statistical analysis

All data are expressed as mean ± SD. The statistical analysis was performed using the χ^2 test for independence, the two-tailed Student's *t*-test, and simple regression analysis. Correlation between two parameters was tested by the Spearman rank correlation matrix.

RESULTS

Clinical features

The clinical features of both groups are shown in Table 1. Most of the clinical features in the FL and NASH patients were not significantly different except BMI, HOMA-IR, serum aspartate aminotransferase (AST), hyaluronic acid, leptin, and adiponectin. The NASH patients had significantly higher BMI (28.8 ± 4.33 *vs* 25.6 ± 1.20), HOMA-IR (5.79 ± 4.15 *vs* 2.26 ± 0.96), AST (59.4 ± 27.0 *vs* 37.5 ± 10.0), hyaluronic acid (62.0 ± 58.6 *vs* 15.0 ± 9.06), and leptin (7.79 ± 4.47 *vs* 2.58 ± 0.43), than the FL patients. On the other hand, significantly lower serum adiponectin levels were observed in the NASH patients compared with the FL patients (5.31 ± 1.70 *vs* 8.25 ± 0.95).

Neovascularization

The typical features of CD34-immunopositive neovessels in the liver are shown in Figure 1A. Apparent CD34-positive vessels could be observed neither in the liver of the healthy subjects nor in the FL patients, whereas marked immunopositive vessels could be observed in NASH livers along with the development of liver fibrosis. In the liver of low-grade fibrosis, neovascularization could

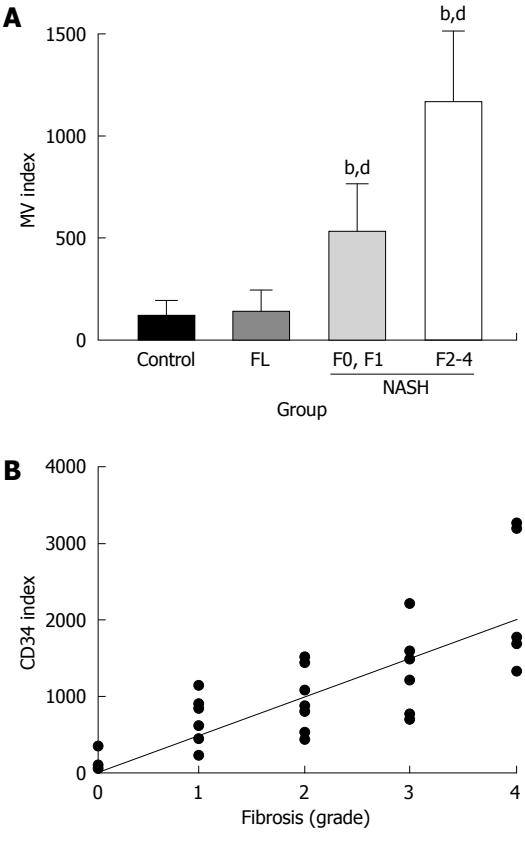

Figure 2 Semi-quantitative analysis of CD34 and possible correlation with liver fibrosis development. A: Semi-quantitative analysis of the CD34-positive neovessels in the liver in non-alcoholic fatty liver disease (NAFLD). There was no difference between the control healthy liver and simple FL. In NASH, a marked augmentation of neovascularization was found in the liver of NASH as compared with FL. The magnitude of neovascularization in high-grade (F2 to F4) liver fibrosis was more than in low-grade (F0, F1) fibrosis. The proportional increase in neovascularization was almost parallel to the development of liver fibrosis. $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* low-grade fibrosis with NASH group. The number of patients in each group was as follows: Control (n = 3), FL (n = 11), low-grade fibrosis (F0 and F1: n = 10), and high-grade fibrosis (F2 to F4: n = 18). MV: Microvessel density; B: The relationship between the development of CD-34-positive vessels and fibrosis grade in NAFLD. The degree of angiogenesis correlated with the development of fibrosis. The equation was y = 509.82x - 6.7958. The correlation coefficient was 0.6288.

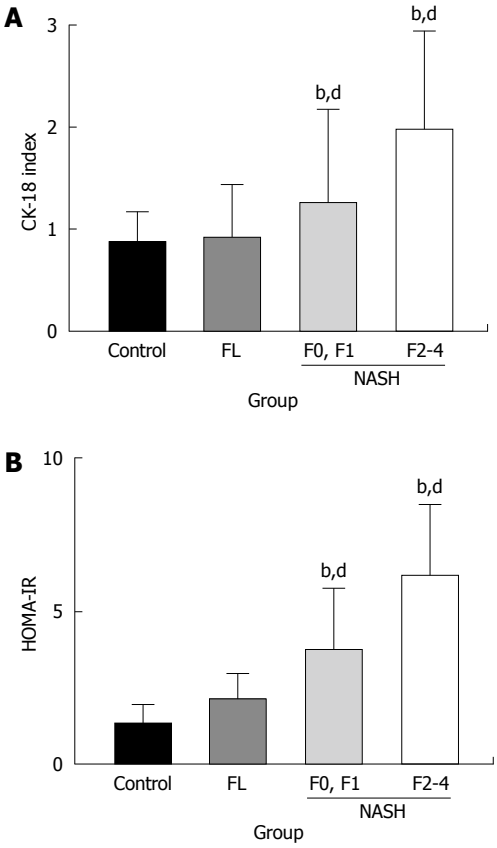

Figure 3 Semi-quantitative analysis of the CK-18-positive cells in the liver (A) and HOMA-IR (B) in NAFLD. Similar to the CD34-positive neovascularization, there was no difference between the control healthy liver and FL. In NASH, a marked augmentation of CK-18 (A) and HOMA-IR (B) was found in the liver of NASH as compared with FL. The magnitude of neovascularization in high-grade (F2 to F4) liver fibrosis was more than in low-grade (F0, F1) fibrosis. The number of patients in each group was as follows; C (n = 3), FL (n = 11), low-grade fibrosis (F0 and F1: n = 10), and high-grade fibrosis (F2 to F4: n = 18). $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* low-grade fibrosis with NASH group.

be observed around the central vein (zone Ⅲ). These neovessels progressed to the portal area (zone Ⅰ) and were also observed along the fibrotic septa in high-grade fibrosis. We next performed a semiquantitative analysis of the CD34-positive neovessels in conjunction with liver fibrosis development. There was no difference between the normal control liver and the FL (Figure 2A), which conformed with the immunohistochemical features. In NASH, a marked augmentation of neovascularization was found in the liver of NASH cases as compared with the FL. The magnitude of neovascularization in high-grade (F2 to F4) liver fibrosis was more than in low-grade (F0, F1) fibrosis. The increase in neovascularization was almost parallel to the development of liver fibrosis. As shown in Figure 2B, the degree of CD34-positive vessels positively correlated with fibrosis development (r^2 = 0.6288).

CK-18 and IR

We next examined CK-18 expression in the liver. The immunopositivity of CK-18 was mainly observed in the hepatocytes (Figure 1B). Similar to CD34, CK-18 expression was significantly increased in the NASH patients as compared with simple FL and healthy liver. Staining of CK-18 was barely visible in the healthy liver and FL, whereas most NASH cases showed positive staining. Among the NASH patients, those with high-grade fibrosis had greater hepatic expression of CK-18 than those with low-grade fibrosis (Figure 3A). We observed that the magnitude of CK-18 expression positively correlated with CD34-positive neovascularization (r^2 = 0.6512, Figure 4A). We also examined the possible role of IR in progression of NASH, and observed that HOMA-IR was significantly higher in NASH patients than in simple FL and healthy liver. The HOMA-IR also increased with the progression of NASH, and there was a positive correlation between HOMA-IR and hepatic neovascularization (Figure 3B and Figure 4B, respectively).

DISCUSSION

Recent studies have revealed that angiogenesis plays an important role in many pathological events, including

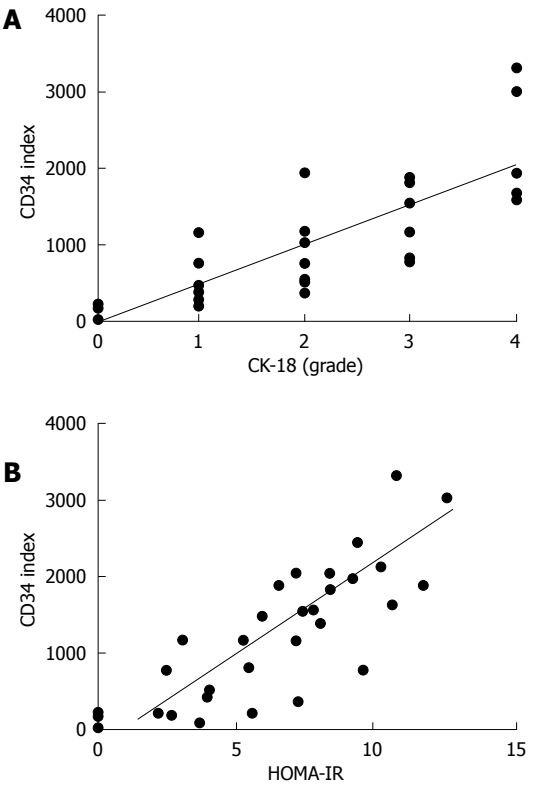

Figure 4 The relationship between angiogenesis, CK-18-positive vessels (A), and HOMA-IR (B). The degree of angiogenesis correlated with the CK-18-positive cells (A) and HOMA-IR (B). A: The magnitude of expression of CK-18 correlated positively with CD34-positive neovascularization. The equation was y = 500.63x + 4.6606. The correlation coefficient was 0.6512; B: Similarly, there was a positive correlation between HOMA-IR and hepatic neovascularization. The equation was y = 233.75x - 339.39. The correlation efficient was 0.6184.

liver fibrosis development. Although previous studies conducted to determine the molecular processes associated with fibrosis and angiogenesis were performed independently, recent studies have revealed that both biological phenomena emerged concomitantly[4]. Angiogenesis in the liver is characterized by capillarization of the sinusoids[3]. It has been shown that capillarization and phenotypic changes of the hepatic sinusoidal endothelial cells (ECs) occur during liver fibrosis development[23]. It has been reported that CD34 is not expressed by healthy ECs, but when ECs alter their phenotype, they can express CD34[24]. Much attention is focused on a possible association with chronic liver diseases[25]. A recent study on chronic hepatitis C (CHC) has shown that the number of CD34-positive new vessels was significantly increased and positively correlated with the fibrosis stage[26]. As well as CHC, in the current study we found a significant development of hepatic CD34-positive neovascularization in NASH, whereas almost no development could be observed in FL. The degree of angiogenesis was almost parallel to the development of liver fibrosis in NASH. These results are consistent with our previous experimental finding that angiogenesis increased stepwise during hepatic fibrosis development in several fibrosis models, including the rodent dietary NASH model[5,7]. We also observed that HOMA-IR was significantly higher in NASH along with liver fibrosis development, and that it positively correlated with the development of neovascularization. We previously reported that the IR status, i.e. co-existence of high glucose and insulin, itself significantly promoted liver fibrosis development in rats along with augmentation of neovascularization. Furthermore, high glucose and insulin stimulated *in vitro* neovascularization, and the combination treatment with glucose and insulin significantly promoted the effect as compared with either agent alone[15]. Collectively, it is likely that IR-mediated neovascularization is involved in the development and progression of NASH.

Because NAFLD is a common manifestation of metabolic syndromes, various adipocytokines are involved in the progression of NASH[27,28]. Among them, adiponectin and leptin are well known to be involved in the pathogenesis of NASH[27,29,30]. Adiponectin administration alleviates NAFLD progression in mice, and liver fibrosis is accelerated in adiponectin knockout mice, indicating the protective effect of adiponectin against liver fibrosis development in NASH[31]. In the NASH patients, the circulating adiponectin is reportedly decreased[30], and we also observed that the serum level of adiponectin in NASH decreased significantly more than in FL. On the other hand, recent reports have revealed that leptin exerts pro-fibrogenic activity. Hepatic fibrogenesis is impaired in leptin- and leptin receptor-deficient animals[29,32]. Leptin also enhances proliferation of activated hepatic stellate cells (HSCs), which play a central role in the development of liver fibrosis[33]. In addition to these direct effects on HSC, recent studies have revealed that leptin possesses angiogenic activity[7,34]. We previously reported that leptin exerted a potent angiogenic effect, and that leptin-mediated neovascularization played an important role in the development of liver fibrosis in the rat NASH model[7]. In the current study, the serum leptin level was significantly higher in NASH than in FL. However, we only measured the serum level of leptin in the current study. The role of leptin in fibrosis development in NASH is still controversial. It has been reported that local leptin plays a more important role than serum leptin in the progression of NASH[35]. Further studies are required to elucidate the local leptin and leptin receptor interaction with *in situ* hybridization in the future.

As well as neovascularization, we observed that the hepatocyte apoptosis marker, CK-18, was also significantly increased in NASH as compared with simple FL. This finding was consistent with recent reports suggesting that the CK-18 expression can detect the presence of NASH[10,12,36]. Uncontrolled hepatocyte apoptosis proved to be an important event triggering liver fibrogenesis[9]. We also observed that the expression of CK-18 was increased along with liver fibrosis development in NASH. Moreover, there was a positive correlation between the CK-18 expression and hepatic neovascularization. These results suggested that there was some crosstalk between CK-18 and angiogenesis in the liver of NASH. As well as being a marker of apoptosis, CK-18 has been found to enhance the migratory and invasive potential of tumor cells[37]. Furthermore, it has been reported

that CK-18 expression was significantly higher in HCC than in CHC[38]. We previously reported that hepatic neovascularization increased in a stepwise manner during hepatocarcinogenesis, and the increase in angiogenesis was mainly observed in the glutathione-S-transferase placental form (GST-P)-positive pre-neoplastic lesions as compared with the adjacent tissues[39,40]. A recent report has shown that CK-18 expression also significantly increased in GST-P-positive lesions[41]. These results indicate that, as well as being a marker of apoptosis, CK-18 may have a direct association with hepatic neovascularization.

It is important to elucidate whether the correlation among these factors is a cause or consequence of NASH. However, in this study, we could not identify what factors were the causes or consequences in the progression of NASH. Although the respective factors interact with each other, further sequential studies are required in the future to determine what factors developed prior to other factors during the progression of NASH. Also, the number of patients was not high enough in the current study. We are acquiring a larger number of patients' files and when the sample of patients with NASH and simple FL is adequate, we will perform an advanced analysis of the current parameters.

In conclusion, we have shown that only the liver of NASH cases had marked neovascularization whereas simple FL and healthy livers did not. The hepatic neovascularization was proportional to the increase in grade of liver fibrosis. CK-18 expression and HOMA-IR were also significantly increased in NASH as compared with FL and healthy liver. Furthermore, CK-18 and HOMA-IR also positively correlated with the degree of neovascularization. These results indicate that the crosstalk between angiogenesis, CK-18, and IR may play an important role in the onset and progression of NASH.

COMMENTS

Background

It has been reported that angiogenesis, cytokeratin-18 (CK-18), and insulin resistance (IR) play important roles in the development of non-alcoholic steatohepatitis (NASH). The aim of the current study was to elucidate the possible crosstalk between angiogenesis, CK-18, and IR especially in patients with NASH.

Research frontiers

Significant development of hepatic neovascularization was observed only in NASH, and the degree of angiogenesis was almost parallel to liver fibrosis development. Similarly, CK-18 expression and IR were significantly increased in NASH. Furthermore, CK-18 and IR also positively correlated with the degree of neovascularization.

Innovations and breakthroughs

Only NASH was associated with marked neovascularization, which was proportional to the increase in grade of liver fibrosis. Moreover, the degree of neovascularization positively correlated with CK-18 and IR in NASH. These results emphasize the new findings that the crosstalk between angiogenesis, CK-18, and IR plays an important role in the onset and progression of NASH.

Applications

The novel findings may lead to a new alternative therapy for NASH in the near future.

Peer review

The manuscript is well written and the conclusions are appropriate for the results.

REFERENCES

1 **Angulo P**. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; **346**: 1221-1231
2 **Reid AE**. Nonalcoholic steatohepatitis. *Gastroenterology* 2001; **121**: 710-723
3 **Carmeliet P**. Angiogenesis in life, disease and medicine. *Nature* 2005; **438**: 932-936
4 **Kalluri R**, Sukhatme VP. Fibrosis and angiogenesis. *Curr Opin Nephrol Hypertens* 2000; **9**: 413-418
5 **Yoshiji H**, Kuriyama S, Yoshii J, Ikenaka Y, Noguchi R, Hicklin DJ, Wu Y, Yanase K, Namisaki T, Yamazaki M, Tsujinoue H, Imazu H, Masaki T, Fukui H. Vascular endothelial growth factor and receptor interaction is a prerequisite for murine hepatic fibrogenesis. *Gut* 2003; **52**: 1347-1354
6 **Medina J**, Caveda L, Sanz-Cameno P, Arroyo AG, Martín-Vílchez S, Majano PL, García-Buey L, Sánchez-Madrid F, Moreno-Otero R. Hepatocyte growth factor activates endothelial proangiogenic mechanisms relevant in chronic hepatitis C-associated neoangiogenesis. *J Hepatol* 2003; **38**: 660-667
7 **Kitade M**, Yoshiji H, Kojima H, Ikenaka Y, Noguchi R, Kaji K, Yoshii J, Yanase K, Namisaki T, Asada K, Yamazaki M, Tsujimoto T, Akahane T, Uemura M, Fukui H. Leptin-mediated neovascularization is a prerequisite for progression of nonalcoholic steatohepatitis in rats. *Hepatology* 2006; **44**: 983-991
8 **Kitade M**, Yoshiji H, Kojima H, Ikenaka Y, Noguchi R, Kaji K, Yoshii J, Yanase K, Namisaki T, Yamazaki M, Tsujimoto T, Moriya K, Kawaratani H, Akahane T, Fukui H. Neovascularization and oxidative stress in the progression of non-alcoholic steatohepatitis. *Mol Med Report* 2008; **1**: 543-548
9 **Canbay A**, Friedman S, Gores GJ. Apoptosis: the nexus of liver injury and fibrosis. *Hepatology* 2004; **39**: 273-278
10 **Wieckowska A**, Zein NN, Yerian LM, Lopez AR, McCullough AJ, Feldstein AE. In vivo assessment of liver cell apoptosis as a novel biomarker of disease severity in nonalcoholic fatty liver disease. *Hepatology* 2006; **44**: 27-33
11 **Ueno T**, Toi M, Linder S. Detection of epithelial cell death in the body by cytokeratin 18 measurement. *Biomed Pharmacother* 2005; **59** Suppl 2: S359-S362
12 **Feldstein AE**, Wieckowska A, Lopez AR, Liu YC, Zein NN, McCullough AJ. Cytokeratin-18 fragment levels as noninvasive biomarkers for nonalcoholic steatohepatitis: a multicenter validation study. *Hepatology* 2009; **50**: 1072-1078
13 **Polyzos SA**, Kountouras J, Zavos C. Nonalcoholic fatty liver disease: the pathogenetic roles of insulin resistance and adipocytokines. *Curr Mol Med* 2009; **9**: 299-314
14 **Ota T**, Takamura T, Kurita S, Matsuzawa N, Kita Y, Uno M, Akahori H, Misu H, Sakurai M, Zen Y, Nakanuma Y, Kaneko S. Insulin resistance accelerates a dietary rat model of nonalcoholic steatohepatitis. *Gastroenterology* 2007; **132**: 282-293
15 **Kaji K**, Yoshiji H, Kitade M, Ikenaka Y, Noguchi R, Yoshii J, Yanase K, Namisaki T, Yamazaki M, Moriya K, Tsujimoto T, Kawaratani H, Akahane T, Uemura M, Fukui H. Impact of insulin resistance on the progression of chronic liver diseases. *Int J Mol Med* 2008; **22**: 801-808
16 **Aithal GP**, Thomas JA, Kaye PV, Lawson A, Ryder SD, Spendlove I, Austin AS, Freeman JG, Morgan L, Webber J. Randomized, placebo-controlled trial of pioglitazone in nondiabetic subjects with nonalcoholic steatohepatitis. *Gastroenterology* 2008; **135**: 1176-1184
17 **Ratziu V**, Giral P, Jacqueminet S, Charlotte F, Hartemann-Heurtier A, Serfaty L, Podevin P, Lacorte JM, Bernhardt C, Bruckert E, Grimaldi A, Poynard T. Rosiglitazone for nonalcoholic steatohepatitis: one-year results of the randomized placebo-controlled Fatty Liver Improvement with Rosiglitazone Therapy (FLIRT) Trial. *Gastroenterology* 2008; **135**: 100-110
18 **Zhang Y**, Proenca R, Maffei M, Barone M, Leopold L,

Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994; **372**: 425-432
19 **Yamauchi T**, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, Sugiyama T, Miyagishi M, Hara K, Tsunoda M, Murakami K, Ohteki T, Uchida S, Takekawa S, Waki H, Tsuno NH, Shibata Y, Terauchi Y, Froguel P, Tobe K, Koyasu S, Taira K, Kitamura T, Shimizu T, Nagai R, Kadowaki T. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* 2003; **423**: 762-769
20 Intraobserver and interobserver variations in liver biopsy interpretation in patients with chronic hepatitis C. The French METAVIR Cooperative Study Group. *Hepatology* 1994; **20**: 15-20
21 **Di Carlo I**, Fraggetta F, Lombardo R, Azzarello G, Vasquez E, Puleo S. CD 34 expression in chronic and neoplastic liver diseases. *Panminerva Med* 2002; **44**: 365-367
22 **Yoshiji H**, Kuriyama S, Yoshii J, Ikenaka Y, Noguchi R, Hicklin DJ, Huber J, Nakatani T, Tsujinoue H, Yanase K, Imazu H, Fukui H. Synergistic effect of basic fibroblast growth factor and vascular endothelial growth factor in murine hepatocellular carcinoma. *Hepatology* 2002; **35**: 834-842
23 **Park YN**, Yang CP, Fernandez GJ, Cubukcu O, Thung SN, Theise ND. Neoangiogenesis and sinusoidal "capillarization" in dysplastic nodules of the liver. *Am J Surg Pathol* 1998; **22**: 656-662
24 **Theuerkauf I**, Zhou H, Fischer HP. Immunohistochemical patterns of human liver sinusoids under different conditions of pathologic perfusion. *Virchows Arch* 2001; **438**: 498-504
25 **Fernández M**, Semela D, Bruix J, Colle I, Pinzani M, Bosch J. Angiogenesis in liver disease. *J Hepatol* 2009; **50**: 604-620
26 **Gabriel A**, Kukla M, Wilk M, Liszka Ł, Petelenz M, Musialik J. Angiogenesis in chronic hepatitis C is associated with inflammatory activity grade and fibrosis stage. *Pathol Res Pract* 2009; **205**: 758-764
27 **Marra F**, Aleffi S, Bertolani C, Petrai I, Vizzutti F. Adipokines and liver fibrosis. *Eur Rev Med Pharmacol Sci* 2005; **9**: 279-284
28 **Larter CZ**, Farrell GC. Insulin resistance, adiponectin, cytokines in NASH: Which is the best target to treat? *J Hepatol* 2006; **44**: 253-261
29 **Leclercq IA**, Farrell GC, Schriemer R, Robertson GR. Leptin is essential for the hepatic fibrogenic response to chronic liver injury. *J Hepatol* 2002; **37**: 206-213
30 **Musso G**, Gambino R, Biroli G, Carello M, Fagà E, Pacini G, De Michieli F, Cassader M, Durazzo M, Rizzetto M, Pagano G. Hypoadiponectinemia predicts the severity of hepatic fibrosis and pancreatic Beta-cell dysfunction in nondiabetic nonobese patients with nonalcoholic steatohepatitis. *Am J Gastroenterol* 2005; **100**: 2438-2446
31 **Kamada Y**, Tamura S, Kiso S, Matsumoto H, Saji Y, Yoshida Y, Fukui K, Maeda N, Nishizawa H, Nagaretani H, Okamoto Y, Kihara S, Miyagawa J, Shinomura Y, Funahashi T, Matsuzawa Y. Enhanced carbon tetrachloride-induced liver fibrosis in mice lacking adiponectin. *Gastroenterology* 2003; **125**: 1796-1807
32 **Honda H**, Ikejima K, Hirose M, Yoshikawa M, Lang T, Enomoto N, Kitamura T, Takei Y, Sato N. Leptin is required for fibrogenic responses induced by thioacetamide in the murine liver. *Hepatology* 2002; **36**: 12-21
33 **Saxena NK**, Ikeda K, Rockey DC, Friedman SL, Anania FA. Leptin in hepatic fibrosis: evidence for increased collagen production in stellate cells and lean littermates of ob/ob mice. *Hepatology* 2002; **35**: 762-771
34 **Sierra-Honigmann MR**, Nath AK, Murakami C, García-Cardeña G, Papapetropoulos A, Sessa WC, Madge LA, Schechner JS, Schwabb MB, Polverini PJ, Flores-Riveros JR. Biological action of leptin as an angiogenic factor. *Science* 1998; **281**: 1683-1686
35 **Ikejima K**, Okumura K, Lang T, Honda H, Abe W, Yamashina S, Enomoto N, Takei Y, Sato N. The role of leptin in progression of non-alcoholic fatty liver disease. *Hepatol Res* 2005; **33**: 151-154
36 **Younossi ZM**, Jarrar M, Nugent C, Randhawa M, Afendy M, Stepanova M, Rafiq N, Goodman Z, Chandhoke V, Baranova A. A novel diagnostic biomarker panel for obesity-related nonalcoholic steatohepatitis (NASH). *Obes Surg* 2008; **18**: 1430-1437
37 **Chu YW**, Runyan RB, Oshima RG, Hendrix MJ. Expression of complete keratin filaments in mouse L cells augments cell migration and invasion. *Proc Natl Acad Sci USA* 1993; **90**: 4261-4265
38 **Athanassiadou P**, Psyhoyiou H, Grapsa D, Gonidi M, Ketikoglou I, Patsouris E. Cytokeratin 8 and 18 expression in imprint smears of chronic viral hepatitis, autoimmune hepatitis and hepatocellular carcinoma. A preliminary study. *Acta Cytol* 2007; **51**: 61-65
39 **Yoshiji H**, Kuriyama S, Yoshii J, Ikenaka Y, Noguchi R, Hicklin DJ, Wu Y, Yanase K, Namisaki T, Kitade M, Yamazaki M, Tsujinoue H, Masaki T, Fukui H. Halting the interaction between vascular endothelial growth factor and its receptors attenuates liver carcinogenesis in mice. *Hepatology* 2004; **39**: 1517-1524
40 **Yoshiji H**, Noguchi R, Kitade M, Kaji K, Ikenaka Y, Namisaki T, Yoshii J, Yanase K, Yamazaki M, Tsujimoto T, Akahane T, Kawaratani H, Uemura M, Fukui H. Branched-chain amino acids suppress insulin-resistance-based hepatocarcinogenesis in obese diabetic rats. *J Gastroenterol* 2009; **44**: 483-491
41 **Kakehashi A**, Inoue M, Wei M, Fukushima S, Wanibuchi H. Cytokeratin 8/18 overexpression and complex formation as an indicator of GST-P positive foci transformation into hepatocellular carcinomas. *Toxicol Appl Pharmacol* 2009; **238**: 71-79

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BRIEF ARTICLE

Antifibrotic effects of green tea on *in vitro* and *in vivo* models of liver fibrosis

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Abstract

AIM: To examine the protective effect of green tea extract (GT) on hepatic fibrosis *in vitro* and *in vivo* in dimethylnitrosamine (DMN)-induced rats.

METHODS: HSC-T6, a rat hepatic stellate cell line, was used as an *in vitro* assay system. Cell proliferation, collagen content, and type 1 collagen expression were examined in activated HSC-T6 cells. Collagen was determined by estimating the hydroxyproline content. In rats with DMN-induced hepatic fibrosis, serum aspartate aminotransferase and alanine aminotransferase concentrations, liver hydroxyproline and lipid peroxides were determined. Pathologic changes were examined by hematoxylin & eosin staining.

RESULTS: GT administration prevented the development of hepatic fibrosis in the rat model of DMN-induced liver fibrosis. These results were confirmed both by liver histology and by quantitative measurement of hepatic hydroxyproline content, a marker of liver collagen deposition. Accordingly, inhibition of proliferation, reduced collagen deposition, and type 1 collagen expression were observed in activated HSC-T6 cells following GT treatment. These results imply that GT reduced the proliferation of activated HSC and down regulated the collagen content and expression of collagen type 1, thereby ameliorating hepatic fibrosis.

CONCLUSION: This study demonstrates that green tea administration can effectively improve liver fibrosis caused by DMN, and may be used as a therapeutic option and preventive measure against hepatic fibrosis.

Key words: Dimethylnitrosamine; Green tea extract; HSC-T6 cell; Liver fibrosis; Rat model; Type 1 collagen

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INTRODUCTION

Hepatic fibrosis is a consequence of severe liver damage and occurs in many forms of chronic liver damage, including virus infection, autoimmune liver diseases and sustained alcohol abuse[1]. Hepatic stellate cells (HSC) are recognized as the primary cellular source of matrix components in chronic liver diseases, and therefore play a critical role in the development and maintenance of liver fibrosis[2]. The key cellular and molecular events involved in the pathogenesis of liver fibrosis include activation of HSC to a myofibroblast-like phenotype, production of excess matrix proteins, and increased cell proliferation[2]. Overproduction of extracellular matrix (ECM) components, particularly collagen, is a characteristic of activated HSC, and activation and proliferation of HSC have been implicated in the pathogenesis of liver fibrosis[3]. Therefore, suppression of HSC activation has been proposed as a therapeutic target against hepatic fibrosis[4].

Acetaldehyde, a highly reactive compound produced by alcohol metabolism, stimulates the deposition of ECM proteins. Acetaldehyde also stimulates type 1 collagen synthesis and gene transcription in cultured rat and human HSC[5] and in human liver fibroblasts[6].

Several studies have shown that lipid peroxidation stimulates collagen production in fibroblasts and HSC[7], and plays an important role in the development of liver

fibrosis. Lipid peroxidation has been shown to stimulate the expression of collagen gene transcripts[8]. It has recently been shown that stellate cells are activated by free radicals as well as by malondialdehyde (MDA), a product of lipid peroxidation[9]. In addition, stellate cell activation by type 1 collagen has been shown to be blocked by antioxidants[9], suggesting that lipid peroxidation may play a role in hepatofibrogenesis.

Green tea, which is a widely consumed drink, has received much attention due to its beneficial biological effects. Polyphenols, often collectively referred to as catechins, account for up to 30% of the dry weight and serve as a major effective component of green tea. The effects of green tea have been widely studied and antioxidative, antiallergic, antimutagenic/anticarcinogenic, and antibacterial effects have been documented[10-12]. It has been shown that an aqueous extract of polyphenols from green tea (*Camellia sinensis*) reduces liver fibrosis in rats induced by bile duct ligation, and epigallocatechin gallate (EGCG), the major component in green tea, was implicated as the main active ingredient[13]. EGCG has been reported to suppress cell proliferation and collagen production in HSC[14]. In addition, the hepatoprotective effects of green tea against carbon tetrachloride, cholestasis and alcohol induced liver fibrosis were reported in many studies[13,15,16]. However, the hepatoprotective effect of green tea in dimethylnitrosamine (DMN)-induced models has not been studied. The DMN-induced liver fibrosis model can reproduce most of the features observed during human liver fibrosis[17]. Furthermore, this model has other advantages such as progressive and remarkable pathological alterations, a high fibrosis reproduction rate, and a low mortality rate in experimental animals[18]. This model is also stable even after termination of DMN administration and is a reliable tool for screening antifibrotic agents[19]. Therefore, the aim of the present study was to examine the protective effect of green tea extract (GT) on hepatic fibrosis in a rat HSC line and in a rat model of DMN-induced hepatic fibrosis.

MATERIALS AND METHODS

Preparation of GT

Green tea, cultivated from Cheju island, Korea, was extracted with 80% methanol and freeze-dried.

In vitro experiment

Cell culture: HSC-T6 cells, an immortalized rat HSC line, were cultured in Dulbecco's minimal essential medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Gibco) and 0.5% antibiotics. Cultures were placed in a humidified atmosphere of 5% CO_2 at 37℃, and the medium was changed twice a week. Acetaldehyde (175 μmol/L) was added to induce collagen type 1 and morphological features of activated stellate cell.

Cell viability: HSC-T6 cells were seeded into 96-well plates at a density of 1.5×10^4 cells/well until 50% confluence. Cells treated with GT (10, 50, 100 μg/mL) for 48 h were incubated with MTT (1 mg/mL) in a medium for 3 h at 37℃. The supernatant was removed and 100 μL of DMSO was added to each well to dissolve the formazan product. Absorbance at 570 nm was measured using a microplate reader.

Hydroxyproline content: Collagen was determined by estimating the hydroxyproline content, an amino acid characteristic of collagen. HSC-T6 cells were lysed after treatment with GT (100 μg/mL) for 24 h. The lysates were hydrolyzed in 6 mol/L HCl for 16 h at 110℃ and evaporated to dryness to remove the acid. The residue, dissolved in distilled water, was mixed with 50% isopropanol and chloramine-T solution and left for 10 min at room temperature. Finally, p-dimethylaminobenzaldehyde in 60% perchloric acid was added and heated to 60℃ for 25 min. The absorbance was measured at 558 nm.

Expression of collagen type 1: The expression of collagen type 1 was observed by ELISA. HSC-T6 cells, seeded on 24-well plates at a density of 1.5×10^5 and cultured until 90% confluency, were treated with serum-free DMEM with or without 175 μmol/L acetaldehyde. Ascorbic acid (50 μg/L), and 3-aminopropionitrile fumarate (100 μg/L) were also added to increase the collagen proline hydroxylation and to prevent collagen cross-linking. After 24 h of treatment with GT (100 μg/L), aliquots of medium were transferred into immunowell plates, and glutaraldehyde (0.01%) was added and incubated at room temperature for 1 h. Collagen type 1 antibody (1:4000, Abcam Co., Cambridge, UK) was added and further incubated for 2 h at 37℃. The antigen-coated plates were blocked with casein and incubated with the secondary antibody (1:8000) linked to peroxidase, and subsequently re-incubated with substrate (TMB 10 mg/mL, 3% H_2O_2, 50 mmol/L sodium acetate buffer, pH 5.1) for 15 min. The enzymatic reaction was stopped by adding 1 mol/L H_2SO_4, and the absorbance at 450 nm was measured with a microplate reader.

In vivo experiment

Animals and treatments: Male albino rats (235-250 g) were purchased from Samtako (Kyunggi-do, Korea) and housed in controlled temperature and relative humidity, and a 12 h light/dark cycle. All experiments were performed according to National guidelines for the use of animals in biomedical research. The rats were randomly assigned to four groups of eight rats each: the normal control group without any treatment (NC), the hepatic fibrosis control group (FC), and hepatic fibrosis with 100 mg/kg GT treated group (FG). Hepatic fibrosis was induced by intraperitoneal injections of 10 mg/kg dimethylnitrosamine (DMN, Sigma, St. Louis, USA) for 3 consecutive days each week over a period of 4 wk. Normal saline was given to NC rats. GT was administered in drinking water which was calculated according to the amount of water consumed the previous day. At the end of the 4 wk experimental period, all rats were killed under ether anesthesia. Blood was obtained from the

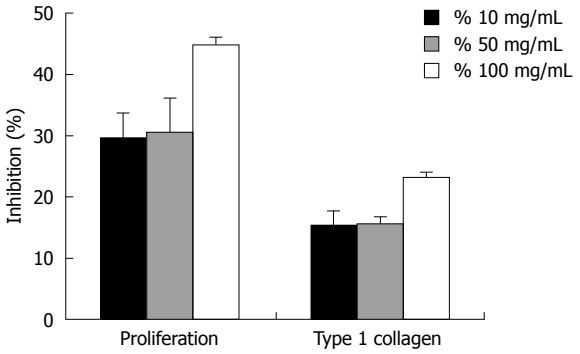

Figure 1 Inhibitory effects of green tea on HSC-T6 cell proliferation and type 1 collagen expression. Green tea suppressed HSC-T6 cell proliferation and type 1 collagen expression in a dose-dependent manner. Data are expressed as mean ± SD.

inferior vena cava, and the liver was excised. The liver was immediately frozen for biochemical measurements or fixed in formalin for histochemical examination.

Hepatotoxicity and lipid peroxidation: Hepatotoxicity was assessed by quantifying the activities of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using a spectrophotometric diagnostic kit (Youngdong Pharmaceutical Co., Korea). Lipid peroxidation in the liver and serum were determined by measuring the levels of MDA, an end product of lipid metabolism. For the serum sample, 3 mol/L sulfuric acid and 100 g/L phosphotungstic acid were added and incubated at room temperature for 10 min, and then centrifuged. For the liver sample, homogenates of liver in potassium phosphate buffer were prepared. MDA contents in the serum and liver samples were determined using a colorimetric reaction with thiobarbituric acid.

Hepatic hydroxyproline content: A portion of liver tissue (200 mg) was homogenized in 10 volumes of 0.5 mol/L potassium phosphate (KP) buffer and hydroxyproline content was measured as described above.

Histology of liver: Liver tissues were fixed in 10% neutral buffered formalin, dehydrated with 50%-100% ethanol, and embedded in paraffin. Five micrometer sections were cut and stained with hematoxylin-eosin.

Statistical analysis

All data were analyzed and expressed as mean ± SD. Comparisons were performed by Student's *t*-test to detect differences between the groups. A level of $P < 0.05$ was considered statistically significant.

RESULTS

Effects on cell proliferation and collagen production

The anti-proliferative activity in HSC-T6 cells was determined by cell viability using the MTT assay. As shown in Figure 1, HSC-T6 cell proliferation was dose-dependently inhibited by GT. GT at 10, 50 and 100 μg/mL caused dose-dependent inhibition of HSC-T6 cell proliferation by 29.5% ± 4.2%, 30.6% ± 5.6%, and 44.8% ± 1.2 %, respectively ($P < 0.05$, Figure 1). The antiproliferative effects were not related to the nonspecific cytotoxic effects of green tea because cells showed normal morphology (data not shown).

To assess the effect of GT on ECM production, hydroxyproline content and type 1 collagen expression, assessed by ELISA, were examined in activated HSC-T6 cells. Serum-starved HSC-T6 cells were cultured with acetaldehyde and GT treatment for 24 h. Treatment with 100 μg/mL GT significantly reduced cell hydroxyproline content by 23.0% ± 2.1% compared to the control group. Furthermore, the expression of type 1 collagen was up-regulated by acetaldehyde stimulation, and GT markedly reduced collagen type 1 expression in a dose-dependent manner. Acetaldehyde at a concentration of 175 μmol/L induced collagen type 1 expression by 17.4% ± 0.1%, and 10, 50 and 100 mg/mL GT reduced collagen type 1 expression by 15.2% ± 2.2%, 15.5% ± 1.3%, and 23.0% ± 1.1%, respectively (Figure 1).

Table 1 Relative organ weights and serum enzyme levels in rats with DMN-induced hepatic fibrosis (n = 10) (mean ± SD)

Group	NC	FC	FG
Liver index	3.1 ± 0.2	2.9 ± 0.2	2.8 ± 0.3
Spleen index	0.2 ± 0.03[a]	0.7 ± 0.1[c]	0.4 ± 0.1
ALT (IU/mL)	254.2 ± 26.3[a]	572.4 ± 83.9[c]	312.8 ± 29.3
AST (IU/mL)	88.2 ± 12.1[a]	451.2 ± 23.1[c]	224.4 ± 24.4

NC: Normal control group; FC: Hepatic fibrosis control group; FG: Hepatic fibrosis with green tea extract (100 mg/kg) treated group. Liver index: Liver weight/body weight × 100; Spleen index: Spleen weight/body weight × 100; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase. [a]$P < 0.05$ *vs* FG; [c]$P < 0.05$ *vs* FG.

Effects on organ weights

As shown in Table 1, the liver index, which is the percent of liver weight at final body weight, was not significantly different among the experimental groups. In contrast, relative spleen weight was increased 3.5-fold by DMN treatment, and GT administration restored the relative spleen weight ($P < 0.05$).

Serum biochemical analysis

AST and ALT concentrations in serum were used as biochemical markers to evaluate hepatic injury. ALT is a cytosolic enzyme, primarily present in the liver. An increase in plasma ALT indicates liver damage more specifically than AST. AST, which is a mitochondrial enzyme present in large quantities in the heart, liver, skeletal muscle, and kidney, in part indicates liver injury. Serum activities of ALT and AST were markedly increased with DMN treatment and GT supplementation attenuated the elevation of AST and ALT activities (Table 1).

Histology and fibrosis marker

Liver fibrosis was evaluated by hematoxylin & eosin staining. The control group showed normal architecture

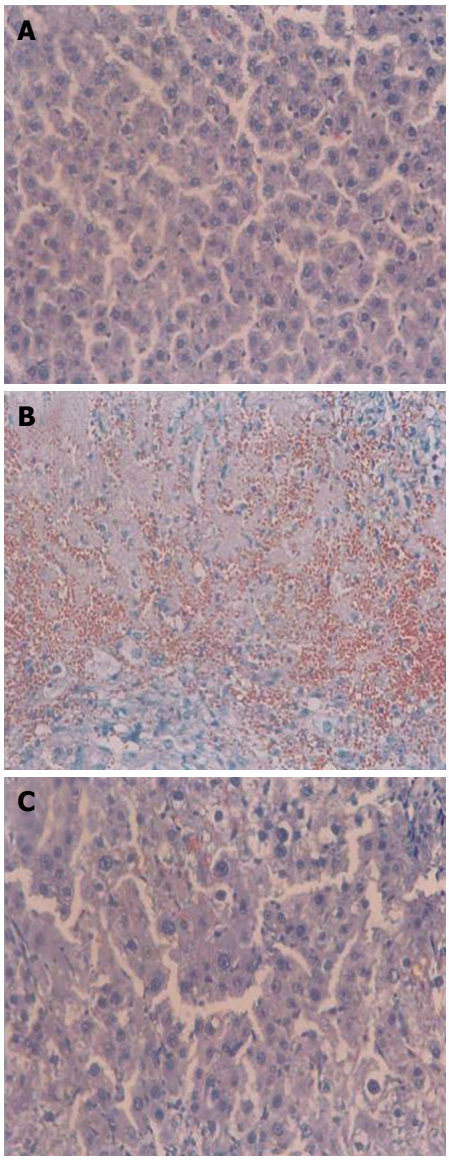

Figure 2 Effects of green tea extract on liver tissue morphology in DMN-induced fibrosis model. Representative pictures of hematoxylin and eosin-stained sections of liver tissue from normal control rat (A), DMN-treated control (B), DMN-treated + green tea extract (100 mg/kg) group (C). Original magnification, × 200.

(Figure 2A), whereas the DMN-treated group exhibited necrosis, congestion, hemorrhage, and destruction of the lobular architecture (Figure 2B). Red blood cells from blood vessels were found in liver tissue due to the collapse of the matrix structure. GT administration exhibited notable recovery effects (Figure 2C).

Hydroxyproline and lipid peroxide content in liver

The histological findings were corroborated by biochemical parameters of liver tissue collagen content determined by hydroxyproline, and lipid peroxide determined by MDA.

Hydroxyproline, a product of collagen metabolism, is an amino acid characteristic of collagen. The total collagen present in liver was, therefore, determined by estimating the hydroxyproline content. As shown in Figure 3, hydroxyproline content was significantly increased following DMN treatment (FC), indicating that the liver fibrosis model was successfully established. GT administration (FG, 100 mg/kg) restored the hydroxyproline content in fibrotic liver. Lipid peroxides, measured in terms of the formation of MDA, were significantly increased in DMN-induced rat liver. GT administration significantly reduced the lipid peroxide level.

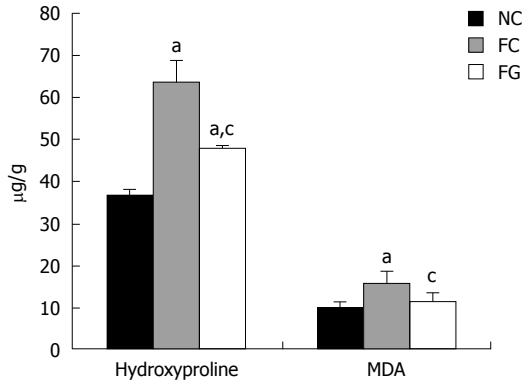

Figure 3 Effects of green tea on hydroxyproline and lipid peroxide (MDA) levels in DMN-treated rat liver. Data are expressed as mean ± SD. MDA: Malondialdehyde. $^{a}P < 0.05$ *vs* normal control (NC), $^{c}P < 0.05$ *vs* fibrosis control (FC).

DISCUSSION

Hepatic fibrosis is characterized by an abnormal accumulation of ECM proteins, particularly collagen[3,4]. When hepatic fibrosis occurs, collagen proliferation, mainly collagen type 1 and 3, accounts for 50% of the total protein in fibrotic liver[20], and collagens are the main components of ECM. Therefore, collagen type 1 is an important parameter reflecting the metabolism of collagen in liver. The main collagen producing cells in the liver are HSC, which proliferate and undergo a process of activation during the development of fibrosis resulting in increased capacity for collagen synthesis[21]. Changes in hydroxyproline content in the liver are considered an index for collagen metabolism and provide valuable information on the biochemical and pathological states of liver fibrosis. The present study demonstrated that consumption of GT prevented the development of hepatic fibrosis in a rat model of DMN-induced liver fibrosis. The results were confirmed both by liver histology and by quantitative measurement of hepatic hydroxyproline content, a marker of collagen deposition in liver.

Accordingly, inhibition of proliferation, reduced collagen content, and type 1 collagen expression were observed in activated HSC-T6 cells following GT treatment. Activated HSC are the main source of ECM when liver fibrosis occurs[22]. Therefore, these results imply that GT inhibit the proliferation of activated HSC and down regulate the collagen content and expression of collagen type 1, thereby inhibiting hepatic fibrosis. The results of the present study are consistent with previous observations showing that EGCG, the major component in green

tea, suppresses collagen production[23], and proliferation[24] in HSC.

Oxidative stress resulting from the increased production of reactive oxygen species and lipid peroxides is suggested to be associated with the proliferation and activation of stellate cells either directly or through paracrine stimulation of neighboring cells including injured hepatocytes[25]. Furthermore, oxidative stress has been shown to modulate collagen gene expression[7]. Therefore, a number of studies have focused on the pathogenetic significance of oxidative stress in liver injury, as well as on the therapeutic intervention of this process with antioxidant and metabolic scavengers. GT administration resulted in a reduction of lipid peroxide in HSC-T6 cells and DMN-treated fibrotic liver. Chen *et al*[26] have also shown that a single-dose of EGCG improved hepatic injury in rats induced by CCl_4 administration through the inhibition of lipid peroxidation.

In conclusion, this study demonstrates that green tea administration can effectively improve liver fibrosis caused by DMN as shown by reduced levels of collagen, lipid peroxidation, HSC proliferation, and type 1 collagen expression in the liver. Therefore, green tea may protect liver cells and reduce the deposition of collagen fibers in the liver. Green tea provides a safe and effective strategy for improving hepatic fibrosis.

COMMENTS

Background

Hepatic stellate cells (HSC) are recognized as the primary cellular source of matrix components in chronic liver diseases, and therefore play a critical role in the development and maintenance of liver fibrosis. Overproduction of extracellular matrix components, particularly collagen, is a characteristic of activated HSC, and activation and proliferation of HSC have been implicated in the pathogenesis of liver fibrosis.

Research frontiers

Hepatoprotective effects of green tea against carbon tetrachloride, cholestasis and alcohol induced liver fibrosis were reported in many studies. However, the hepatoprotective effect of green tea in dimethylnitrosamine (DMN)-induced models has not been studied.

Innovations and breakthroughs

The present study demonstrates that consumption of green tea prevents the development of hepatic fibrosis in a rat model of DMN-induced liver fibrosis. These results were confirmed both by liver histology and by quantitative measurement of hepatic hydroxyproline content, a marker of collagen deposition in the liver. Accordingly, inhibition of proliferation, reduced collagen content, and type 1 collagen expression were observed in activated HSC cells following green tea treatment.

Applications

This study demonstrates that green tea may protect liver cells and reduces the deposition of collagen fibers in the liver. Green tea provides a safe and effective strategy for improving hepatic fibrosis.

Terminology

HSC are the main collagen producing cells in the liver, which proliferate and undergo a process of activation during the development of fibrosis resulting in an increased capacity for collagen synthesis.

Peer review

This study examined the protective effect of green tea extract on hepatic fibrosis in a HSC line and in a rat model of DMN-induced liver fibrosis. Green tea administration prevents the development of hepatic fibrosis in the rat model of liver fibrosis. Furthermore, inhibition of proliferation, reduced collagen deposition, and type 1 collagen expression were observed in activated HSC cells following green tea treatment. The results imply that green tea may protect liver cells and reduce the deposition of collagen fibers in the liver. Green tea provides a safe and effective strategy for improving hepatic fibrosis.

REFERENCES

1 **Bissell DM**. Hepatic fibrosis as wound repair: a progress report. *J Gastroenterol* 1998; **33**: 295-302
2 **Wu J**, Zern MA. Hepatic stellate cells: a target for the treatment of liver fibrosis. *J Gastroenterol* 2000; **35**: 665-672
3 **Bataller R**, Brenner DA. Liver fibrosis. *J Clin Invest* 2005; **115**: 209-218
4 **Lotersztajn S**, Julien B, Teixeira-Clerc F, Grenard P, Mallat A. Hepatic fibrosis: molecular mechanisms and drug targets. *Annu Rev Pharmacol Toxicol* 2005; **45**: 605-628
5 **Casini A**, Cunningham M, Rojkind M, Lieber CS. Acetaldehyde increases procollagen type I and fibronectin gene transcription in cultured rat fat-storing cells through a protein synthesis-dependent mechanism. *Hepatology* 1991; **13**: 758-765
6 **Savolainen ER**, Leo MA, Timpl R, Lieber CS. Acetaldehyde and lactate stimulate collagen synthesis of cultured baboon liver myofibroblasts. *Gastroenterology* 1984; **87**: 777-787
7 **Parola M**, Pinzani M, Casini A, Albano E, Poli G, Gentilini A, Gentilini P, Dianzani MU. Stimulation of lipid peroxidation or 4-hydroxynonenal treatment increases procollagen alpha 1 (I) gene expression in human liver fat-storing cells. *Biochem Biophys Res Commun* 1993; **194**: 1044-1050
8 **Lyons BL**, Schwarz RI. Ascorbate stimulation of PAT cells causes an increase in transcription rates and a decrease in degradation rates of procollagen mRNA. *Nucleic Acids Res* 1984; **12**: 2569-2579
9 **Lee KS**, Buck M, Houglum K, Chojkier M. Activation of hepatic stellate cells by TGF alpha and collagen type I is mediated by oxidative stress through c-myb expression. *J Clin Invest* 1995; **96**: 2461-2468
10 **Erba D**, Riso P, Bordoni A, Foti P, Biagi PL, Testolin G. Effectiveness of moderate green tea consumption on antioxidative status and plasma lipid profile in humans. *J Nutr Biochem* 2005; **16**: 144-149
11 **Shiozaki T**, Sugiyama K, Nakazato K, Takeo T. [Effect of tea extracts, catechin and caffeine against type-I allergic reaction] *Yakugaku Zasshi* 1997; **117**: 448-454
12 **Cabrera C**, Artacho R, Giménez R. Beneficial effects of green tea--a review. *J Am Coll Nutr* 2006; **25**: 79-99
13 **Zhong Z**, Froh M, Lehnert M, Schoonhoven R, Yang L, Lind H, Lemasters JJ, Thurman RG. Polyphenols from Camellia sinenesis attenuate experimental cholestasis-induced liver fibrosis in rats. *Am J Physiol Gastrointest Liver Physiol* 2003; **285**: G1004-G1013
14 **Sakata R**, Ueno T, Nakamura T, Sakamoto M, Torimura T, Sata M. Green tea polyphenol epigallocatechin-3-gallate inhibits platelet-derived growth factor-induced proliferation of human hepatic stellate cell line LI90. *J Hepatol* 2004; **40**: 52-59
15 **Xiao J**, Lu R, Shen X, Wu M. [Green tea extracts protected against carbon tetrachloride-induced chronic liver damage and cirrhosis] *Zhonghua Yufang Yixue Zazhi* 2002; **36**: 243-246
16 **Arteel GE**, Uesugi T, Bevan LN, Gäbele E, Wheeler MD, McKim SE, Thurman RG. Green tea extract protects against early alcohol-induced liver injury in rats. *Biol Chem* 2002; **383**: 663-670
17 **Wasser S**, Tan CE. Experimental models of hepatic fibrosis in the rat. *Ann Acad Med Singapore* 1999; **28**: 109-111
18 **Jézéquel AM**, Mancini R, Rinaldesi ML, Ballardini G, Fallani M, Bianchi F, Orlandi F. Dimethylnitrosamine-induced cirrhosis. Evidence for an immunological mechanism. *J Hepatol* 1989; **8**: 42-52
19 **George J**, Rao KR, Stern R, Chandrakasan G. Dimethylnitrosamine-induced liver injury in rats: the early deposition of collagen. *Toxicology* 2001; **156**: 129-138
20 **Gressner AM**. The cell biology of liver fibrogenesis - an imbalance of proliferation, growth arrest and apoptosis of

myofibroblasts. *Cell Tissue Res* 1998; **292**: 447-452
21 **Friedman SL**. Cellular sources of collagen and regulation of collagen production in liver. *Semin Liver Dis* 1990; **10**: 20-29
22 **Alcolado R**, Arthur MJ, Iredale JP. Pathogenesis of liver fibrosis. *Clin Sci* (Lond) 1997; **92**: 103-112
23 **Nakamuta M**, Higashi N, Kohjima M, Fukushima M, Ohta S, Kotoh K, Kobayashi N, Enjoji M. Epigallocatechin-3-gallate, a polyphenol component of green tea, suppresses both collagen production and collagenase activity in hepatic stellate cells. *Int J Mol Med* 2005; **16**: 677-681
24 **Chen A**, Zhang L. The antioxidant (-)-epigallocatechin-3-gallate inhibits rat hepatic stellate cell proliferation in vitro by blocking the tyrosine phosphorylation and reducing the gene expression of platelet-derived growth factor-beta receptor. *J Biol Chem* 2003; **278**: 23381-23389
25 **Svegliati Baroni G**, D'Ambrosio L, Ferretti G, Casini A, Di Sario A, Salzano R, Ridolfi F, Saccomanno S, Jezequel AM, Benedetti A. Fibrogenic effect of oxidative stress on rat hepatic stellate cells. *Hepatology* 1998; **27**: 720-726
26 **Chen JH**, Tipoe GL, Liong EC, So HS, Leung KM, Tom WM, Fung PC, Nanji AA. Green tea polyphenols prevent toxin-induced hepatotoxicity in mice by down-regulating inducible nitric oxide-derived prooxidants. *Am J Clin Nutr* 2004; **80**: 742-751

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BRIEF ARTICLE

Factors relating to the short term effectiveness of percutaneous biliary drainage for hilar cholangiocarcinoma

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Abstract

AIM: To identify factors that were related to the short term effectiveness of percutaneous transhepatic biliary drainage in cholangiocarcinoma patients and to evaluate the impact of palliative drainage on their survival.

METHODS: Seventy-four patients with hilar cholangiocarcinoma who underwent percutaneous biliary drainage were enrolled in the study. The demographic and laboratory data as well as the imaging characteristics were retrospectively analyzed to correlate with the bile output and reduction rate of serum bilirubin 1 wk after drainage.

RESULTS: Patients with more bile duct visualized on percutaneous transhepatic cholangiography or absence of multiple liver metastases on imaging studies had more bile output after biliary drainage [odds ratio (OR): 8.471, P = 0.010 and OR: 1.959, P = 0.022, respectively]. Patients with prolonged prothrombin time had a slow decrease in serum bilirubin (OR: 0.437, P = 0.005). The median survival time was not significantly different in patients with low or high bile output (75 d *vs* 125 d, P = 0.573) or in patients with slow or rapid reduction of serum bilirubin (88 d *vs* 94 d, P = 0.576).

CONCLUSION: The short term effectiveness of percutaneous biliary drainage was related to patient's prothrombin time or the extent of tumor involvement. It, however, had no impact on survival.

Key words: Cholangiocarcinoma; Percutaneous biliary drainage; Treatment effectiveness

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INTRODUCTION

Cholangiocarcinoma is the second most common primary liver cancer, after hepatocellular carcinoma and the incidence is increasing[1,2]. Hilar cholangiocarcinoma with the tumor involving the biliary confluence or the right or left intrahepatic ducts is most common and accounts for 40%-60% of all cholangiocarcinomas[3]. About 80% of patients with hilar cholangiocarcinoma are unsuitable for curative surgical resection due to severe comorbidity for major surgery, metastases or advanced loco-regional disease[4]. Percutaneous or endoscopic biliary drainage is usually performed as a palliative treatment to relieve these patients from jaundice, pain, and cholangitis[5].

A fluent bile output after percutaneous transhepatic biliary drainage (PTBD) reduces the biliary pressure and therefore, alleviates the cholangitis and pain of patients. The reduction of serum bilirubin is usually the hallmark of successful biliary drainage. Nonetheless, despite the drainage catheter being correctly positioned in the bile duct, there are still some patients who have scanty bile output and persistent elevation of serum bilirubin[6]. We retrospectively analyzed the clinical and imaging characteristics of these patients in an attempt to identify the factors related to bile output and reduction of serum bilirubin after PTBD. In addition, we also compared

the survival of patients with different bile output and reduction rates of bilirubin after PTBD to investigate if the short term effectiveness of biliary drainage had any impact on the long term survival.

MATERIALS AND METHODS

Patients

From January 1998 to June 2007, 74 consecutive patients with hilar cholangiocarcinoma who underwent PTBD in our hospital, a tertiary transferring center, were enrolled. The diagnosis of cholangiocarcinoma was confirmed either by pathologic diagnosis (n = 39) or by imaging studies plus clinical follow-up which illustrated further tumor progression (n = 35). These patients included 39 males and 35 females with a mean age of 66.1 ± 12.8 and 66.5 ± 12.5 years, respectively. All of the patients, irrespective of future treatment modality, underwent PTBD due to the presence of jaundice and a dilated biliary system and were further observed for at least 1 wk to evaluate the effectiveness of PTBD. Of them, 54 patients were unsuitable for surgical intervention on the basis of their comorbidity and/or tumor extent and were enrolled for survival analysis.

Clinical characteristics

The medical records of each patient were retrospectively reviewed. Data collected from all patients on the day of, or 1 d before, biliary drainage included the initial serum levels of albumin, bilirubin, alanine aminotransferase (ALT), alkaline phosphatase and prothrombin time. The average daily bile output and the serum total bilirubin level 1 wk after drainage were recorded for the evaluation of drainage effectiveness. The overall survival time of patients who did not undergo surgical resection was also checked.

Imaging characteristics analysis

Percutaneous transhepatic cholangiography (PTC), contrast enhanced computed tomography (CT) and/or magnetic resonance (MR) imaging were reviewed for imaging characteristics analysis. The liver tumors were classified as type Ⅰ, Ⅱ, Ⅲa, Ⅲb, and Ⅳ according to the system of Bismuth *et al*[7]. The maximal diameter of the tumors was estimated on CT or MR imaging either by direct measurement of the tumor size if its margin could be clearly defined or indirect measurement of the distance between dilated ducts if the tumor was difficult to visualize. The maximal diameter of the intrahepatic duct punctured for biliary drainage was measured on CT, MR, or PTC imaging. The number of visualized intrahepatic bile ducts (first branches of right and left intrahepatic ducts) was counted later on a follow-up PTC as it may depend on the injection pressure of contrast and the radiologist usually injected less contrast media to avoid risk of cholangitis when performing PTBD. A follow-up PTC taken several days after the biliary drainage, although it may still have some limitation, should carry a lower risk of cholangitis and allow more contrast injection. In addition, CT or MR imaging was checked for multiple liver metastases, locoregional lymphadenopathy and peritoneal involvement.

Percutaneous transhepatic biliary drainage procedure

Before PTBD, the bleeding profile was checked and treated if abnormal, and antibiotic therapy was commenced. If CT or MR images showed dilated ducts were confined to a single lobe, PTBD was performed *via* that lobe. If the ducts of both lobes were dilated, our radiologists preferred to approach from the left side unless it was atrophied due to tumor invasion of the ipsilateral portal vein. The duct selected for drainage depended on the decision of the radiologist, who selected the most feasible duct to approach. PTBD was performed as a standard procedure. In brief, the biliary system was punctured and a guide wire (0.035 inch) was introduced into the bile duct. Through the guide wire, the puncture tract was dilated using a bougie, followed by the insertion of an 8-French pigtail catheter.

The effectiveness of biliary drainage

The average daily bile output during the first week after PTBD was calculated. The reduction rate of serum bilirubin was calculated by dividing the reduction of total bilirubin level after 1 wk of drainage by the original level. For differentiating the effectiveness of biliary drainage, an average bile output of more or less than 300 mL/d and a reduction rate of serum bilirubin of higher or lower than 20% (i.e. the integer of median value of patient distribution) were chosen to further divide patients into two groups with similar sample size.

Statistical analysis

Except for patient survival which was presented as median survival days, all the other data were expressed as mean ± SD. The differences in demographic, biochemical, and imaging characteristics between patient groups were compared by Independent-Samples t test or χ^2 test as appropriate. Variables that achieved statistical significance ($P < 0.05$) or were close to significance ($P < 0.1$) in the univariate analysis were subsequently included in a multivariate analysis using a stepwise forward logistic regression. The survival of patients with different effectiveness of biliary drainage was compared by using the log-rank test. The statistical calculations were computed using the SPSS 12.0 program for Windows (SPSS Inc., Chicago, IL, US).

RESULTS

Events associated with the biliary drainage

There was no procedure associated mortality or significant morbidity, except two cases of transient hemobilia and two cases of cholangitis. Most of our patients who did not undergo surgical intervention were maintained on PTBD and only 12 (16.2%) of them were switched to external-internal biliary drainage. The drainage was further revised if the initial PTBD drainage had unsatisfactory function during the follow up. Of the 26 patients

Table 1 The clinical characteristics of patients with high and low bile output (mean ± SD)

	Bile output		*P*-value[1]
	> 300 mL (*n* = 33)	< 300 mL (*n* = 41)	
Age (yr)	64.5 ± 10.3	67.7 ± 14.2	0.25
Sex (male:female)	20:13	19:22	0.35
Biochemical data			
Albumin (g/dL)	3.4 ± 0.6	3.0 ± 0.5	0.01
Total bilirubin (mg/dL)	12.2 ± 7.4	12.0 ± 8.5	0.92
ALT (U/L)	132.5 ± 184.5	142.6 ± 115.2	0.77
Prolonged prothrombin time (s)	1.0 ± 1.1	1.2 ± 1.3	0.42
Bilirubin reduction rate (%)	22.5 ± 41.2	13.6 ± 29.7	0.31
Alkaline phosphatase (U/L)	463.9 ± 301.8	552.9 ± 373.8	0.297
Imaging characteristics			
Visualized bile ducts on PTC	2.7 ± 1.2	1.7 ± 1.0	0.001[2]
Tumor size (cm)	5.3 ± 2.9	4.6 ± 2.5	0.31
Diameter of bile duct (mm)	10.5 ± 6.1	11.1 ± 5.9	0.65
Approach side (right:left)	9:24	10:31	0.79
Bismuth type Ⅰ/Ⅱ/Ⅲa/Ⅲb/Ⅳ[3]	8/6/5/3/7	2/6/11/11/5	0.03
Lymphadenopathy	10	9	0.59
Liver metastasis	4	14	0.03[2]
Peritoneal involvement	7	8	1.00

[1]By Independent-Samples *T* test and χ^2 test as appropriate; [2]Only the absence of multiple liver metastases and more intrahepatic bile ducts visualized on PTC were statistically significant in multivariate analysis; [3]Eleven patients with ambiguous imaging that cannot be confidently classified were not included for analysis. PTC: Percutaneous transhepatic cholangiography.

Table 2 Clinical characteristics between the patients who had rapid and slow bilirubin reduction rates after drainage (mean ± SD)

	Reduction		*P*-value[1]
	> 20% (*n* = 37)	< 20% (*n* = 33)	
Age (yr)	67.6 ± 12.5	65.4 ± 12.3	0.515
Sex (male:female)	19:18	18:15	1.000
Biochemical data			
Albumin (g/dL)	3.4 ± 0.5	3.0 ± 0.6	0.016
Total bilirubin (mg/dL)	10.6 ± 5.9	15.0 ± 5.1	0.022
ALT (U/L)	172.3 ± 190.2	102.1 ± 70.0	0.048
Prolonged prothrombin time (s)	0.7 ± 0.7	1.7 ± 1.4	0.001[2]
Alkaline phosphatase (U/L)	499.5 ± 268.6	543.8 ± 428.8	0.630
Imaging characteristics			
Visualized bile ducts on PTC	2.2 ± 1.2	2.1 ± 1.2	0.815
Tumor size (cm)	4.9 ± 2.6	4.9 ± 3.0	0.968
Diameter of bile duct (mm)	11.0 ± 5.9	10.4 ± 5.9	0.670
Approach side (right:left)	10:27	8:25	1.000
Bismuth type Ⅰ/Ⅱ/Ⅲa/Ⅲb/Ⅳ[3]	5/7/10/5/6	5/5/6/9/5	0.657
Lymphadenopathy	11	7	0.583
Liver metastasis	12	5	0.159
Peritoneal involvement	10	5	0.379

[1]Abbreviations as in Table 1; [2]In multivariate analysis, only the prothrombin time was significantly associated with bilirubin reduction; [3]Abbreviations as in Table 1.

who underwent palliative treatment only and survived longer than 90 d, 20 patients (77%) received at least one drainage revision.

Bile output after drainage

There were 33 patients with and 41 patients without an average bile output of more than 300 mL/d. As shown in Table 1, among the biochemical data, only a higher serum albumin concentration was associated with higher bile output in patients undergoing PTBD. Higher bile output was, however, not associated with a more rapid reduction of serum bilirubin as it was not significantly different between both groups. Patients who had fewer intrahepatic bile ducts shown on PTC, hence a more advanced Bismuth classification, or multiple liver metastases had lower bile output after biliary drainage. The size of tumor, the diameter of bile duct, the approach side of PTBD, and the presence of lymphadenopathy or peritoneum involvement were all unrelated to the amount of bile output.

In multivariate analysis, the absence of liver metastases and a greater number of intrahepatic bile ducts visualized on PTC were still significantly associated with a higher bile output. The odds ratios (ORs) were 8.471, (95% CI: 1.675-42.835, $P = 0.010$) for negative metastasis and 1.959, (95% CI: 1.103-3.481, $P = 0.022$) for more intrahepatic bile ducts on PTC.

Reduction rate of serum bilirubin after drainage

Of the 70 patients available for analysis of bilirubin reduction (four patients did not have a serum bilirubin check 1 wk after drainage), 37 patients had a bilirubin reduction of more than 20% after 1 wk of drainage and 33 patients did not. The reduction rate of bilirubin was significantly more rapid in patients who had a less prolonged prothrombin time or a better serum biochemical profile including higher serum albumin, lower serum bilirubin or ALT (Table 2). None of the imaging characteristics such as tumor size, Bismuth classification, liver metastasis and lymphadenopathy had an impact on the rate of serum bilirubin reduction after biliary drainage.

In multivariate analysis, only prolonged prothrombin time was significantly associated with a slower reduction rate of serum bilirubin. The OR was 0.437 (95% CI: 0.245-0.780, $P = 0.005$), compared to those with normal prothrombin time.

Short term effectiveness of biliary drainage and patients' survival

Most of the patients who were unsuitable for surgical resection died of cholangitis and/or liver failure. Only one patient was still alive at the time of analysis. The median survival time of these patients was 94 d. Although 12 patients received additional drainage due to scanty biliary output or persistent hyperbilirubinemia, the median survival of patients with bilateral biliary drainage was similar to that of patients who received only unilateral drainage (66 d *vs* 94 d, $P = 0.358$). In addition to biliary drainage, 10 of the 54 patients who had unresectable tumors also underwent external beam radiation therapy. The median survival was, however, not significantly longer

in these patients (103 d) than in those who received drainage only (88 d, $P = 0.493$). These patients were therefore, combined for survival analysis.

The serum bilirubin reduction rate, whether it was more rapid or slower than 20% did not affect the median survival time of patients (94 d *vs* 88 d, $P = 0.576$). The median survival time was also similar between patients with a bile output less than 300 mL (75 d) and those with a bile output more than 300 mL (125 d, $P = 0.573$).

DISCUSSION

As shown in our previous and current studies, bile output was not correlated with the reduction rate of bilirubin[8]. Both liver cells and ductular cells contribute to the formation of bile[9]. Biliary obstruction results in proliferation of bile ducts and ductules. The increased amount of bile after relief of obstruction can be caused by excretion of water and electrolytes from the proliferated biliary epithelial cells (i.e. the secretin choleresis)[9]. Nevertheless, the reduction of serum bilirubin is dependent on bilirubin excretion by liver cells which can be impaired as a result of cholestasis[10]. Prolonged biliary obstruction in these patients results in bile duct proliferation and hepatocyte damage, which explains the discrepancy between bile output and bilirubin reduction rate. A high bile output is therefore, not associated with a rapid reduction in serum bilirubin if most of the bile comes from the bile ducts rather than liver cells.

Cholangiocarcinoma with more advanced Bismuth classification implies a complicated biliary obstruction and is frequently found to have fewer intrahepatic bile ducts depicted on PTC[7]. Liver metastases also cause biliary obstruction by compressing the bile ducts externally. The presence of multi-site biliary obstruction, either caused by intra-luminal obstruction or external compression of the tumor, leads to fewer sources of bile flowing into the drained bile duct and was therefore, associated with less bile output after PTBD in our observations.

Accumulation of bile salts within the liver can cause necrosis and apoptosis of liver cells[10-13]. The liver may need more time to recover in cases with long lasting biliary obstruction. Prolonged prothrombin time in cholangiocarcinoma patients can be due to either vitamin K deficiency or impaired liver synthesis of coagulation factors, both sequelae of prolonged cholestasis. Weston reported that patients with prolonged prothrombin time took longer for bilirubin reduction after endoscopic biliary stenting[14]. Similarly, patients with a prolonged prothrombin time in our study also had a slow reduction of serum bilirubin.

Surgical resection is the standard treatment for intrahepatic cholangiocarcinoma[15]. Patients unsuitable for surgical resection live a significantly shorter time than those undergo curative tumor resection. Previous reports indicate that the method of drainage has no impact on patient survival[4]. Our data further showed that patient's survival was not related to the bile output or the rate of bilirubin reduction after biliary drainage. This may be because even though the drainage provides good short term success in palliating symptoms, it is however associated with significant morbidity in the long term follow up including catheter clogging, catheter dislodgement, cholangitis, and liver failure[16]. As seen in our study, most of the patients who survived more than 90 d received multiple drainage revisions and as a consequence of tumor progression, most of them still died of cholangitis and liver failure. Therefore, the short term effectiveness of PTBD adds little benefit to survival unless other effective methods such as photodynamic therapy are available to retard the tumor progress and keep the biliary systems patent[17,18].

In conclusion, our study identified factors that were related to the short term effectiveness of PTBD in patients with hilar cholangiocarcinoma. After biliary drainage, patients may have less bile output in the presence of multiple sites of biliary obstruction, and slower reduction of serum bilirubin if the prothrombin time is prolonged. A higher bile output was not associated with a more rapid bilirubin reduction. Although we only observed the drainage effect at 1 wk which may not reflect the ultimate result of drainage, we did find that a short term relief of biliary obstruction by PTBD was not associated with a better survival in patients with unresectable cancer.

COMMENTS

Background

Biliary drainage is performed as a palliative treatment of hilar cholangiocarcinoma. The reduction of serum bilirubin is usually the hallmark of successful biliary drainage. However, some patients may have persistent jaundice or scanty bile output after biliary drainage.

Research frontiers

This paper analyzed factors relating to bile output and reduction of serum bilirubin after percutaneous biliary drainage. Furthermore, the impact of short term effectiveness of biliary drainage on long term survival was investigated.

Innovations and breakthroughs

After percutaneous biliary drainage, high bile output was not associated with a rapid reduction of serum bilirubin. The bilirubin reduction was related to patient's prothrombin time and the bile output, to the extent of tumor involvement. Neither the amount of bile output nor the rate of bilirubin reduction had an impact on survival.

Applications

The effectiveness of percutaneous biliary drainage can be properly estimated before the procedure. Patient should be observed for daily bile output as well as the reduction of serum bilirubin. An initially well functioning biliary drainage does not link to a longer survival of patients and further efforts to maintain biliary patency are required.

Peer review

Chen *et al* found that the short term effectiveness of percutaneous biliary drainage was related to patient's prothrombin time or the extent of tumor involvement, but it had no impact on survival. The paper is informative.

REFERENCES

1 **Nakeeb A**, Pitt HA, Sohn TA, Coleman J, Abrams RA, Piantadosi S, Hruban RH, Lillemoe KD, Yeo CJ, Cameron JL. Cholangiocarcinoma. A spectrum of intrahepatic, perihilar, and distal tumors. *Ann Surg* 1996; **224**: 463-473; discussion 473-475

2 **Blechacz BR**, Gores GJ. Cholangiocarcinoma. *Clin Liver Dis* 2008; **12**: 131-150, ix

3 **Jarnagin WR**, Fong Y, DeMatteo RP, Gonen M, Burke EC, Bodniewicz BS J, Youssef BA M, Klimstra D, Blumgart LH. Staging, resectability, and outcome in 225 patients with hilar cholangiocarcinoma. *Ann Surg* 2001; **234**: 507-517; discussion 517-519

4 **Singhal D**, van Gulik TM, Gouma DJ. Palliative management of hilar cholangiocarcinoma. *Surg Oncol* 2005; **14**: 59-74

5 **Weber A**, Landrock S, Schneider J, Stangl M, Neu B, Born P, Classen M, Rösch T, Schmid RM, Prinz C. Long-term outcome and prognostic factors of patients with hilar cholangiocarcinoma. *World J Gastroenterol* 2007; **13**: 1422-1426

6 **Rerknimitr R**, Kladcharoen N, Mahachai V, Kullavanijaya P. Result of endoscopic biliary drainage in hilar cholangiocarcinoma. *J Clin Gastroenterol* 2004; **38**: 518-523

7 **Bismuth H**, Nakache R, Diamond T. Management strategies in resection for hilar cholangiocarcinoma. *Ann Surg* 1992; **215**: 31-38

8 **Chen CY**, Shiesh SC, Lin XZ. Indicators of liver excretory function in patients undergoing biliary decompression for obstructive jaundice. *Hepatogastroenterology* 1998; **45**: 786-790

9 **Alpini G**, Lenzi R, Sarkozi L, Tavoloni N. Biliary physiology in rats with bile ductular cell hyperplasia. Evidence for a secretory function of proliferated bile ductules. *J Clin Invest* 1988; **81**: 569-578

10 **Malhi H**, Gores GJ. Cellular and molecular mechanisms of liver injury. *Gastroenterology* 2008; **134**: 1641-1654

11 **Miyoshi H**, Rust C, Roberts PJ, Burgart LJ, Gores GJ. Hepatocyte apoptosis after bile duct ligation in the mouse involves Fas. *Gastroenterology* 1999; **117**: 669-677

12 **Faubion WA**, Guicciardi ME, Miyoshi H, Bronk SF, Roberts PJ, Svingen PA, Kaufmann SH, Gores GJ. Toxic bile salts induce rodent hepatocyte apoptosis via direct activation of Fas. *J Clin Invest* 1999; **103**: 137-145

13 **Sodeman T**, Bronk SF, Roberts PJ, Miyoshi H, Gores GJ. Bile salts mediate hepatocyte apoptosis by increasing cell surface trafficking of Fas. *Am J Physiol Gastrointest Liver Physiol* 2000; **278**: G992-G999

14 **Weston BR**, Ross WA, Wolff RA, Evans D, Lee JE, Wang X, Xiao LC, Lee JH. Rate of bilirubin regression after stenting in malignant biliary obstruction for the initiation of chemotherapy: how soon should we repeat endoscopic retrograde cholangiopancreatography? *Cancer* 2008; **112**: 2417-2423

15 **Blechacz B**, Gores GJ. Cholangiocarcinoma: advances in pathogenesis, diagnosis, and treatment. *Hepatology* 2008; **48**: 308-321

16 **Stoker J**, Laméris JS, van Blankenstein M. Percutaneous metallic self-expandable endoprostheses in malignant hilar biliary obstruction. *Gastrointest Endosc* 1993; **39**: 43-49

17 **Shim CS**, Cheon YK, Cha SW, Bhandari S, Moon JH, Cho YD, Kim YS, Lee LS, Lee MS, Kim BS. Prospective study of the effectiveness of percutaneous transhepatic photodynamic therapy for advanced bile duct cancer and the role of intraductal ultrasonography in response assessment. *Endoscopy* 2005; **37**: 425-433

18 **Zoepf T**, Jakobs R, Arnold JC, Apel D, Riemann JF. Palliation of nonresectable bile duct cancer: improved survival after photodynamic therapy. *Am J Gastroenterol* 2005; **100**: 2426-2430

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BRIEF ARTICLE

Effect of electro-acupuncture on substance P, its receptor and corticotropin-releasing hormone in rats with irritable bowel syndrome

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Abstract

AIM: To investigate the effect and mechanism of electro-acupuncture (EA) at ST25 and ST37 on irritable bowel syndrome (IBS) of rats.

METHODS: A total of 21 male Sprague-Dawley rats were randomly divided into normal group, model group and EA group. A rat model of IBS was established by constraining the limbs and distending the colorectum of rats. Rats in EA group received bilateral EA at ST25 and ST37 with a sparse and intense waveform at a frequency of 2/50 Hz for 15 min, once a day for 7 d as a course. Rats in normal and model groups were stimulated by distending colorectum (CR). An abdominal withdrawal reflex (AWR) scoring system was used to evaluate improvements in visceral hypersensitivity. Toluidine blue-improved method, immunohistochemistry and radioimmunoassay were used to observe mucosal mast cells (MC), changes of substance P (SP) and substance P receptor (SPR) in colon and change of corticotropin-releasing hormone (CRH) in hypothalamus.

RESULTS: The threshold of visceral sense was significantly lower in model group than in normal group, and significantly higher in EA group than in model group. The number of mucosal MC was greater in model group than in normal group and significantly smaller in EA group than in model group. The CRH level in hypothalamus of rats was significantly higher in model group than in normal group, which was remarkably decreased after electro-acupuncture treatment. The SP and SPR expression in colon of rats in model group was decreased after electro-acupuncture treatment.

CONCLUSION: EA at ST25 and ST37 can decrease the number of mucosal MC and down-regulate the expression of CRH in hypothalamus, and the expression of SP and SPR in colon of rats with IBS.

Key words: Electro-acupuncture; Corticotropin-releasing hormone; Irritable bowel syndrome; Substance P; Substance P receptor

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INTRODUCTION

Irritable bowel syndrome (IBS) is a common bowel disorder characterized by recurrent abdominal pain or discomfort associated with altered bowel habits in the absence of structural pathology[1]. Since IBS is diagnosed based on its symptoms and its pathophysiology is unclear, its treatment outcome remains unsatisfactory[2,3]. Our previous study showed that electro-acupuncture (EA) is effective against IBS[4]. However, its mechanism of action needs to be further studied.

IBS patients often describe a correlation between

stressful life events and the onset or exacerbation of their gastrointestinal symptoms, and seem more susceptible to stressful events in daily life[5]. The central nervous system response to stressful events modulates the autonomic nervous system outflow and activates the hypothalamic-pituitary-adrenal axis[6]. Dysfunction of these systems has been proposed to be an etiological factor for IBS[7,8]. It has been reported that there is a difference in hormone level involving stress response between IBS patients and healthy subjects[8]. Central release of corticotropin-releasing hormone (CRH) plays an important role in the stress response[9], inducing a higher adrenocorticotropic hormone (ACTH) level and a more profound enhancement of colonic motility in IBS patients than in healthy controls[10]. It has been shown that CRH increases rectal sensitivity[11]. Thus, alterations in neuroendocrine response to stress may be of importance in the pathophysiology of IBS[12].

Visceral hypersensitivity is highly prevalent in IBS patients, and activation of intestinal mast cells (MC) may play a role in visceral hypersensitivity because they are in close proximity to gastrointestinal mucosal sensory nerve terminals containing neuropeptides, and a bidirectional pathway connecting the central nervous system, gut and MC have been demonstrated. MC at the ileocecal junction may be a mediator of the gut and nervous system in IBS[13] and substance P (SP) is a gastrointestinal peptide hormone. Both of them reside in the gastrointestinal tract and central nervous system. SP is also an interactive signaling molecule between the nervous and immune systems[14] and can modulate the function of intestinal mucosal MC by regulating neurosecretion and paracrine secretion.

This study was to explore the effect of EA at ST25 and ST37 on IBS by observing the MC count, the CRH level in hypothalamus, and the expression of SP and SPR in colon of rats.

MATERIALS AND METHODS

Animals

Twenty-one male Sprague-Dawley rats (SPF class), weighing 185-215 g, were supplied by Experimental Animal Center of Shanghai University of TCM, and randomly divided into normal group, model group and EA group according to their weights, 7 in each group. All rats were housed at a constant temperature and a humidity environment with free access to food and water. All studies were performed in accordance with the proposals of the Committee for Research and Ethical Issues of the International Association and approved by the Committee on the Use of Human and Animal Subjects in Teaching and Research, Shanghai University of TCM.

Establishment of rat model of IBS

An experimental rat model of IBS was established as previously described[15,16]. On the second day after the rats were fasted, experiment was begun. Rats in the normal group were given grabbing around the anus, while rats in the other two groups were stimulated by distending colorectum (CR). Limbs of the rats were fixed with medical adhesive tapes to limit their movements. The fixed rats could crawl without using their rear limbs. CR was distended before the limbs of rats were constrained and after the medical adhesive tapes were removed. The finger of a disposable rubber glove was tightly fixed onto the end of a polyethylene tube with 4 holes (0.5 cm apart) using medical silk thread as a 4 cm-long balloon. The other end of the tube was connected to a 10 cm-long rubber tube with a tri-channel valve connected to a syringe and sphygmomanometer. Vaseline was smeared on surface of the balloon which was slowly inserted into 5 cm of the rat anus along the physical curve of CR. The fixed time was 2 h each day, and CR was distended for 3 min, once every other day for 8 d. The whole modeling time was 15 d.

Table 1 AWR[14] scoring criteria

Score 0	No behavioral response to CRD
Score 1	Immobile during distension of CR and occasional clicking the head at onset of the stimulus
Score 2	A mild contraction of abdominal muscles, but no lifting of abdomen off the plattorm
Score 3	A strong contraction of abdominal muscles and lifting of abdomen off the platform, no lifting of pelvic structure off the platform
Score 4	Arching body and lifting of pelvic structure and scrotum

AWR: Abdominal withdrawal reflex; CRD: Colorectal distension; CR: Colorectum.

Treatment

Rats in the EA group were treated with EA at bilateral ST 25 and ST 37, once a day for 7 d as a course. Needles were pricked 0.3 cm in depth with a dense-sparse waveform at a frequency of 2/50 Hz and retained for 15 min. Rats in the normal and model groups received no EA treatment.

Contraction reaction in rat abnormal scoring test

The abdominal withdrawal reflex (AWR) scoring criteria[14] are shown in Table 1. Rats in the model group were fasted in afternoon of the previous day. Vaseline was smeared on surface of the balloon which was slowly inserted into 5 cm of the rat anus according to the physical curve of CR and retained for 5 min. The test was begun when the rats became adapted.

After air was added into the balloon with a syringe, the rat rectum was stimulated and different degrees of contraction reaction were observed. The pressure (mmHg) during behavior response scored as 1, 2, 3, and 4 was recorded and expressed as the threshold of sensitivity. Each score was tested three times, and each rat was tested by two persons not participating in this research. Means were calculated (6 values in total). Three-minute intervals were set between each two tests for the full adaptation of rats.

Table 2 Threshold pressure of rat contraction reaction in different groups (n = 7) (mean ± SD)

Group	Threshold pressure (mmHg)			
	Score 1	Score 2	Score 3	Score 4
Normal group	23.38 ± 3.15	41.26 ± 3.58	68.00 ± 8.97	86.35 ± 10.01
Model group	15.50 ± 3.25[b]	23.76 ± 3.91[b]	37.43 ± 6.75[b]	57.95 ± 5.45[b]
EA group	21.81 ± 1.93[d]	34.02 ± 3.87[b,d]	50.50 ± 7.28[b,d]	63.23 ± 6.24[b,d]

[b]P < 0.01 *vs* normal group; [d]P < 0.01 *vs* model group.

Table 3 Corticotropin-releasing hormone level in hypothalamus of rats and MC count in colonic membrane of rats in different groups (n = 7) (mean ± SD)

Group	CRH level (pg/mg)	MC count in each visional field
Normal group	42.68 ± 4.39	2.19 ± 0.31
Model group	66.63 ± 18.19[a]	10.0 ± 1.21[a]
EA group	42.81 ± 7.44[c]	4.81 ± 0.63[a,c]

[a]P < 0.05 *vs* normal group; [c]P < 0.05 *vs* model group. EA: Electro-acupuncture; MC: Mast cells..

Observation using toluidine blue-improved method

Samples were taken from the descending colon (5 cm above anus) and cecum, cleaned with normal saline, fixed with 10% formalin, dehydrated, paraffin-embedded, cut into sections and stained with toluidine blue-improved (TBI) method, deparaffinized and rehydrated, dipped in toluidine blue for 30 min. Two or three drops of glacial acetic acid were added into the samples until the presence of pretty clear nuclei and granulation. The samples were dried with cold air, cleaned in xylene, mounted onto Permount or Histoclad, and observed under a microscope (Olympus-BH2, × 100 and × 400). Three high-power fields (× 400) were randomly selected and the number of MC was counted and expressed as mean.

Radioimmunoassay for CRH

Sample preparation: All rats were killed by dislocating cervical vertebra, with their brain taken out and hypothalamus isolated in ice bath. The hypothalamus was rinsed with 0.9% sodium chloride and restored in a liquid nitrogen container. The hypothalamus was taken out from the liquid nitrogen container, weighed and labeled. One milliliter 1 mol/L glacial acetic acid was added and homogenized for 100 min, then 0.8 mL 1 mol/L NaOH was added and centrifuged for 20 min at 4000 r/min. The supernatant was stored at -20℃ for radioimmunoassay. The sample (50 μL) was incubated for 24 h at 4℃. Then, 500 μL separating-medium was added into each tube, incubated at room temperature for 45 min, centrifuged for 20 min at 4000 r/min. The supernatant was aspirated and the results were calculated.

Immunohistochemistry for SP/SPR: Sample sections were deparaffinized in xylene for 10 min, and dehydrated in 95%, 90%, 70% ethanol for 2 min. Primary antibody was bound to the specific rabbit anti-rat antigen diluted at 1:150, at 4℃ for 18 h. The samples stained with the envision immunohistochemistry method served as a positive control, while PBS-alternated primary antibody served as a negative control. Brown and dark brown granulation was observed with a background of purple blue. The positive expressing areas of SP and SPR under three fields were averaged.

Statistical analysis

Experimental data were expressed as mean ± SD. Statistical analyses were performed using SPSS 13.0 (SPSS Inc. Wacker Drive, Chicago, Illinois). Differences in mean were compared by one way ANOVA. P < 0.05 was considered statistically significant.

RESULTS

Contraction reaction in rat abnormal scoring test

The threshold pressure was remarkably lower in model group than in normal group, and obviously higher in EA group than in model group (P < 0.01, Table 2).

Effect of EA on CRH in hypothalamus of rats

The CRH level was significantly higher in hypothalamus of rats in model group than in normal group (P < 0.05), which was significantly decreased after EA treatment (P < 0.05). No significant difference was found in CRH level between normal and EA groups (Table 3).

MC in rat colonic membrane

The number of MC was greater in model group than in normal group (P < 0.05, Table 3) and smaller in EA group than in model group (P < 0.05). The plasma of MC was stained purple, while nuclei were stained dark blue, scattered in mucous and submucous layers, or gathered into groups or lined up. The cells were round, oval, shuttle-like, and erose in shape. Small cells had little plasma and were clear in shape, while big cells had more plasma and were unclear in shape.

SP and SPR expression in colon tissue of rats

The expression level of SP and SPR was higher in model group than in normal group (P < 0.05), which was decreased after EA treatment (P < 0.05, Table 4, Figure 1).

DISCUSSION

IBS is a prevalent functional gastrointestinal (GI) disorder

Table 4 SP and SPR expression in colonic membrane of rats in different groups (n = 7) (mean ± SD)

Group	SP expression		SPR expression	
	Optical density	Expressing area (μm^2)	Optical density	Expressing area (μm^2)
Normal group	14.21 ± 0.64	1772.77 ± 176.34	14.86 ± 0.48	362.65 ± 41.96
Model group	18.21 ± 1.07[a]	3157.31 ± 304.95[a]	16.36 ± 1.14[a]	532.83 ± 105.60[a]
EA group	16.29 ± 0.95[a,c]	2020.09 ± 116.31[a,c]	13.71 ± 0.70[a,c]	340.02 ± 29.61[c]

[a]P < 0.05 *vs* normal group; [c]P < 0.05 *vs* model group. SP: Substance P; SPR: Substance P receptor.

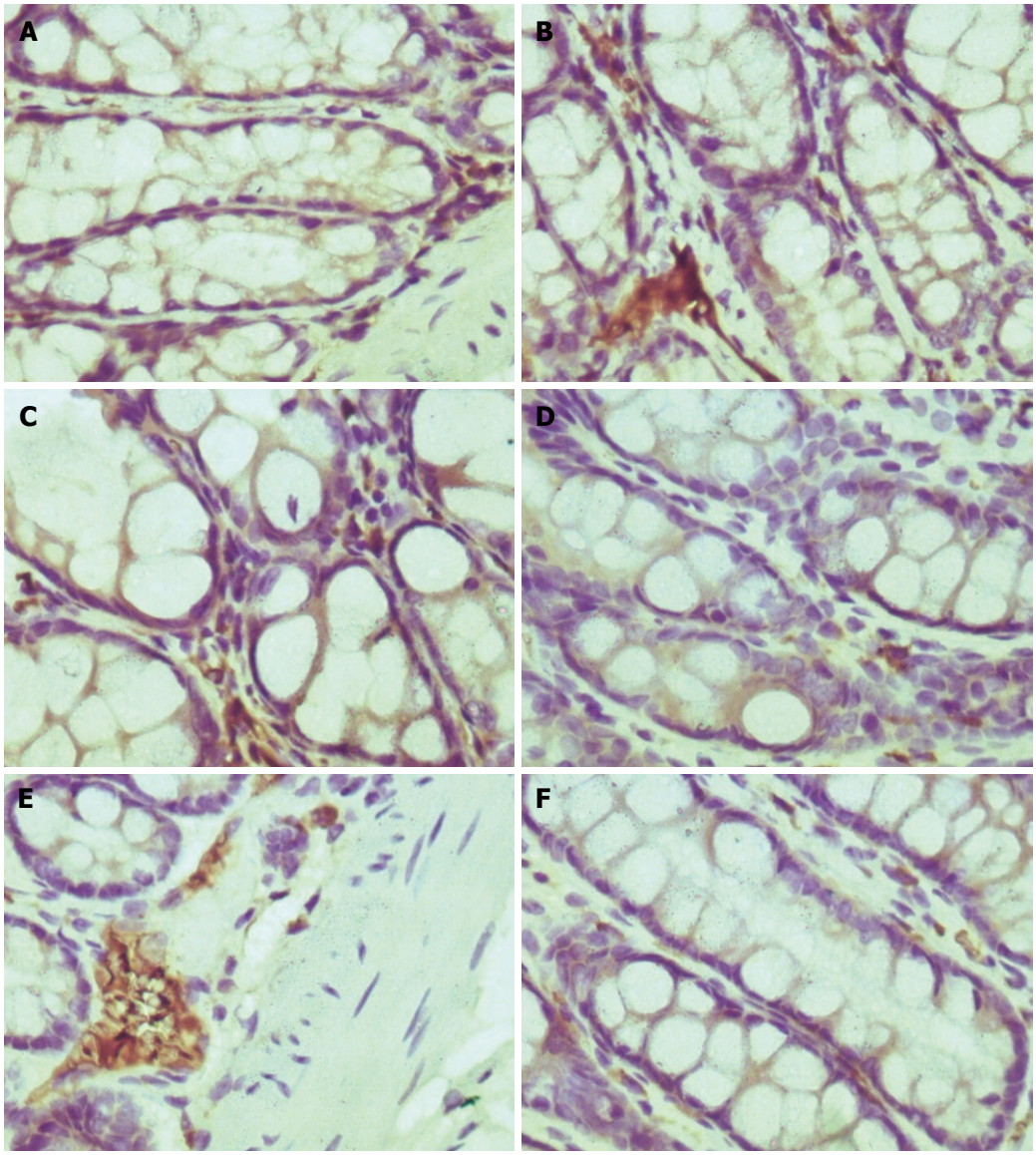

Figure 1 Expression of substance P and its receptor in colonic tissue of rats in normal group (A, D), model group (B, E) and EA group (C, F) (× 400).

characterized by chronic or recurrent abdominal pain or discomfort associated with altered bowel habits[16].

IBS is presumed to be a disorder of the brain-gut link[17]. Psychological stress induces colonic segmental contractions which are exaggerated in IBS patients[18,19]. Stress can alter GI function. However, the mechanism underlying stress-induced intestinal response is still unclear. Epidemiological data show that psychological stress is one of the most important etiological factors for IBS. Mental stress is one of the factors for the induction or aggravation of the symptoms of IBS[20]. Visceral hypersensitivity and dysregulation of central pain perception in the brain-gut axis play a pivotal role in the pathophysiology of IBS.

The central nervous system response to stress modulates the autonomic nervous system outflow and activates the hypothalamic-pituitary-adrenal axis[6]. Dysfunction of these systems has been proposed to be an etiological factor for IBS[7,8]. In addition, CRH, which plays an important role in the stress response[9], induces

a higher adrenocorticotropic hormone (ACTH) level and a more profound enhancement of colonic motility in IBS patients than in healthy controls[10]. It has been shown that CRH increases rectal sensitivity[11]. Thus, alterations in neuroendocrine response to stress may be of importance in the pathophysiology of IBS[12].

It was reported that CRH injected into the intracerebral ventricle of rats exerts a stimulatory effect on colonic motor function by inducing spike burst activities in the proximal colon, accelerating transit, and inducing defecation[21-23]. Use of nonselective CRH1 (NBI-359565) receptor antagonists showed that colonic motor function induced by CRH is delayed in rats, suggesting that central CRH combined with CRH1 receptor can regulate the colon function[23]. In the study by Fukudo *et al*[10], the descending colon motility induced by CRH was greater in IBS patients than in health subjects, CRH produced duodenal phase Ⅲ motor activities in 80% of healthy subjects and duodenal dysmotility in 40% of IBS patients, the time of abdominal symptoms evoked by CRH was significantly longer in IBS patients than in healthy subjects, the plasma ACTH level induced by CRH was significantly higher in IBS patients than in healthy subjects, indicating that human intestinal motility is probably modulated by exogenous CRH. The brain-gut in IBS patients may have an exaggerated response to CRH. Intravenous injection of CRH can promote the viscera sensibility in rats, which can be inhibited by CRH1 receptor antagonists[23]. This study showed that the CRH expression level in hypothalamus of rats was significantly higher in model group than in normal group ($P < 0.05$), which was remarkably decreased ($P < 0.05$) after EA treatment. No distinct difference in CRH expression was found between normal and EA groups, suggesting that EA therapy can inhibit the expression of CRH in hypothalamus of rats.

Recently, the role of probiotics in intestinal ecosystems has received great attention because of their beneficial effects on human and animal gut health[24]. It has been shown that probiotics can improve inflammation in some IBS patients and alleviate IBS symptoms such as pain[25]. It has been demonstrated in animal studies that neonatal intervention with probiotics can protect against short and long term consequences of impaired intestinal barrier function and gut-associated immune dysfunction induced by neonatal stress, reduce elevated corticosterone levels in pups with early psychological trauma (maternal deprivation), suggesting that normalization of HPA-axis activity is mediated by the effect of probiotics on gut function[26-30]. Further study is needed to explore the relation between acupuncture and probiotics used in treatment of IBS.

The pathological mechanism of IBS is not clear, but it is believed to be associated with alterations in mentality, GI motility, and visceral sensitivity, *etc*. Recently, researchers have suggested the role of inflammatory cells in the pathogenesis of IBS[31]. Mucosal MC are located throughout the gut in close proximity to enteric nerves, and secrete numerous inflammatory substances including histamine, cytokines, proteases, and eicosanoids that are known to sensitize visceral sensory nerve fibers. That is why some researchers have become interested in them.

SP is closely related with the pathological change in IBS, which plays a role in stress, intestinal infection, and visceral hypersensitivity in the development of IBS[31,32]. Meanwhile, SP is a gastrointestinal peptide hormone existing in the central nervous system and gastrointestinal tract, and a signaling molecule connecting the nervous system to the immune system. Wang *et al*[33] reported that the expression of SP and c-fos protein in the enteric and central nervous systems of the rat model of constipation-predominant IBS is abnormal, suggesting that abnormal changes in SP may be involved in the pathogenesis of IBS, and SP containing the neural pathway may be one of the neural pathways that play an important role in the regulation of gastrointestinal function.

SP in the intestinal tract is mainly produced by nerve terminal and endocrine cells such as MC. SP in combination with its receptor exerts its effect on the homologous effector cells of stomach and intestine, leading to complicated physiologic functions such as gastrointestinal motility, sensibility, secretion and absorption. In the enteric nervous system, SP, as an enteric nervous system of neurotransmitters, can increase gastrointestinal motility, promote contraction of alimentary tract smooth muscle, reinforce colon progradation, and stimulate water and electrolyte secretion in small intestine and colon. Some researchers believe that mucosal MC can restore the function and paresthesia of intestinal tract, while others hold that there is an amplifying ring among SP, MC and sensory neurofibra. Releasing of neuropeptides from sensory nerve ending, such as SP, has a direct effect on target organs. SP in combination with its special receptor on the surface of mucosal MC can activate and degranulate MC, releasing histamine and influencing sensorineural function, which promotes SP and local blood vessels to release nerve growth factor. In this study, the increased expression of SP was closely related with the number of MC in lamina propria of rats with IBS. It was reported that MC are associated with neurofibra by membrane-membrane touch[34]. The number of MC and degranulated MC is greater in IBS patients than in healthy subjects and the activated MC are adjacent to the inner-intestinal neuroplexus[13,35]. Our previous study showed that MC in colonic mucosa and c-fos positive cells are significantly increased, EA at ST-25 and Tegaserod injected into stomach can inhibit the proliferation and activation of MC in the colon, and regulate the secretion of SP, SPR, VIP, and VIPR, but the effect of EA is obviously better than that of Tegaserod[36]. In this study, the number of MC, the optical density and positive expressing areas of SP, SPR were greater in rats with IBS than in normal rats, indicating that MC, SP and SPR are closely related with the development of IBS ($P < 0.05$). However, MC, SP and SPR were decreased after EA treatment ($P < 0.05$), suggesting that EA at ST25 and ST37 can effectively

adjust the dysfunction of MC and down-regulate the expression of SP and SPR.

In conclusion, dysfunction of the central and enteric nervous systems leads to IBS. EA at ST25 and ST37 can decrease the number of MC, the expression of SP and SPR in colon, and the CRH level in hypothalamus of rats.

COMMENTS

Background

Irritable bowel syndrome (IBS) is a common disorder in clinical practice, but its pathophysiology has not been completely elucidated, which makes its treatment difficult. The authors' previous study showed that the general therapeutic rate of electro-acupuncture (EA) at ST-25 for IBS is 84.90%. However, the regulatory effect of EA on IBS is still unknown.

Research frontiers

More and more data show that IBS is closely related with the brain-gut axis, which has becoming a hot spot of study.

Innovations and breakthroughs

The results of the authors' study have proved that EA at ST25 and ST37 is effective against IBS. EA at ST25 and ST37 exerts its effect on IBS by decreasing the number of mucosal mast cells and down-regulating the expression of substance P, substance P receptor in colon and corticotropin-releasing hormone in hypothalamus of rats.

Applications

The experimental and clinical data can be used in further study on EA in treatment of IBS.

Peer review

This study is interesting. Its findings are useful for the treatment of IBS.

REFERENCES

1 **Jones J**, Boorman J, Cann P, Forbes A, Gomborone J, Heaton K, Hungin P, Kumar D, Libby G, Spiller R, Read N, Silk D, Whorwell P. British Society of Gastroenterology guidelines for the management of the irritable bowel syndrome. *Gut* 2000; **47** Suppl 2: ii1-ii19

2 **Torii A**, Toda G. Management of irritable bowel syndrome. *Intern Med* 2004; **43**: 353-359

3 **Talley NJ**. Irritable bowel syndrome. *Intern Med J* 2006; **36**: 724-728

4 **Liu HR**, Yang Y, Wu HG. Clinical Study on Acupuncture in Treating Diarrhea-predominant Irritable Bowel Syndrome. *J Acupunct Tuina Sci* 2008; **6**: 360-362

5 **Whitehead WE**, Crowell MD, Robinson JC, Heller BR, Schuster MM. Effects of stressful life events on bowel symptoms: subjects with irritable bowel syndrome compared with subjects without bowel dysfunction. *Gut* 1992; **33**: 825-830

6 **Mayer EA**. The neurobiology of stress and gastrointestinal disease. *Gut* 2000; **47**: 861-869

7 **Aggarwal A**, Cutts TF, Abell TL, Cardoso S, Familoni B, Bremer J, Karas J. Predominant symptoms in irritable bowel syndrome correlate with specific autonomic nervous system abnormalities. *Gastroenterology* 1994; **106**: 945-950

8 **Munakata J**, Mayer EA, Chang L, Schmulson M, Liu M, Tougas G, Kamath M, Naliboff B. Autonomic and neuroendocrine responses to recto-sigmoid stimulation. *Gastroenterology* 1998; **114**: A808

9 **Taché Y**, Martinez V, Million M, Rivier J. Corticotropin-releasing factor and the brain-gut motor response to stress. *Can J Gastroenterol* 1999; **13** Suppl A: 18A-25A

10 **Fukudo S**, Nomura T, Hongo M. Impact of corticotropin-releasing hormone on gastrointestinal motility and adrenocorticotropic hormone in normal controls and patients with irritable bowel syndrome. *Gut* 1998; **42**: 845-849

11 **Lembo T**, Plourde V, Shui Z, Fullerton S, Mertz H, Tache Y, Sytnik B, Munakata J, Mayer E. Effects of the corticotropin-releasing factor (CRF) on rectal afferent nerves in humans. *Neurogastroenterol Motil* 1996; **8**: 9-18

12 **Mayer EA**, Naliboff BD, Chang L, Coutinho SV. V. Stress and irritable bowel syndrome. *Am J Physiol Gastrointest Liver Physiol* 2001; **280**: G519-G524

13 **Yang Y**, Zhou D, Zhang W. [Mast cells of ileocecal junction in irritable bowel syndrome] *Zhonghua Neike Zazhi* 1997; **36**: 231-233

14 **Ansel JC**, Brown JR, Payan DG, Brown MA. Substance P selectively activates TNF-alpha gene expression in murine mast cells. *J Immunol* 1993; **150**: 4478-4485

15 **Al-Chaer ED**, Kawasaki M, Pasricha PJ. A new model of chronic visceral hypersensitivity in adult rats induced by colon irritation during postnatal development. *Gastroenterology* 2000; **119**: 1276-1285

16 **Williams CL**, Villar RG, Peterson JM, Burks TF. Stress-induced changes in intestinal transit in the rat: a model for irritable bowel syndrome. *Gastroenterology* 1988; **94**: 611-621

17 **Videlock EJ**, Chang L. Irritable bowel syndrome: current approach to symptoms, evaluation, and treatment. *Gastroenterol Clin North Am* 2007; **36**: 665-685, x

18 **Fukudo S**, Suzuki J. Colonic motility, autonomic function, and gastrointestinal hormones under psychological stress on irritable bowel syndrome. *Tohoku J Exp Med* 1987; **151**: 373-385

19 **Fukudo S**, Nomura T, Muranaka M, Taguchi F. Brain-gut response to stress and cholinergic stimulation in irritable bowel syndrome. A preliminary study. *J Clin Gastroenterol* 1993; **17**: 133-141

20 **Frank L**, Kleinman L, Rentz A, Ciesla G, Kim JJ, Zacker C. Health-related quality of life associated with irritable bowel syndrome: comparison with other chronic diseases. *Clin Ther* 2002; **24**: 675-689; discussion 674

21 **Okano S**, Nagaya H, Ikeura Y, Natsugari H, Inatomi N. Effects of TAK-637, a novel neurokinin-1 receptor antagonist, on colonic function in vivo. *J Pharmacol Exp Ther* 2001; **298**: 559-564

22 **Martínez V**, Wang L, Rivier JE, Vale W, Taché Y. Differential actions of peripheral corticotropin-releasing factor (CRF), urocortin II, and urocortin III on gastric emptying and colonic transit in mice: role of CRF receptor subtypes 1 and 2. *J Pharmacol Exp Ther* 2002; **301**: 611-617

23 **Williams CL**, Peterson JM, Villar RG, Burks TF. Corticotropin-releasing factor directly mediates colonic responses to stress. *Am J Physiol* 1987; **253**: G582-G586

24 **Barouei J**, Adams MC, Hodgson DM. Prophylactic role of maternal administration of probiotics in the prevention of irritable bowel syndrome. *Med Hypotheses* 2009; **73**: 764-767

25 **Andresen V**, Camilleri M. Irritable bowel syndrome: recent and novel therapeutic approaches. *Drugs* 2006; **66**: 1073-1088

26 **Gareau MG**, Jury J, MacQueen G, Sherman PM, Perdue MH. Probiotic treatment of rat pups normalises corticosterone release and ameliorates colonic dysfunction induced by maternal separation. *Gut* 2007; **56**: 1522-1528

27 **García-Ródenas CL**, Bergonzelli GE, Nutten S, Schumann A, Cherbut C, Turini M, Ornstein K, Rochat F, Corthésy-Theulaz I. Nutritional approach to restore impaired intestinal barrier function and growth after neonatal stress in rats. *J Pediatr Gastroenterol Nutr* 2006; **43**: 16-24

28 **Gareau MG**, Jury J, Perdue MH. Neonatal maternal separation of rat pups results in abnormal cholinergic regulation of epithelial permeability. *Am J Physiol Gastrointest Liver Physiol* 2007; **293**: G198-G203

29 **Eutamene H**, Bueno L. Role of probiotics in correcting abnormalities of colonic flora induced by stress. *Gut* 2007; **56**: 1495-1497

30 **Barbara G**, De Giorgio R, Stanghellini V, Cremon C, Corinaldesi R. A role for inflammation in irritable bowel syndrome? *Gut*

2002; **51** Suppl 1: i41-i44
31 **Ringel Y**, Sperber AD, Drossman DA. Irritable bowel syndrome. *Annu Rev Med* 2001; **52**: 319-338
32 **Dong WZ**, Zou DW, Li ZS, Zou XP, Zhu AY, Xu GM, Yin N, Gong YF, Sun ZX, Man XH. Study of visceral hypersensitivity in irritable bowel syndrome. *Chin J Dig Dis* 2004; **5**: 103-109
33 **Wang WF**, Yang YS, Peng LH, Sun G. Alternation of substance P-containing neural pathways in a rat model of irritable bowel syndrome with rectal distension. *Chin J Dig Dis* 2006; **7**: 211-218
34 **Dong WZ**, Zhou XP, Zhou DW, Li ZS, Xu GM, Zhou AY, Gong YF. The relationship between mast cell and neuropeptide fibre of colonic mucous membrane in irritable bowel syndrome. *Dier Junyi Daxue Xuebao* 2003; **24**: 147-150
35 **Park CH**, Joo YE, Choi SK, Rew JS, Kim SJ, Lee MC. Activated mast cells infiltrate in close proximity to enteric nerves in diarrhea-predominant irritable bowel syndrome. *J Korean Med Sci* 2003; **18**: 204-210
36 **Wu HG**, Jiang B, Zhou EH, Shi Z, Shi DR, Cui YH, Kou ST, Liu HR. Regulatory mechanism of electroacupuncture in irritable bowel syndrome: preventing MC activation and decreasing SP VIP secretion. *Dig Dis Sci* 2008; **53**: 1644-1651

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CASE REPORT

Biloma: An unusual complication in a patient with pancreatic cancer

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Abstract

The term biloma describes an encapsulated collection of bile within the abdomen, usually secondary to bile duct disruption. The commonest causes reported in the literature are iatrogenic (secondary to hepatobiliary surgery), trauma or complications due to choledocholithiasis. A few cases have been reported as complications of cholangiocarcinoma or acute cholecystitis. We report the case of a 64-year-old man initially diagnosed with a non-obstructive malignancy of the pancreas, who developed a spontaneous intrahepatic biloma 8 mo later. This was identified following a 1-wk history of fever, rigors and icterus. The biloma was identified on computed tomography and subsequently drained under ultrasound guidance. Forty-eight hours later, a stent was inserted endoscopically into his common bile duct and he made an uneventful in-hospital recovery. We believe this is the first documented case of spontaneous intrahepatic biloma to occur secondary to pancreatic malignancy.

Key words: Obstructive jaundice; Endoscopic retrograde cholangiopancreatography; Computed tomography; Choledocholithiasis; Bile duct diseases

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INTRODUCTION

In 1979, Gould and Patel[1] described the case of a 32-year-old man who was found to have a large encapsulated collection of bile outside the biliary tree, secondary to a tear in the right hepatic duct. This followed a traumatic insult to the upper abdomen. The patient proceeded to have this collection drained and a T-tube was inserted to permit bile drainage. Since the initial description, there have been a few documented cases of spontaneous biloma formation, usually in the context of choledocholithiasis. The current case is sufficiently exceptional with regard to location, etiology and mode of presentation to merit a report.

CASE REPORT

A 64-year-old man was referred to the gastroenterology clinic by his general practitioner (GP) with constant upper abdominal pain that was worse after eating. In addition, the patient had been suffering from alternating constipation and loose stools. He denied the passage of blood in his motions or any weight loss.

A full blood count revealed normocytic anemia (hemoglobin 123 g/L; mean corpuscular volume: 88.4 fL). Renal and liver function tests were normal. Given the features on presentation, a colonoscopy was suggested, however, he was reluctant to go ahead with the procedure. As an alternative, a computed tomography (CT) pneumocolon was arranged.

Although no significant polyps or other colonic abnormalities were seen, there was an irregular 3 cm × 4.5 cm mass arising from the neck/body of the pancreas, with multiple lymph nodes in the peripancreatic region. Further assessment with a dual-phase CT scan of the pancreas was performed (Figure 1). As a result of encasement

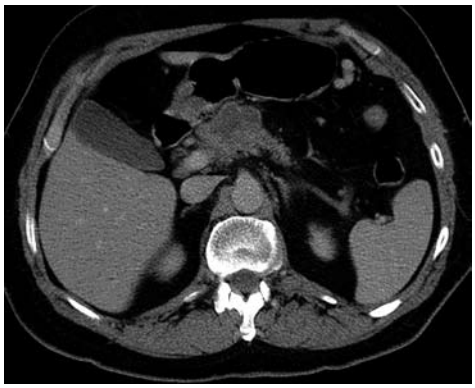

Figure 1 CT pneumocolon, which suggested the presence of advanced pancreatic malignancy.

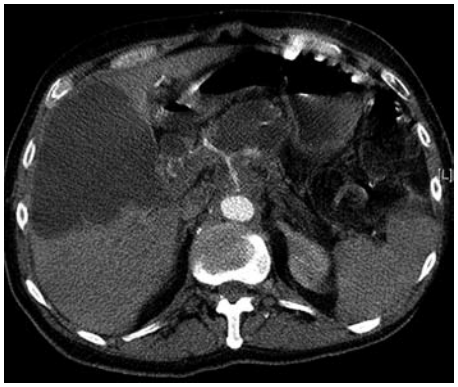

Figure 2 Large intrahepatic cystic lesion with bile duct compression caused by enlargement of the pancreatic neoplasm.

of the superior mesenteric artery and portal veins, the tumor was deemed inoperable. An ultrasound-guided biopsy of the lesion was undertaken, and histology confirmed adenocarcinoma of the pancreas.

The patient went on to complete three courses of gemcitabine chemotherapy but further staging CT demonstrated slowly progressive disease over a 3-mo period.

Six months later, the patient was referred by his GP to the on-call medical team with a week-long history of nausea, anorexia and new-onset jaundice. Upon examination, he was tender in the epigastrium and right upper quadrant, with no overt peritonism. His blood tests were as follows: bilirubin, 164 μmol/L; alanine aminotransferase, 84 IU/L; alkaline phosphatase, 5567 IU/L; albumin, 35 g/L; C-reactive protein (CRP), > 160 mg/L; prothrombin time, 16.3 s; and white blood cell count, 12.9×10^9/L.

Repeat CT of the pancreas was undertaken, which demonstrated a dilated common bile duct (CBD) with external compression from enlargement of the pancreatic tumor, and a large, intrahepatic cystic lesion that measured 12 cm × 9 cm, adjacent to the gallbladder (Figure 2).

Ultrasound-guided drainage was performed and yielded frank bile in keeping with an intrahepatic biloma. Microbiological analysis of the fluid did not reveal the presence of any pathogenic organisms. Endoscopic retrograde cholangiopancreatography (ERCP) demonstrated

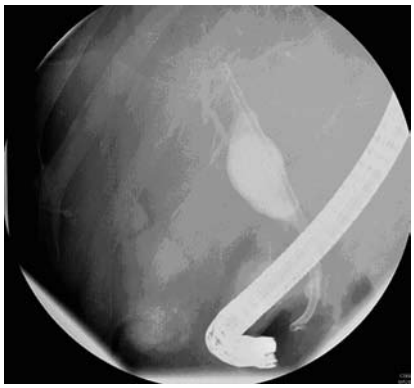

Figure 3 ERCP demonstrating a smooth stricture in the distal CBD, with dilated common hepatic ducts above. Contrast material was seen to flow within the biloma collection, which implied communication with the intrahepatic ducts.

a smooth stricture of the distal CBD and a dilated common hepatic duct above. Contrast material was seen to be flowing into the biloma, which implied a connection with the intrahepatic ducts (Figure 3). The stricture was stented and drainage from the biloma ceased within 48 h following insertion.

DISCUSSION

The first reported case of a biloma was reported by Gould and Patel in 1979[1]. They reported extrahepatic bile leakage post trauma to the right upper quadrant of the abdomen. The bile accumulated in an encapsulated form. Although originally described as a bilious collection outside the liver, the term biloma has been extended to include any such lesion that may be intrahepatic but anatomically outside the biliary tree[2]. The majority of bilomas are iatrogenic and follow transhepatic cholangiography, liver biopsy, ERCP or cholecystectomy. Biloma also has been recognized to arise following external trauma[3]. Spontaneous biloma is exceedingly rare, and the majority occur secondary to choledocholithiasis or cholangiocarcinoma[4]. Rarer causes have been reported in the context of sickle cell disease[5] or as a complication of hepatic infarction and abscess formation. To the best of our knowledge, biloma that occurs secondary to primary pancreatic malignancy has not been reported previously in the literature.

As diagnostic techniques continue to evolve, an increasing number of cases have been identified, but the exact mechanism behind spontaneous biloma formation is still unknown. Postulated pathogenic mechanisms are Sphincter of Oddi spasm, CBD tumor or calculus obstruction that results in increased intraductal pressure, bile duct necrosis and rupture of the bile duct. As a result of the relatively slow onset of ductal obstruction that occurs in the context of a pancreatic neoplasm, such an acute elevation in biliary pressure is unusual (cf. impacted CBD stone). The size and location of biloma is influenced by the cause of rupture, location and size of bile leak, and rate of absorption by the peritoneum.

Most bilomas are secondary to CBD rather than hepatic duct perforation[6].

There is no difference in the incidence between males and females, but the condition is found more often in the sixth to seventh decades of life. The age predominance may reflect that of the underlying etiological factor rather than that of developing the complication. Presentation is nonspecific, with abdominal pain, usually in the right upper quadrant (although a few reported cases of bile migration to the left subphrenic space have been documented, which has given rise to a predominance of pain on the left side). Fever may be accompanied by jaundice and abdominal distension. Extreme cases that result in bilious ascites also have been reported[7]. In our case, there was no history of recent hepatobiliary intervention (pancreatic biopsy had been performed 6 mo prior to presentation, but no evidence of biloma was seen on interval scanning). There were complaints of fever, rigors, anorexia and scleral icterus. Blood tests may show neutrophil leukocytosis, elevated CRP and obstructive liver function tests. Blood cultures may show Gram-negative bacteremia. Biloma can be picked up on ultrasound, CT or magnetic resonance imaging. Despite advancing imaging modalities, biloma may be difficult to differentiate from large cystic metastasis, seroma, angioma or lymphocele. Ultrasound becomes useful in this situation, with a definitive diagnosis being made following radiologically guided aspiration. Once fluid is obtained, microbiological testing is mandatory to exclude the presence of coexisting infection. ERCP is particularly useful in diagnosing an active leak; this may also allow therapeutic intervention[8]. Precise location of the biloma allows for percutaneous drainage, which negates the need for surgical intervention. Endoscopic intervention includes sphincterotomy with stone extraction, if appropriate, to lower biliary pressure. Placement of a stent in more distal lesions is an option, as this reduces the pressure gradient into the duodenum and facilitates forward flow of bile. This also relieves obstruction from lesions that narrow the biliary tree. In our case, the latter approach was used to overcome the obstruction caused by the large pancreatic tumor.

Surgical management remains contentious but can be useful in cases of ongoing leakage despite endoscopic therapy. The goals are to halt abdominal contamination from bile by means of peritoneal drainage, surgical closure of active leaks, and T-tube drainage[7-9].

REFERENCES

1 **Gould L**, Patel A. Ultrasound detection of extrahepatic encapsulated bile: "biloma". *AJR Am J Roentgenol* 1979; **132**: 1014-1015

2 **Kuligowska E**, Schlesinger A, Miller KB, Lee VW, Grosso D. Bilomas: a new approach to the diagnosis and treatment. *Gastrointest Radiol* 1983; **8**: 237-243

3 **Vazquez JL**, Thorsen MK, Dodds WJ, Quiroz FA, Martinez ML, Lawson TL, Stewart ET, Foley WD. Evaluation and treatment of intraabdominal bilomas. *AJR Am J Roentgenol* 1985; **144**: 933-938

4 **Fujiwara H**, Yamamoto M, Takahashi M, Ishida H, Ohashi O, Onoyama H, Takeyama Y, Kuroda Y. Spontaneous rupture of an intrahepatic bile duct with biloma treated by percutaneous drainage and endoscopic sphincterotomy. *Am J Gastroenterol* 1998; **93**: 2282-2284

5 **Middleton JP**, Wolper JC. Hepatic biloma complicating sickle cell disease. A case report and a review of the literature. *Gastroenterology* 1984; **86**: 743-744

6 **Kang SB**, Han HS, Min SK, Lee HK. Nontraumatic perforation of the bile duct in adults. *Arch Surg* 2004; **139**: 1083-1087

7 **Hartle RJ**, McGarrity TJ, Conter RL. Treatment of a giant biloma and bile leak by ERCP stent placement. *Am J Gastroenterol* 1993; **88**: 2117-2118

8 **Lilly JR**, Weintraub WH, Altman RP. Spontaneous perforation of the extrahepatic bile ducts and bile peritonitis in infancy. *Surgery* 1974; **75**: 664-673

9 **Jackson BT**, Saunders P. Perforated choledochus cyst. *Br J Surg* 1971; **58**: 38-42

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CASE REPORT

Ectopic papilla of Vater in the pylorus

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Abstract

The major papilla of Vater is usually located in the second portion of the duodenum, to the posterior medial wall. Sometimes the mouth of the biliary duct is located in other areas. Drainage of the common bile duct into the pylorus is extremely rare. A 73-year old man, with a history of duodenal ulcer, was admitted to hospital with the diagnosis of cholangitis. Dilatation of the extrahepatic biliary duct was observed by abdominal ultrasonography, and endoscopic retrograde cholangiopancreatography (ERCP) was performed. No area suggesting the presence of the papilla of Vater was found within the second duodenal portion. Finally the major papilla was located in the theoretical pyloric duct. Cholangiography was performed and choledocholithiasis was found in the biliary tree. The patient underwent dilatation of the papilla with a balloon tyre and removal of a 7 mm stone using a Dormia basket, which solved the problem without further complications. This anomaly increased the difficulty of performing therapeutic interventions during ERCP. This alteration in anatomy may increase the risk of complications during papillotomy, with a theoretically higher risk of perforation. Dilatation using a balloon was the chosen therapeutic technique both in our case and in the literature, due to its low rate of complications.

Key words: Ectopic common bile duct; Endoscopic dilatation; Endoscopic retrograde cholangiopancreatography; Papilla of Vater; Papillotomy; Pyloric drainage

Peer reviewer: Dr. Aydin Karabacakoglu, Assistant Professor, Department of Radiology, Meram Medical Faculty, Selcuk University, Konya 42080, Turkey

Guerra I, Rábago LR, Bermejo F, Quintanilla E, García-Garzón S. Ectopic papilla of Vater in the pylorus. *World J Gastroenterol* 2009; 15(41): 5221-5223 Available from: URL: http://www.wjgnet.com/1007-9327/15/5221.asp DOI: http://dx.doi.org/10.3748/wjg.15.5221

INTRODUCTION

The major papilla of Vater is usually located in the second portion of the duodenum, to the posterior medial wall. Both the common bile duct and the main pancreatic duct empty into it. Sometimes the mouth of the biliary duct is located in other areas along the duodenum, mainly within the third or the fourth portion[1-3], although it has also been found with a much lower frequency in the duodenal bulb[4-6]. In such cases it is common to find a previous duodenal ulcerous pathology[7]. Very rarely has the papilla been found in the stomach[8-10], although the frequent use of endoscopic retrograde cholangiopancreatography (ERCP) has increased its recognition. In the literature we found a case in the 1930's describing common bile duct drainage into the pylorus[2], and another more recent case of drainage into the duodenal wall adjacent to the pylorus[11], however, no more cases have been published. We describe a case in which the mouth of the biliary duct was found in the pyloric channel.

CASE REPORT

A 73-year-old patient, with a history of digestive bleeding secondary to duodenal ulcer 10 years previously, presented to hospital due to high temperature and pain in the right hypochondrium. His C-reactive protein level was 190 mg/dL, alanine-aminotransferase 40 IU/L, leucocytes 15 500/mm^3 and total bilirubin 2.62 mg/dL. Abdominal ultrasonography showed dilatation of the extrahepatic biliary duct, with no lithiasis. After the diagnosis of cholangitis with increased bilirubin and dilatation of the biliary duct was made, ERCP was performed. No area suggesting the presence of the papilla of Vater was found within the second duodenal portion. Finally the major papilla was located in the theoretical pyloric duct (Figure 1), since the patient showed a duodenal bulb which was deformed and post-ulcerous, having disappeared almost completely. The papilla did not show clear anatomical signs which made it inadvisable to perform a papillotomy. Cholangiography was carried out and choledocholithiasis

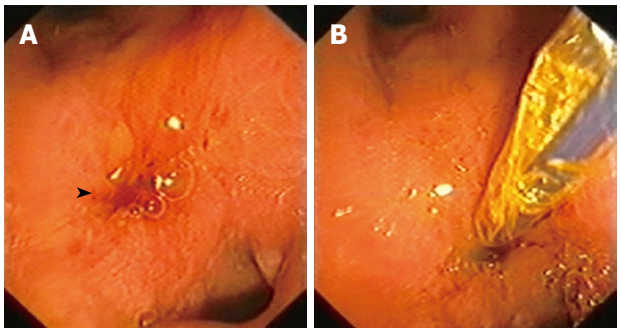

Figure 1 Endoscopic retrograde cholangiopancreatography. A: View of the papilla located in the pylorus (arrow head); B: Piping of the papilla.

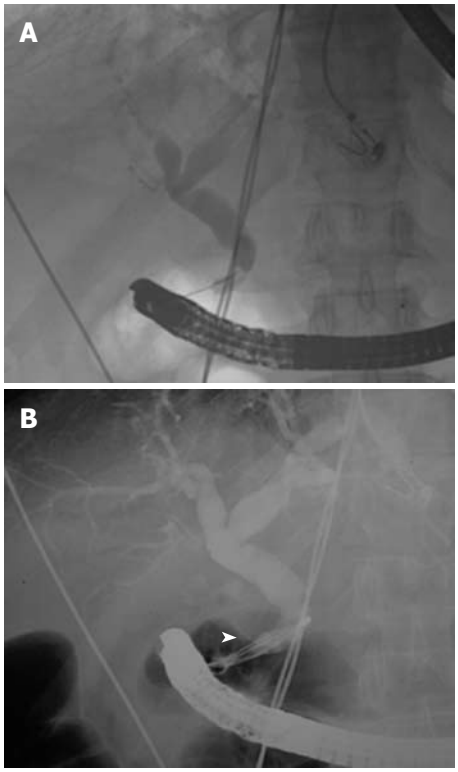

Figure 2 Cholangiography. A: Common bile duct ending in a hook shape, common in proximal drainage of the papilla; B: Removal of choledocholithiasis with Dormia basket (arrow head).

was found in the biliary tree (Figure 2A). The patient underwent dilatation of the papilla with a balloon tyre and removal of a 7 mm stone using a Dormia basket, which solved the problem without further complications (Figures 2B and 3).

DISCUSSION

The papilla of Vater is a bulge in the duodenal mucosa into which both the common bile duct and the Wirsung empty, sometimes shaping into a Y, or they can be separated by a mucosa layer making them independent. The most common location of the papilla of Vater is within the posterior medial wall of the second portion of the duodenum. The frequent use of ERCP has allowed better observation of papillae in an ectopic location.

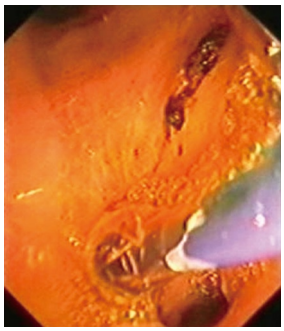

Figure 3 Endoscopic retrograde cholangiopancreatography. Balloon dilatation of the ectopic papilla.

Although the diagnosis of ectopic papillae have been described by other types of radiological studies such as computed tomography[4], X-ray of the esophagus-gastroduodenal tract[12], intraoperative cholangiography[1] and echoendoscopy[5], most ectopic papillae are identified by ERCP. An ectopic location distal to the second duodenal portion, within the third and fourth duodenal portions, has been described frequently, and has a frequency rate of 5.6% to 23%[13]. Ectopic papillae are much less frequent in a proximal location, and a few cases have been located in the gastric, pylorus and duodenal bulb areas[14]. Filippini in 1931 described the first case of papilla located in the pylorus[2], although Quintana and Labat[2] mentioned 3 cases of dual drainage in the duodenum and pylorus. Another recent case has been described where the papilla was located in the posterior duodenal wall below the pylorus[11]. In our subject the papilla was located in the pylorus, with a duodenal bulb which was deformed due to previous ulcerous pathology.

A theory on the origin of ectopic papillae has been suggested which describes their occurrence during embryonic formation. The liver originates in the hepatic diverticulum, which is divided into the hepatic pars and the cystic pars during embryogenesis. The hepatic pars then develops into both the liver and the hepatic ducts, while the cystic pars develops into the gall-bladder and the cystic duct. The common bile duct originates in the hepatic antrum, which is the common area of the hepatic diverticulum. Boyden[15] claimed that an earlier subdivision of the hepatic diverticulum could cause the common bile duct to empty into different locations other than the usual location.

Ectopic papillae located in the bulb may be secondary to an ulcerous duodenal pathology, which could cause, due to contiguity, anomalous drainage in the duodenum[11]. In our patient the location of the papilla in the pyloric channel might be related to the patient's previous ulcerous duodenal pathology. Nevertheless, since we did not find signs of the papilla in the second duodenal portion, his condition was probably due to a congenital malformation with biliary and pancreatic drainage in the posterior pyloric area, which could have caused the later development of a duodenal ulcer. In our subject we observed the diagnosis requirements for an ectopic papilla as described by Lee *et al*[14], for a location in the bulb which were: (1) an orifice was observed in the bulb by duodenoscopy or upper endoscopy, and the bile duct and/or the pancreatic duct were directly visualized radiographically, when contrast

was injected *via* this opening; (2) there was direct drainage of the common bile duct into the duodenal bulb without evidence of any other drainage into the duodenum on cholangiography; and (3) there was no evidence of a papilla-like structure in the second or third duodenal portion on duodenoscopic examination. Fistula secondary to ulcer disease or choledocholithiasis, spontaneous or iatrogenic surgical fistula, and surgical choledochoenteric diversion should be included in the differential diagnosis[8].

The clinical importance of the ectopic location of the papilla means that there is a tendency for the development of choledocholithiasis through anomalous bile drainage, due to the lack of a sphincter mechanism. Likewise, it can also lead to mucosal damage in the area, with swelling and ulcer formation, due to the action of biliary pancreatic secretion[14]. The clinical symptoms may include recurrent abdominal pain, which could explain the high percentage of patients undergoing cholecystectomy described in some series[13]. The absence of a sphincter would allow passage of the gastroduodenal contents into the main bile duct, possibly causing cholangitis in association with biliary obstruction[7,13,14]. In a recent study by Disibeyaz *et al*[13] on 39 patients where the papilla was located in the bulb, they detected episodic abdominal pain in 95% of patients and cholangitis in 59% of patients. The predominance of male sex and an association with ulcerous duodenal pathology in 61.5% of the patients should be emphasized. In our case, the patient had a history of duodenal ulcer and was admitted to hospital suffering from cholangitis, which led us to think that this patient had a papilla in an ectopic location when we failed to find it in its usual location, making it necessary to check both the duodenal bulb and the stomach.

This anomaly increased the difficulty of performing therapeutic interventions during ERCP. This alteration in anatomy, when there are no clear anatomic signs, may increase the risk of complications during papillotomy, with a theoretically higher risk of perforation, as has been described in the literature[13]. Balloon dilatation may be the chosen technique for these patients when there is a biliary obstruction. Its effectiveness has recently been suggested, especially when gallstones need to be removed, without relevant complications[13]. Stent installation may be necessary when balloon dilatation is unsuccessful and the patient has comorbidities which make surgery inadvisable. The surgical option should only be taken in these cases when endoscopic treatment is not effective.

In conclusion, ectopic location of the papilla is a rare finding, however, the frequent use of ERCP may increase the number of cases diagnosed. Although a distal location is found most frequently, a proximal location must be taken into account, especially in patients with a history of recurrent abdominal pain, cholangitis, ulcerous duodenal pathology and biliary obstruction in whom the papilla cannot be found in its usual location during ERCP. Although further studies are necessary, balloon dilatation was the chosen therapeutic technique both in our case and in the literature, due to its low rate of complications. Technical difficulty and a higher probability of perforation when performing a papillotomy, due to the lack of anatomic reference, make its use inadvisable.

REFERENCES

1 **Lindner HH**, Peña VA, Ruggeri RA. A clinical and anatomical study of anomalous terminations of the common bile duct into the duodenum. *Ann Surg* 1976; **184**: 626-632
2 **Quintana EV**, Labat R. Ectopic drainage of the common bile duct. *Ann Surg* 1974; **180**: 119-123
3 **Doty J**, Hassall E, Fonkalsrud EW. Anomalous drainage of the common bile duct into the fourth portion of the duodenum. Clinical sequelae. *Arch Surg* 1985; **120**: 1077-1079
4 **Lee HJ**, Ha HK, Kim MH, Jeong YK, Kim PN, Lee MG, Kim JS, Suh DJ, Lee SG, Min YI, Auh YH. ERCP and CT findings of ectopic drainage of the common bile duct into the duodenal bulb. *AJR Am J Roentgenol* 1997; **169**: 517-520
5 **Krstic M**, Stimec B, Krstic R, Ugljesic M, Knezevic S, Jovanovic I. EUS diagnosis of ectopic opening of the common bile duct in the duodenal bulb: a case report. *World J Gastroenterol* 2005; **11**: 5068-5071
6 **Ozaslan E**, Saritaş U, Tatar G, Simşek H. Ectopic drainage of the common bile duct into the duodenal bulb: report of two cases. *Endoscopy* 2003; **35**: 545
7 **Song MH**, Jun DW, Kim SH, Lee HH, Jo YJ, Park YS. Recurrent duodenal ulcer and cholangitis associated with ectopic opening of bile duct in the duodenal bulb. *Gastrointest Endosc* 2007; **65**: 324-325; discussion 325
8 **Pereira-Lima J**, Pereira-Lima LM, Nestrowski M, Cuervo C. Anomalaous location of the papilla of vater. *Am J Surg* 1974; **128**: 71-74
9 **Kanematsu M**, Imaeda T, Seki M, Goto H, Doi H, Shimokawa K. Accessory bile duct draining into the stomach: case report and review. *Gastrointest Radiol* 1992; **17**: 27-30
10 **Katsinelos P**, Papaziogas B, Paraskevas G, Chatzimavroudis G, Koutelidakis J, Katsinelos T, Paroutoglou G. Ectopic papilla of vater in the stomach, blind antrum with aberrant pyloric opening, and congenital gastric diverticula: an unreported association. *Surg Laparosc Endosc Percutan Tech* 2007; **17**: 434-437
11 **Sung HY**, Kim JI, Park YB, Cheung DY, Cho SH, Park SH, Han JY, Kim JK. The papilla of Vater just below the pylorus presenting as recurrent duodenal ulcer bleeding. *Intern Med* 2007; **46**: 1853-1856
12 **Holz K**. [Atypical orifice of the common bile duct during localization of the Vater papilla on the rear wall of the duodenal bulb] *Fortschr Geb Rontgenstr Nuklearmed* 1970; **112**: 409-410
13 **Disibeyaz S**, Parlak E, Cicek B, Cengiz C, Kuran SO, Oguz D, Güzel H, Sahin B. Anomalous opening of the common bile duct into the duodenal bulb: endoscopic treatment. *BMC Gastroenterol* 2007; **7**: 26
14 **Lee SS**, Kim MH, Lee SK, Kim KP, Kim HJ, Bae JS, Kim HJ, Seo DW, Ha HK, Kim JS, Kim CD, Chung JP, Min YI. Ectopic opening of the common bile duct in the duodenal bulb: clinical implications. *Gastrointest Endosc* 2003; **57**: 679-682
15 **Boyden EA**. The problem of the double ductus choledochus (an interpretation of an accessory bile duct found attached to the pars superior of the duodenum). *Anat Rec* 1932; **55**: 71-94

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CASE REPORT

Gastrointestinal stromal tumor causing small bowel intussusception in a patient with Crohn's disease

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Abstract

We report a case of jejunoileal intussusception in a 42-year-old patient with Crohn's disease caused by a gastrointestinal stromal tumor. The patient complained of vague diffuse abdominal pain for a period of 4 mo. Intussusception was suspected at computer tomography and magnetic resonance imaging scans. Segmental resection of the small intestine was performed. Pathological examination of the surgical specimen revealed a gastrointestinal stromal tumor as well as aphthous ulcerations and areas of inflammation, which were characteristic of Crohn's disease. This is the first report of small bowel intussusception due to a gastrointestinal stromal tumor coexisting with Crohn's disease.

Key words: Gastrointestinal stromal tumor; Crohn's disease; Intussusception

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INTRODUCTION

Intussusception, defined as the telescoping of a segment of the gastrointestinal tract into an adjacent one, is extremely rare in the stomach, but more common in the small intestine, ileocecal junction and colon. It is the leading cause of intestinal mechanical obstruction and the second most common surgical emergency in children[1]. However, it is rather infrequent in adults, accounting for 0.1% of all surgical admissions and 1%-5% of mechanical bowel obstructions[2]. In these cases, it is frequently related to malignancy.

Gastrointestinal stromal tumors (GISTs) are a subset of mesenchymal tumors of varying differentiation. They are rare clinical entities, constitute less than 3% of all gastrointestinal malignant neoplasms and represent only 20% of small-bowel malignant neoplasms (excluding lymphoma)[3]. With the improvement on immunohistochemical staining techniques and ultrastructural evaluation, GISTs are recognized as a distinct group of mesenchymal tumors now[4].

In the current report, we present a case of a 45-year-old man with Crohn's disease complaining of intermittent vague abdominal pain for a period of 4 mo. Enteroclysis, computer tomography (CT) and magnetic resonance imaging (MRI) abdominal scans showed small bowel intussusception, a diagnosis which was confirmed after surgical exploration of the abdomen where a tumor causing a jejunoileal intussusception was identified. Pathological examination of the surgical specimen revealed a gastrointestinal stromal tumor as well as inflammation and aphthous ulcerations, which were characteristic of Crohn's disease involving small bowel. Coexistence of these clinical entities resulting in intussusception has never been reported in the literature.

CASE REPORT

A 45-year-old man with symptoms of bowel obstruction was admitted to the surgical department of our hospital in April, 2008. He complained of diffuse blunt abdominal pain preceded by two episodes of vomiting and obstipation. The patient reported similar attacks of milder episodes, consisting of intermittent abdominal pain and bloating for the past 4 mo. He was diagnosed as Crohn's disease following gastroenterological evaluation with colonoscopy and terminal ileum biopsies at sites of ileitis 1 year ago on the grounds of chronic diarrhea, cramp abdominal pain attacks and mild anemia. The patient did not receive any regular medication. Physical examination showed mild abdominal distention with slight tenderness and hypoactive bowel sounds. No mass was palpated and rectal examination did not reveal any blood or malignancy. Routine laboratory tests and chest films were normal. However, abdominal plain radiographs showed some small bowel air-fluid levels. The patient underwent a CT scan with *po* and intravenous contrast of 5-mm thick slice, which showed thickening of the distal ileum wall and an intraluminal mass resulting in partial obstruction of the small intestine. Sagittal fast spin echo (FSE) T2, FSE T2 with fat suppression and axial FSE T1 with 5-mm thick slice abdominal MRI (Figure 1) fortified the suspicion of intussusception since it showed a pathognomonic bowel within bowel configuration. In order to secure the diagnosis, an enteroclysis was performed, which showed a jejunoileal stricture caused by invagination of the jejunum into the ileum and proximal to the stenosis bowel dilatation (Figure 2).

Taking into account the high possibility of a malignant mass causing bowel intussusception in an adult patient, laparoscopic approach was not considered in this case, in order to achieve the best possible oncologic clearance at the surgical intervention that followed. So, the patient underwent a laparotomy which identified a jejunoileal intussusception (Figure 3). An approximately 20-cm partial small bowel resection including wide margins and a wedge resection of the respective mesentery up to the beginning of the feeding vessels were performed. The bowel lumen was longitudinally opened immediately following removal of the specimen and an exophytic round-shaped mass of smooth circumference approximately 6 cm in maximum diameter, which was hard at palpation, was easily appreciated. Pathological examination of the surgical specimen proved the lesion to be a stromal tumor with an immunohistochemical profile of c-kit (+), S-100 (-), SMA (-), desmin (-), EMA (-), CD34 (-), AE1/AE3 CK8.18 (-), NSE (-), and synaptophysin (-). The tumor was invading the muscularis propria layer at the point of the intussusception (Figure 4A and B). No lymph node infiltration was identified. Microscopic examination of the remaining specimen disclosed findings consistent with Crohn's disease. The lesion contained aphthous ulcerations and showed severe polymorphonuclear and lymphocytic infiltration as well as eosinophils and intraluminal abscesses.

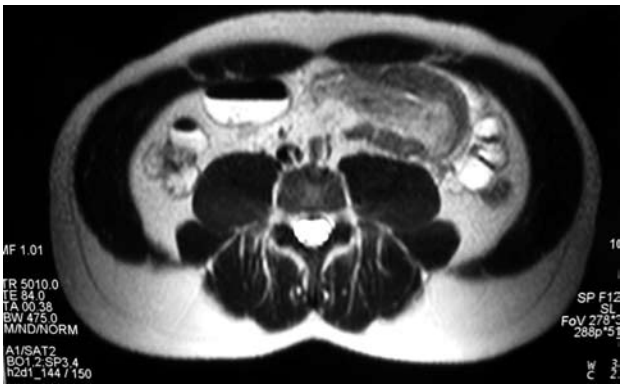

Figure 1 Abdominal MRI scan (Sagittal fast spin echo T2) revealing the jejunoileal intussusception.

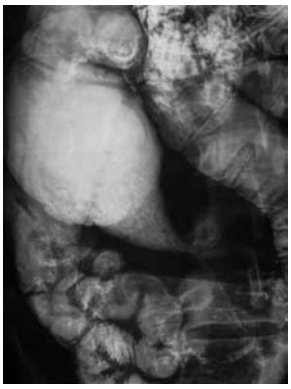

Figure 2 Enteroclysis showing jejunoileal stricture caused by invagination of the jejunum into the ileum and proximal to the stenosis bowel dilatation.

The continuity of the small bowel was restored with a side-to-side entero-enteral anastomosis. The patient, discharged on the 7th postoperative day, following an uncomplicated recovery, was subjected to follow-up with clinical examinations and CT scans at 6-mo intervals and had no clinical or radiologic recurrence at the time when we wrote this paper.

DISCUSSION

Tumors of the small intestine are rare and usually benign. The majority of them are leiomyomas, but adenocarcinomas and GISTs, although uncommon, may also appear. Conventional histologic methods could not produce correct diagnoses and most tumors referred to as leiomyomas and leiomyosarcomas in the older medical literature are actually GISTs. On the basis of immunohistochemical and ultrastructural studies, GISTs are mesenchymal tumors of the gastrointestinal tract that may show myogenic and/or neurogenic characteristics[5], and are usually asymptomatic, but they have been reported to present with bleeding, obstruction or perforation in some cases[6].

Intussusception is the invagination of one bowel loop and its mesentery into the bowel lumen distal to it. It occurs when a proximal segment of intestine (intussusceptum) telescopes into the intestinal segment distal to it (intussuscipiens), and is the second most common complication when tumors locate in the ileum[7]. Invaginations of the lumen may also cause gastrointestinal

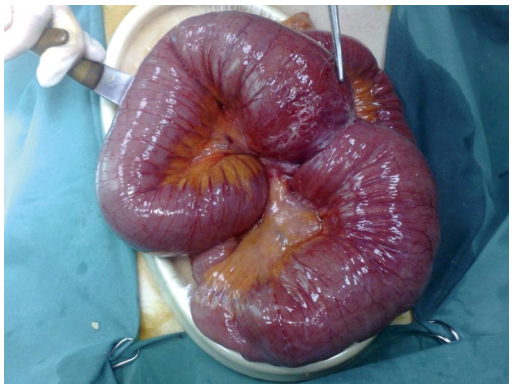

Figure 3 Intraoperative appearance of the jejunoileal intussusception.

bleeding or necrosis of the tumor. Usually patients have slightly enlarging tumors with no rapid onset. Their complaints are usually non specific and it is finally risky to miss the diagnosis. Our patient experienced vague abdominal pain for a period of 4 mo which led him to undergo a colonoscopy that came up normal.

Intussusception is correctly diagnosed preoperatively in only one-third of cases[7]. An accurate diagnosis of intussusception should include a good and specific medical history, a thorough physical examination, radiography, CT, MRI and enteroclysis or even an endoscopic ultrasound or capsule endoscopy. Ultrasound is useful in confirming obstruction and may sometimes identify the cause[8]. However, it is highly operator-dependent. Taking into account the rarity of such findings, it requires an experienced radiologist. In our case, ultrasound was not diagnostic of intussusception. It has been recently reported that CT and MRI offer great help in establishing the preoperative diagnosis of intussusception[9]. On CT, one can diagnose a bowel intussusception within its configuration, thickening of the wall and compressed mesenteric fat and vessels[9]. Although not routinely reported in such cases, an abdominal MRI, like in our report, can aid to establish the preoperative diagnostic suspicion of intussusception.

In contrast to childhood where intussusception is idiopathic in 90% of cases and the basic underlying cause of intussusception is the hypertrophy of Payer's patches activity, adult intussusception has a definable pathologic lesion in over 90% of cases, with neoplasms considered to be the cause in 65% of them[10]. Any intraluminal lesion, especially polyps, which irritates and alters normal peristaltic activity, is able to trigger an intraluminal invagination finally causing an intussusception. Subsequent peristaltic bowel activity produces an area of sequence constriction and relaxation, thus telescoping the leading point through the distal bowel lumen[11]. The malignancy is more likely to be located in the colon rather than in the small bowel. Less common etiologies of intussusception in adults include postoperative factors (adhesions, suture lines, *etc.*), polyps, Meckel's disease, sprue, cecal duplication and intramural hematoma[1]. The presentation of adult intussusception is usually subacute or chronic[2]. The most common symptoms are crampy abdominal pain, nausea, vomiting, abdominal distention

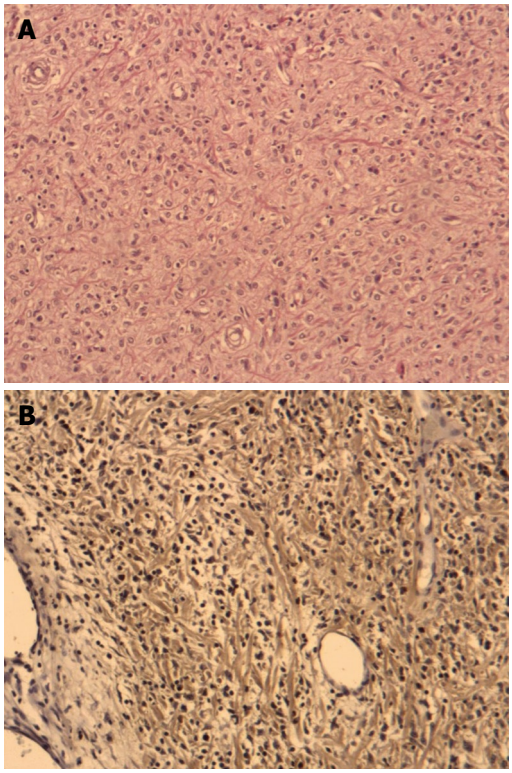

Figure 4 Histological examination (A) (HE, × 100) and c-kit immunohistochemical positive staining (B) (HE, × 40) of the gastrointestinal stromal tumor.

or tenderness. Only up to 20% of all cases present with complete bowel obstruction and acute onset[10]. Moreover, it has been reported that a palpable mass is present in 7%-42% of the cases[11].

In the literature, five cases of small bowel intussusception from a stromal tumor in adults have been described[5,6,10,12,13]. The most recent one was an intraluminal leiomyoma of the small intestine in a 72-year-old female patient, which resulted in invagination and partial obstruction of the jejunum[12]. Another recent report presented a case of a 60-year-old woman with a jejunoileal intussusception due to a myxoid stromal tumor[10]. Others described a GIST as the cause of intussusception in a 32-year-old man and emphasized the role of ultrasound in preoperative diagnosis of the disease[13]. In an older report[5], a case of a 42-year-old patient with intussusception of the lower jejunum was presented and the patient was admitted with progressive anemia, massive melena and lower abdominal pain. Finally, a malignant GIST of the small intestine causing small bowel obstruction due to ileal invagination has also been described[6].

Coincidence of Crohn's disease and gastrointestinal stromal tumor is extremely rare and only two cases have been reported to date[14,15]. The first one described a polypoid tumor within Meckel's diverticulum in an 81-year-old male patient with Crohn's disease and the tumor was immunohistochemically proved to be a GIST[14]. The second report presented a 51-year-old patient with a high risk GIST of the terminal ileum within an area of Crohn's ileitis[15].

Conclusively, in the present report we describe a case of a 42-year-old man with no significant medical history other than a 4-mo intermittent dull abdominal pain who presented with symptoms of acute bowel obstruction. Laparotomy revealed a jejunoileal intussusception and histological examination of the surgical specimen indicated a GIST tumor causing the intussusception as well as patchy areas of inflammation and erosions similar to those identified in Crohn's disease. Coexistence of these pathologically distinct and extremely rare entities has not been reported and represents a unique finding.

REFERENCES

1 **Akçay MN**, Polat M, Cadirci M, Gencer B. Tumor-induced ileo-ileal invagination in adults. *Am Surg* 1994; **60**: 980-981
2 **Tan KY**, Tan SM, Tan AG, Chen CY, Chng HC, Hoe MN. Adult intussusception: experience in Singapore. *ANZ J Surg* 2003; **73**: 1044-1047
3 **Nishida T**, Hirota S. Biological and clinical review of stromal tumors in the gastrointestinal tract. *Histol Histopathol* 2000; **15**: 1293-1301
4 **Kim CJ**, Day S, Yeh KA. Gastrointestinal stromal tumors: analysis of clinical and pathologic factors. *Am Surg* 2001; **67**: 135-137
5 **Hoshino N**, Murata T, Oka K, Kawakami K, Hoshino K, Sekoguchi S, Suga T, Nishimura A, Kato M, Hamada M. Gastrointestinal stromal tumors of the small intestine that expressed c-kit protein. *Intern Med* 2000; **39**: 914-919
6 **Occhionorelli S**, Mitaritonno M, Pennella A, Carella M, Presicce L, Pellegrini D, Fiore MG, Pollinzi Fonte V. Gastrointestinal stromal tumor (GIST): case report. *G Chir* 2001; **22**: 65-69
7 **Badreldina R**, Bowling T. Disorders of the small bowel. *Surgery* (Oxford) 2008; **26**: 338-342
8 **Nylund K**, Ødegaard S, Hausken T, Folvik G, Lied GA, Viola I, Hauser H, Gilja OH. Sonography of the small intestine. *World J Gastroenterol* 2009; **15**: 1319-1330
9 **Sundaram B**, Miller CN, Cohan RH, Schipper MJ, Francis IR. Can CT features be used to diagnose surgical adult bowel intussusceptions? *AJR Am J Roentgenol* 2009; **193**: 471-478
10 **Akçay MN**, Aydinli B, Gürsan N, Balik AA, Onbaş O, Oztürk G. Intussusception in an adult patient with mild intermittent abdominal pain. *Dig Dis Sci* 2005; **50**: 1127-1129
11 **Haas EM**, Etter EL, Ellis S, Taylor TV. Adult intussusception. *Am J Surg* 2003; **186**: 75-76
12 **Sunamak O**, Karabicak I, Aydemir I, Aydogan F, Guler E, Cetinkaya S, Korman MU. An intraluminal leiomyoma of the small intestine causing invagination and obstruction: a case report. *Mt Sinai J Med* 2006; **73**: 1079-1081
13 **Vijay S**, Dahiya N, Sudha P. Radiology for the surgeon: soft-tissue case 56. Small-bowel intussusception. *Can J Surg* 2004; **47**: 453-455
14 **Mijandrusić Sincić B**, Kovac D, Jasić M, Grbas H, Uravić M, Depolo A. Crohn's disease and a gastrointestinal stromal tumor in an 81-year-old man--a rare coincidence. *Zentralbl Chir* 2005; **130**: 597-599
15 **Pfeffel F**, Stiglbauer W, Depisch D, Oberhuber G, Raderer M, Scheithauer W. Coincidence of Crohn's disease and a high-risk gastrointestinal stromal tumor of the terminal ileum. *Digestion* 1999; **60**: 363-366

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CASE REPORT

A case of advanced intrahepatic cholangiocarcinoma successfully treated with chemosensitivity test-guided systemic chemotherapy

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Abstract

Intrahepatic cholangiocarcinoma (ICC) is a relatively rare and highly fatal neoplasm that arises from the biliary epithelium. Prognosis is generally poor and survival is limited to a few months. Here we present a case of advanced ICC successfully treated by chemosensitivity test-guided systemic chemotherapy combining S-1 and cisplatin (CDDP). A 65-year-old woman with a liver tumor was referred to our hospital on November 21, 2007. Abdominal ultrasonography and computed tomography (CT) showed low-density masses of 50 and 15 mm in diameter, respectively in segment Ⅷ of the liver and in the enlarged lymph node in the para-aorta. Ultrasonography-guided fine needle biopsy diagnosed the tumors as ICC. Since the patient was inoperable for lymph node metastasis, she underwent systemic chemotherapy with gemcitabine. Six months after initiation of chemotherapy, CT revealed ICC progression in the liver and pleural dissemination with pleural effusion. The patient was admitted to our hospital for anticancer drug sensitivity testing on June 9, 2008. Based on the sensitivity test results, we elected to administer systemic chemotherapy combining S-1 and CDDP. Two months into the second chemotherapy treatment, CT revealed a reduction of the tumors in the liver and lymph node and a decrease in pleural effusion. After eight cycles of the second chemotherapy, 17 mo after ICC diagnosis, she is alive and well with no sign of recurrence. We conclude that chemosensitivity testing may effectively determine the appropriate chemotherapy regimen for advanced ICC.

Key words: Chemosensitivity testing; Cholangiocarcinoma; Cisplatin; Liver neoplasms; Gemcitabine; S-1; Systemic chemotherapy

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Abe K, Wakatsuki T, Katsushima F, Monoe K, Kanno Y, Takahashi A, Yokokawa J, Ohira H. A case of advanced intrahepatic cholangiocarcinoma successfully treated with chemosensitivity test-guided systemic chemotherapy. *World J Gastroenterol* 2009; 15(41): 5228-5231 Available from: URL: http://www.wjgnet.com/1007-9327/15/5228.asp DOI: http://dx.doi.org/10.3748/wjg.15.5228

INTRODUCTION

Intrahepatic cholangiocarcinoma (ICC) is a relatively rare and highly fatal neoplasm that arises from the biliary epithelium. Radical surgery is currently the optimal therapy for ICC with curative potential. However, most patients present with advanced disease at the time of diagnosis. Prognosis in these patients is poor and survival is limited to a few months[1].

Biliary tract carcinoma (BTC) has traditionally been divided into cancers of the gallbladder, the extrahepatic bile ducts, ampulla of Vater, whereas ICC has been classified as liver cancer. Lately, however the term BTC has been used to include the gallbladder, the extrahepatic bile ducts, ICC and the ampulla of Vater.

Chemotherapy has been performed in cases of unresectable advanced ICC and postoperative recurrence of ICC. However, a standard chemotherapeutic regimen has not yet been established for ICC. There are phase Ⅱ

trials that support the following combinations: gemcitabine/cisplatin (CDDP), gemcitabine/oxaliplatin, gemcitabine/capecitabine, and 5-fluorouracil in unresectable or metastatic ICC[2].

Chemosensitivity testing using surgical material is an established method to evaluate tumor response prior to chemotherapy[3-5]. Sensitivity, specificity and accuracy of chemosensitivity testing were reportedly 82.7%, 70.7% and 73.6%, respectively[6]. Several methods are established to measure cancer cell viability[7]. Recently, chemosensitivity testing for gastric cancer treatment has been approved in Japan. However, it has seldom been performed for ICC, since ICC occurs more rarely than other gastrointestinal malignancies. The adenosine triphosphate (ATP) assay is a highly sensitive and precise method for measuring cell viability, and only a few dozen cells are necessary for the ATP assay[8,9]. Very few reports of chemosensitivity testing used surgical material from ICC. This is the first report concerning chemosensitivity testing using the ATP assay for patients with unresectable ICC. We present a case of advanced ICC successfully treated by chemosensitivity test-guided systemic chemotherapy combining S-1 and CDDP.

CASE REPORT

A 65-year-old woman was examined in a follow-up visit at a local hospital for fatty liver 1 year post-diagnosis on October 30, 2007. Although she exhibited no symptoms, abdominal ultrasonography revealed tumors in the liver, and she was referred to our hospital on November 21, 2007. Her past medical and family histories were not remarkable. She did not consume alcohol. On admission, her conjunctivae were not jaundiced, and heart and respiratory sounds were normal. The liver, spleen and tumor were not palpable.

Laboratory findings on admission were as follows: aspartate aminotransferase 24 IU/L (normal, 13-33 IU/L), alanine aminotransferase 39 IU/L (normal, 6-27 IU/L), γ-glutamyl transpeptidase 26 IU/L (normal, 10-47 IU/L) and alkaline phosphatase 240 IU/L (normal, 115-359 IU/L). Assays for hepatitis B surface antigen and hepatitis C virus antibody were negative. All tumor markers tested showed normal values: specifically, 1.8 ng/mL for CEA (criterion, < 5.0), 5.8 U/mL for CA 19-9 (criterion, < 37), 6.2 ng/mL for AFP (criterion, < 10.0), 10 mAU/mL for PIVKAII (criterion, < 40).

Abdominal ultrasound and computed tomography (CT) scan with contrast enhancement revealed a low-density mass, measuring 50 and 15 mm in diameter, in segment Ⅷ of the liver. The tumor did not show enhancement during the arterial phase but did show peripheral rim enhancement during the portal phase. The CT scan also revealed an enlarged lymph node in the para-aorta (Figure 1). The portal vein was intact until the third branch. Chest X-ray and a CT scan revealed no pleural metastasis.

Ultrasonography-guided fine needle biopsy was performed at the main tumor of the liver on November 22, 2007. Microscopic examinations showed tubular adenocarcinoma (Figure 2). Gastroendoscopy and colonoscopy did not show any other malignancy. Subsequently, the tumor was diagnosed as ICC with intrahepatic metastasis and lymph node metastasis. Since the patient was inoperable for lymph nodes metastasis, she underwent systemic chemotherapy with gemcitabine (800 mg/m^2, 30 min iv infusion), which was administered on days 1, 8 and 15, and repeated every 4 wk. Six months after initiation of chemotherapy, CT revealed ICC progression in the liver and pleural dissemination with pleural effusion (Figures 3 and 4). The patient was admitted to our hospital for anticancer drug sensitivity testing on June 9, 2008. Cultures and ATP assays were performed as described previously[5]. Cell viability was evaluated by measuring the intracellular ATP level using bioluminescence as described by Kangas *et al*[10]. Based on the sensitivity test results, we elected to administer systemic chemotherapy combining S-1 and CDDP (S-1 80 mg/m^2 per day orally administered for 3 wk,

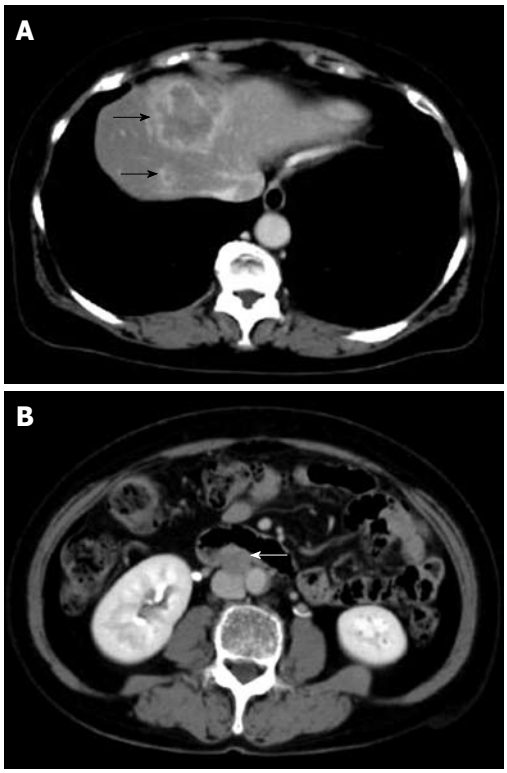

Figure 1 Computed tomography (CT) of the tumor on first admission. CT shows a low-density lesion with rim enhancement in segment Ⅷ of the liver (black arrows) (A) and enlarged lymph node in the para-aorta (white arrow) (B).

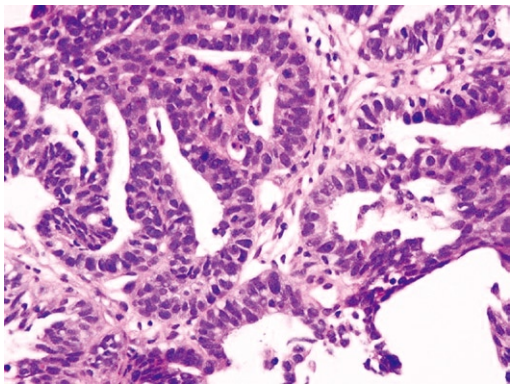

Figure 2 Hematoxylin-eosin staining. Cuboidal cancer cells with chromatin-rich nuclei had proliferated invasively, forming indistinct glandular structures.

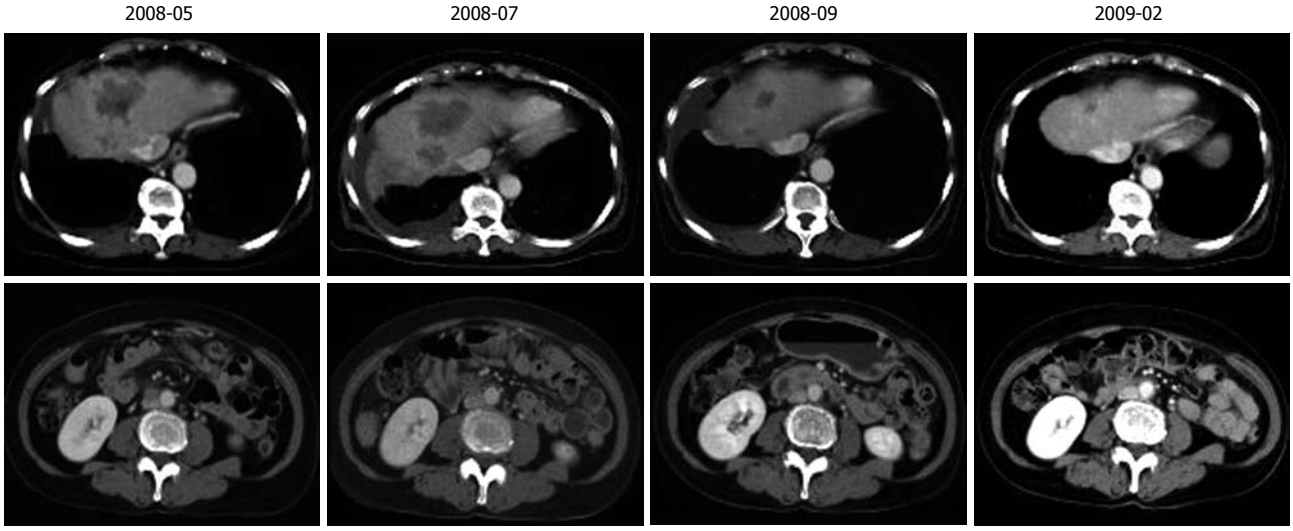

Figure 3 Reduction in tumors in the liver and lymph node following chemotherapy.

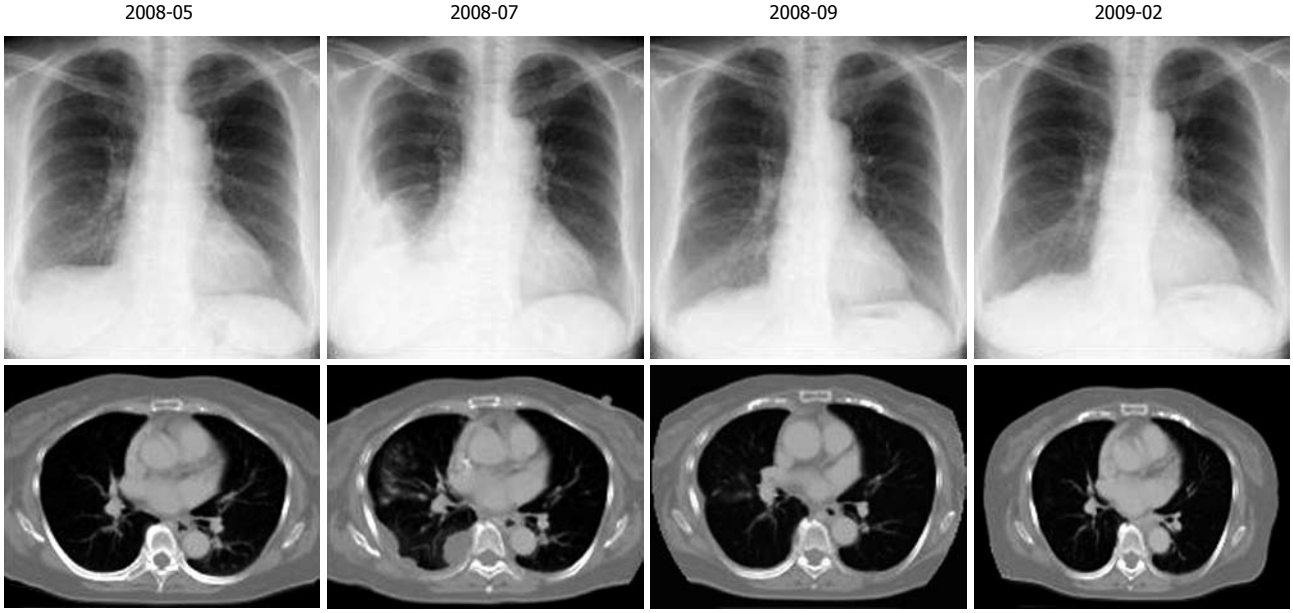

Figure 4 Decreased pleural metastases following chemotherapy.

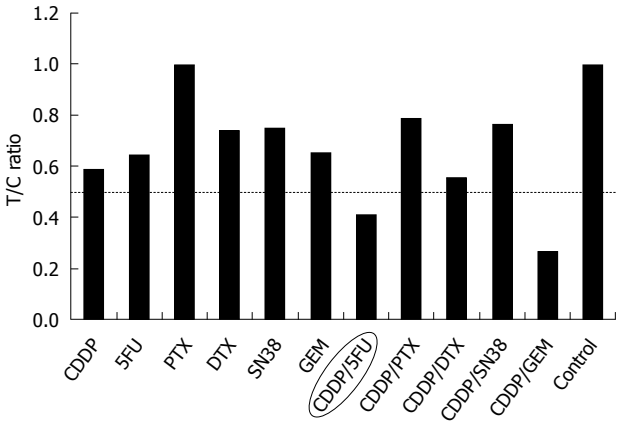

Figure 5 Cell viability evaluated by measuring the intracellular ATP level using bioluminescence. The T/C (treated/control) ratio, or the ratio of ATP quantity of a tumor sample treated with anticancer drugs to that of the control, was used as the index of chemosensitivity.

CDDP 60 mg/m^2 iv infusion administered on day 8, and repeated every 5 wk) (Figure 5). She had symptoms of cough in July 2008. Two months into the second chemotherapy treatment, she had no symptoms and CT revealed a reduction in the tumors of the liver and lymph node and a decrease in pleural effusion (Figures 3 and 4). Side effects and complications of chemotherapy were tolerable, and she experienced only grade-1 nausea. CDDP added to S-1 might worsen nausea. After eight cycles of the second chemotherapy regime, 17 mo after ICC diagnosis, she is alive and well with no sign of recurrence.

DISCUSSION

Although radical surgery is considered the most effective therapy for ICC, only 20% of patients present with resectable disease[11]. Without surgery, ICC is a rapidly fatal disease with a 5-year survival rate of less than 5%[12], while

in curative resections the 5-year survival rate approaches 20%-35% in cases with negative surgical margins[13]. In the present case, to our surprise, the patient with unresectable ICC survived more than 17 mo with chemotherapy. The role of systemic chemotherapy in unresectable ICC is undefined. Although chemotherapy has been reported to be more beneficial than the best supportive care[14], no standard chemotherapy regimen has yet been identified. Most promising approaches involve the use of single agents such as gemcitabine, which has been shown to be an effective therapy for BTC in phase Ⅱ trials[15,16]. Response rates for gemcitabine ranged from 8% to 36% and overall survival times from 6.3 to 16 mo. S-1 is a novel oral fluoropyrimidine agent, which contains tegafur, gimeracil and oteracil potassium. Gimeracil is a competitive inhibitor of dihydropyrimidine dehydrogenase and achieves higher concentrations of 5-fluorouracil in plasma and tumor tissues[17]. Fluoropyrimidines have known synergistic effects with CDDP[18], and combinations of S-1 and CDDP are reportedly effective therapies for patients with advanced BTC in phase Ⅱ trials[19]. In the present case, we selected gemcitabine as first line chemotherapy, because gemcitabine has been extensively evaluated in patients with metastatic BTC.

Chemosensitivity testing using surgical material is an established method for evaluation of tumor response prior to chemotherapy. Several methods have been established for measurement of cancer cell viability. However, it has seldom been performed in ICC, since ICC occurs more rarely than gastrointestinal malignancies and the prognosis of ICC is poor. There have been few reports of chemosensitivity testing using surgical material for ICC without the ATP assay[20-22]. The ATP assay is a highly sensitive and precise method used to measure cell viability. The ATP assay has been shown to be a good predictor of response to chemotherapy in other tissue types, such as breast cancer, ovarian cancer, colorectal adenocarcinoma, melanoma and lung cancer. The assay predicts that anticancer drugs with a lower treated/control (T/C) ratio (< 0.6) are more sensitive. In the present case, based on the results of chemosensitivity testing, we selected systemic chemotherapy combining S-1 and CDDP. Ultimately, *in vitro* ATP chemosensitivity testing was useful. Since 5-fluorouracil has considerable toxic effects and entails the inconvenience of continuous iv infusions, we selected S-1 as an alternative to 5-fluorouracil. Although the T/C ratio combining gemcitabine and CDDP was more sensitive than that combining S-1 and CDDP, we did not select the former combination since gemcitabine administration on its own did not halt disease progression.

REFERENCES

1 **Sirica AE**. Cholangiocarcinoma: molecular targeting strategies for chemoprevention and therapy. *Hepatology* 2005; **41**: 5-15
2 **Hezel AF**, Zhu AX. Systemic therapy for biliary tract cancers. *Oncologist* 2008; **13**: 415-423
3 **Furukawa T**, Kubota T, Suto A, Takahara T, Yamaguchi H, Takeuchi T, Kase S, Kodaira S, Ishibiki K, Kitajima M. Clinical usefulness of chemosensitivity testing using the MTT assay. *J Surg Oncol* 1991; **48**: 188-193
4 **Yamaue H**, Tanimura H, Noguchi K, Hotta T, Tani M, Tsunoda T, Iwahashi M, Tamai M, Iwakura S. Chemosensitivity testing of fresh human gastric cancer with highly purified tumour cells using the MTT assay. *Br J Cancer* 1992; **66**: 794-799
5 **Kawamura H**, Ikeda K, Takiyama I, Terashima M. The usefulness of the ATP assay with serum-free culture for chemosensitivity testing of gastrointestinal cancer. *Eur J Cancer* 1997; **33**: 960-966
6 **Kondo T**, Kubota T, Tanimura H, Yamaue H, Akiyama S, Maehara Y, Tanigawa N, Kitajima M, Takagi H. Cumulative results of chemosensitivity tests for antitumor agents in Japan. Japan Research Society for Appropriate Cancer Chemotherapy. *Anticancer Res* 2000; **20**: 2389-2392
7 **Carmichael J**, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of radiosensitivity. *Cancer Res* 1987; **47**: 943-946
8 **Crouch SP**, Kozlowski R, Slater KJ, Fletcher J. The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. *J Immunol Methods* 1993; **160**: 81-88
9 **Andreotti PE**, Cree IA, Kurbacher CM, Hartmann DM, Linder D, Harel G, Gleiberman I, Caruso PA, Ricks SH, Untch M. Chemosensitivity testing of human tumors using a microplate adenosine triphosphate luminescence assay: clinical correlation for cisplatin resistance of ovarian carcinoma. *Cancer Res* 1995; **55**: 5276-5282
10 **Kangas L**, Grönroos M, Nieminen AL. Bioluminescence of cellular ATP: a new method for evaluating cytotoxic agents in vitro. *Med Biol* 1984; **62**: 338-343
11 **Jarnagin WR**, Fong Y, DeMatteo RP, Gonen M, Burke EC, Bodniewicz BS J, Youssef BA M, Klimstra D, Blumgart LH. Staging, resectability, and outcome in 225 patients with hilar cholangiocarcinoma. *Ann Surg* 2001; **234**: 507-517; discussion 517-519
12 **Farley DR**, Weaver AL, Nagorney DM. "Natural history" of unresected cholangiocarcinoma: patient outcome after noncurative intervention. *Mayo Clin Proc* 1995; **70**: 425-429
13 **Patel T**, Singh P. Cholangiocarcinoma: emerging approaches to a challenging cancer. *Curr Opin Gastroenterol* 2007; **23**: 317-323
14 **Glimelius B**, Hoffman K, Sjödén PO, Jacobsson G, Sellström H, Enander LK, Linné T, Svensson C. Chemotherapy improves survival and quality of life in advanced pancreatic and biliary cancer. *Ann Oncol* 1996; **7**: 593-600
15 **Penz M**, Kornek GV, Raderer M, Ulrich-Pur H, Fiebiger W, Lenauer A, Depisch D, Krauss G, Schneeweiss B, Scheithauer W. Phase II trial of two-weekly gemcitabine in patients with advanced biliary tract cancer. *Ann Oncol* 2001; **12**: 183-186
16 **Tsavaris N**, Kosmas C, Gouveris P, Gennatas K, Polyzos A, Mouratidou D, Tsipras H, Margaris H, Papastratis G, Tzima E, Papadoniou N, Karatzas G, Papalambros E. Weekly gemcitabine for the treatment of biliary tract and gallbladder cancer. *Invest New Drugs* 2004; **22**: 193-198
17 **Ueno H**, Okusaka T, Ikeda M, Takezako Y, Morizane C. Phase II study of S-1 in patients with advanced biliary tract cancer. *Br J Cancer* 2004; **91**: 1769-1774
18 **Scanlon KJ**, Newman EM, Lu Y, Priest DG. Biochemical basis for cisplatin and 5-fluorouracil synergism in human ovarian carcinoma cells. *Proc Natl Acad Sci USA* 1986; **83**: 8923-8925
19 **Kim YJ**, Im SA, Kim HG, Oh SY, Lee KW, Choi IS, Oh DY, Lee SH, Kim JH, Kim DW, Kim TY, Kim SW, Heo DS, Yoon YB, Bang YJ. A phase II trial of S-1 and cisplatin in patients with metastatic or relapsed biliary tract cancer. *Ann Oncol* 2008; **19**: 99-103
20 **Shimada M**, Takenaka K, Kawahara N, Yamamoto K, Shirabe K, Maehara Y, Sugimachi K. Chemosensitivity in primary liver cancers: evaluation of the correlation between chemosensitivity and clinicopathological factors. *Hepatogastroenterology* 1996; **43**: 1159-1164
21 **Ieta K**, Tanaka F, Utsunomiya T, Kuwano H, Mori M. CEACAM6 gene expression in intrahepatic cholangiocarcinoma. *Br J Cancer* 2006; **95**: 532-540
22 **He XR**, Wu XP. Difference in biological characteristics and sensitivity to chemotherapy and radiotherapy between intrahepatic and extrahepatic cholangiocarcinoma cells in vitro. *Chin Med Sci J* 2008; **23**: 54-59

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CASE REPORT

Recurrent massive bleeding due to dissecting intramural hematoma of the esophagus: Treatment with therapeutic angiography

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Abstract

Spontaneous or traumatic intramural bleeding of the esophagus, which is often associated with overlying mucosal dissection, constitutes a rare spectrum of esophageal injury called dissecting intramural hematoma of the esophagus (DIHE). Chest pain, swallowing difficulty, and minor hematemesis are common, which resolve spontaneously in most cases. This case report describes a patient with spontaneous DIHE with recurrent massive bleeding which required critical management and highlights a potential role for therapeutic angiography as an alternative to surgery.

Key words: Esophagus; Intramural hematoma; Therapeutic angiography

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INTRODUCTION

Dissecting intramural hematoma of the esophagus (DIHE) is a rare but well-known esophageal injury[1,2]. It is characterized by a concentric or eccentric intramural hematoma associated with dissection of the esophageal wall[2]. Forceful vomiting, mechanical insult, and underlying coagulopathy are common causes. It can also occur spontaneously without any evident cause[3]. DIHE is considered a benign disease. The hemorrhage from DIHE does not have clinically significant consequences, although in rare cases massive bleeding with hypovolemic shock can occur. We report a case of DIHE presenting as recurrent massive intraluminal bleeding that was treated by transarterial embolization.

CASE REPORT

A 57-year-old man visited the emergency room complaining of a sore throat and difficulty swallowing for 5 d. He had a history of alcoholic liver cirrhosis (Child-Pugh class B), but he continued drinking. Laryngoscopy and cervical computed tomography (CT) revealed severe laryngopharyngitis. Upper gastrointestinal endoscopy showed marked laryngeal edema and a small ulceration in the upper esophageal sphincter. There was no intraluminal mass, except for small varices in the distal esophagus. The gastric mucosa was moderately congested. The patient denied any history of cervical trauma or instrumentation. He was treated medically with antibiotics, and his initial symptoms subsided within 5 d.

One week later, the patient complained that he regurgitated blood from the throat. He continued to spit up small volumes of fresh blood repeatedly. His hemoglobin dropped from 13.9 to 6.5 g/dL, and 4 U of packed red cells were transfused. Emergency endoscopy revealed a small opening in the cervical esophagus and

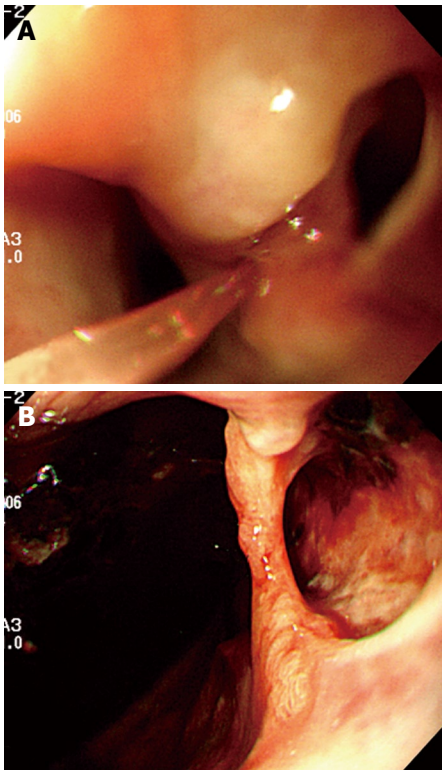

Figure 1 Endoscopic view. A: A small opening in the cervical esophagus; B: Mucosal bridging with a large mucosal defect in the esophagogastric junction.

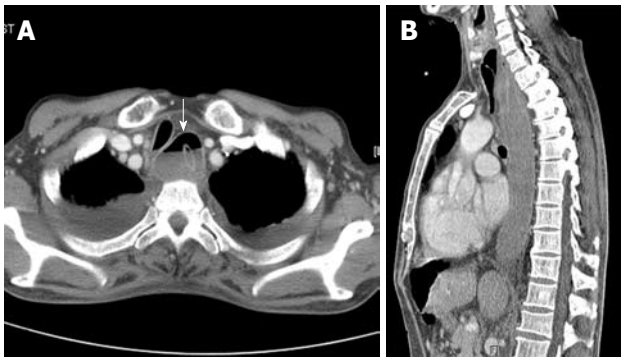

Figure 2 Chest CT images. A: The transverse view of the proximal esophagus shows a concentric intramural hematoma and mucosal dissection with an air-fluid level in the false lumen (arrow). Bilateral pleural effusions are seen; B: The sagittal view shows an extensive intramural hematoma of the esophagus.

mucosal bridging with a large mucosal defect around the esophagogastric junction (Figure 1). Active bleeding was detected from a vessel exposed on the ulcer base in the cardia. After hemostasis with endoscopic clipping, his vital signs and hemoglobin stabilized. Chest CT performed because of the esophageal lesions revealed dissection of the wall and a large circumferential intramural hematoma in the esophagus (Figure 2). Based on the endoscopy and chest CT findings, DIHE with cardiac ulcer bleeding was diagnosed. The patient was treated conservatively without oral intake.

Eight days later, the hematemesis recurred. Conservative treatment with massive transfusion of packed red cells and fresh frozen plasma was ineffective. Endoscopy showed no bleeding at the previously treated site. However, continuous oozing of blood from the distal esophagus was observed. His vital signs were unstable.

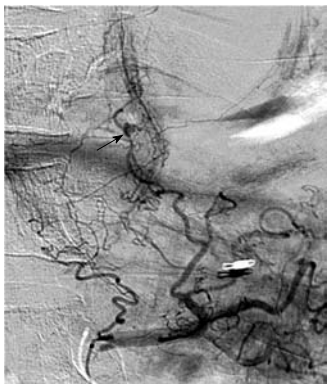

Figure 3 Celiac angiography showing a hyperstaining pseudoaneurysmal lesion (arrow) in an esophageal branch of the left gastric artery. The branch was embolized using glue and lipiodol.

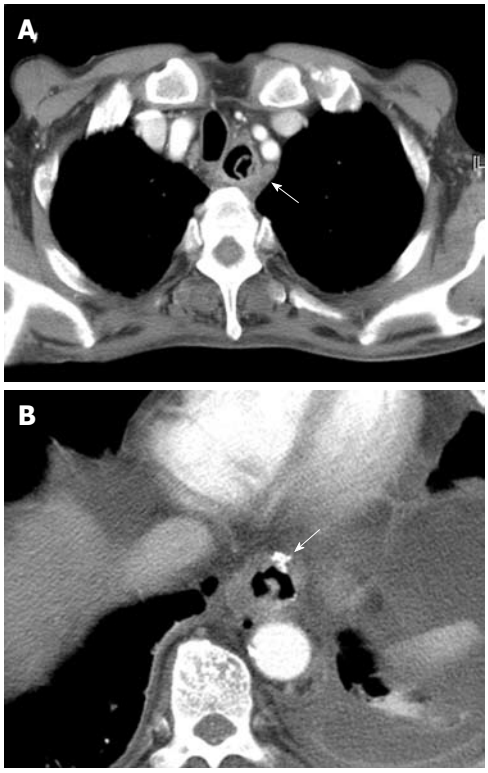

Figure 4 Follow-up chest CT. A: The transverse view of the proximal esophagus showing a resolved intramural hematoma and improved dissection (arrow); B: The embolized lesion is seen as focal lipiodol uptake in the distal esophagus (arrow).

Since surgery was very risky due to underlying liver cirrhosis, less invasive celiac angiography was performed for hemostasis. A small hyperstaining pseudoaneurysmal lesion was observed at an esophageal branch of the left gastric artery (Figure 3). The branch was embolized using glue and lipiodol. After the procedure, the patient's vital signs and hemoglobin stabilized. However, he experienced a few bouts of minor hematemesis 10 d later. Transarterial embolization using glue and coils was reattempted, and no further bleeding occurred. Follow-up chest CT 4 wk later showed a resolving hematoma and double lumens separated by the dissected mucosa. The embolization site was seen as focal lipiodol uptake in the distal esophagus (Figure 4). Endoscopic examination

Table 1 Summary of 11 patients with dissecting intramural hematoma of the esophagus presenting major hemorrhage

Ref.	Sex/age (yr)	Underlying diseases	Precipitating factor	Primary symptoms	Treatment	Outcome
Atefi *et al*[9]	M/29	Diabetes Pneumonia Hemodialysis	Retching	Hematemesis	Conservative	Died
Kerr[15]	F/72	Hypertension	None	Epigastric pain	Conservative	Resolved
Ortiz[16]	F/80	None	Food (rice)	Chest pain, dysphagia	Surgical	Died
Freeman *et al*[14]	F/59	None	None	Chest pain	Conservative	Resolved
de Vries *et al*[12]	M/88	None	Minor head trauma	Retrosternal pain	Conservative	Resolved
Folan *et al*[13]	M/58	Alcoholism	Vegetable (broccoli)	Dysphagia	Surgical	Resolved
Takaoka *et al*[17]	F/87	Suppurative cholangitis	Nasobiliary catheter	Hematemesis	Conservative	Resolved
Cullen *et al*[11]	F/74	Hypertension	Heparin IV	Chest pain, dysphagia	Conservative	Resolved
Yamashita *et al*[18]	F/67	Cerebral aneurysm	Heparin IV Retching	Hematemesis	Conservative	Resolved
Bandyopadhyay *et al*[10]	M/86	Hypertension Ischemic heart disease	Warfarin	Hematemesis	Conservative	Died
Present case	M/57	Liver cirrhosis	None	Hematemesis	Therapeutic angiography	Resolved

was declined. Supportive care with parenteral nutrition was maintained. The patient was discharged without complications after 6 wk.

DISCUSSION

The esophagus is susceptible to various extrinsic injuries (from ingested foods, instruments, and bougienage) or intrinsic sheering forces induced by retching, vomiting, or coughing. DIHE lies in the spectrum of esophageal injuries between a mucosal tear (Mallory-Weiss syndrome) and a transmural laceration (Boerhaave's syndrome)[2,4]. Although these syndromes are usually associated with severe vomiting, DIHE is not always caused by emesis[4]. One out of five patients reports no history of trauma. However, an underlying coagulopathy is found in many patients with so-called spontaneous DIHE[1]. Portal hypertension and endoscopic variceal sclerotherapy are also associated with DIHE in cirrhotic patients[5]. Although the direct cause was not clear in this present case, the cervical esophageal ulcer and underlying portal hypertension may have been precipitating factors.

Acute chest pain is a common presenting symptom and should be differentiated from acute myocardial infarction and aortic dissection[6]. Hematemesis and difficulty swallowing may ensue, and these are helpful for differentiating from other critical diseases. The typical triad of DIHE (chest pain, dysphagia, and hematemesis) is evident only in one third of cases[1]. Therefore, the rarity of DIHE and atypical symptoms can delay correct diagnosis. In our case, repeated hematemesis with hypovolemia was the only clinical feature.

DIHE is generally benign. Most patients recover fully with conservative management[1]. Esophageal obstruction and major bleeding constitute two major complications of DIHE. Esophageal obstruction by the hematoma may cause or aggravate difficulty swallowing. Successful endoscopic decompression or surgical treatment have been reported in such cases[7,8].

A major intraluminal hemorrhage seems to be caused by overlying mucosal rupture and evacuation of the hematoma, however, the exact mechanism remains unclear. Although half of patients are reported to experience hematemesis, the volume of bleeding is usually small, and major bleeding with hypovolemic shock is uncommon.

We reviewed 119 patients who were presented in 87 English-language reports since 1968. Major bleeding was defined as more than 500 mL hematemesis with hypovolemic shock or bleeding that required transfusion of at least 4 U. Eleven cases (9.2%), including the present case, were collected and summarized in Table 1[9-18]. Seven cases were elderly patients (five females and two males). The bleeding was attributed to anticoagulation therapy in three cases[10,11,18]. The majority were treated conservatively. However, this type of treatment was not always effective. An elderly patient who was treated conservatively died after 5 d[10]. Two patients were treated with surgery[13,16]; one of them had a 5-cm longitudinal tear with a pulsatile bleeding vessel between 25 and 30 cm from the alveolar ridge on endoscopy, and the active arterial bleeder was identified and treated with an emergency thoracotomy[13]. In our case, angiography located and treated the bleeding site at an esophageal branch of the left gastric artery. Extensive DIHE and major bleeding might be caused by a small bleeding artery. In this situation, therapeutic angiography may be effective.

Therapeutic angiography has been used to control major non-variceal gastrointestinal bleeding and has been reported to be safe, effective, and durable[19]. It is usually considered when endoscopic therapy has failed or when emergency surgery carries a high risk of mortality. It has also been effective in the hemostasis of uncontrolled Mallory-Weiss syndrome[20] and in the treatment of a giant gastric intramural hematoma[21].

In conclusion, massive intraluminal bleeding can be a complication of DIHE. Although most bleeding in DIHE can be managed medically, prompt, appropriate treatment should be attempted in hemodynamically unstable patients. In such cases, therapeutic angiography may be a useful treatment alternative to surgery.

REFERENCES

1 **Cullen SN**, McIntyre AS. Dissecting intramural haematoma of the oesophagus. *Eur J Gastroenterol Hepatol* 2000; **12**: 1151-1162
2 **Restrepo CS**, Lemos DF, Ocazionez D, Moncada R, Gimenez CR. Intramural hematoma of the esophagus: a pictorial essay. *Emerg Radiol* 2008; **15**: 13-22
3 **Sanaka M**, Kuyama Y, Hirama S, Nagayama R, Tanaka H, Yamanaka M. Spontaneous intramural hematoma localized in the proximal esophagus: truly "spontaneous"? *J Clin Gastroenterol* 1998; **27**: 265-266
4 **Hiller N**, Zagal I, Hadas-Halpern I. Spontaneous intramural hematoma of the esophagus. *Am J Gastroenterol* 1999; **94**: 2282-2284
5 **Low DE**, Patterson DJ. Complete esophageal obstruction secondary to dissecting intramural hematoma after endoscopic variceal sclerotherapy. *Am J Gastroenterol* 1988; **83**: 435-438
6 **Ho CL**, Young TH, Yu CY, Chao YC. Intramural hematoma of the esophagus: ED diagnosis and treatment. *Am J Emerg Med* 1997; **15**: 322-323
7 **K C S**, Kouzu T, Matsutani S, Hishikawa E, Saisho H. Early endoscopic treatment of intramural hematoma of the esophagus. *Gastrointest Endosc* 2003; **58**: 297-301
8 **Kamphuis AG**, Baur CH, Freling NJ. Intramural hematoma of the esophagus: appearance on magnetic resonance imaging. *Magn Reson Imaging* 1995; **13**: 1037-1042
9 **Atefi D**, Horney JT, Eaton SB, Shulman M, Whaley W, Galambos JT. Spontaneous intramural of hematoma of esophagus. *Gastrointest Endosc* 1978; **24**: 172-174
10 **Bandyopadhyay P**, Patel CU. Spontaneous intramural dissection of the oesophagus. *Int J Clin Pract* 2001; **55**: 564-566
11 **Cullen SN**, Chapman RW. Dissecting intramural haematoma of the oesophagus exacerbated by heparin therapy. *QJM* 1999; **92**: 123-124
12 **de Vries RA**, Kremer-Schneider MM, Otten MH. Intramural hematoma of the esophagus caused by minor head injury 6 hours previously. *Gastrointest Radiol* 1991; **16**: 283-285
13 **Folan RD**, Smith RE, Head JM. Esophageal hematoma and tear requiring emergency surgical intervention. A case report and literature review. *Dig Dis Sci* 1992; **37**: 1918-1921
14 **Freeman AH**, Dickinson RJ. Spontaneous intramural oesophageal haematoma. *Clin Radiol* 1988; **39**: 628-634
15 **Kerr WF**. Spontaneous intramural rupture and intramural haematoma of the oesophagus. *Thorax* 1980; **35**: 890-897
16 **Ortiz H**. Intramural rupture and intramural haematoma of the oesophagus. *Br J Surg* 1986; **73**: 595
17 **Takaoka M**, Kubota Y, Yamaguchi T, Fujimura K, Tani K, Ogura M, Tsuji K, Itoh T, Irie T, Mizuno T. Intramural rupture of the esophagus: a rare complication associated with nasobiliary catheter placement. *Intern Med* 1993; **32**: 185-188
18 **Yamashita K**, Okuda H, Fukushima H, Arimura Y, Endo T, Imai K. A case of intramural esophageal hematoma: complication of anticoagulation with heparin. *Gastrointest Endosc* 2000; **52**: 559-561
19 **Aina R**, Oliva VL, Therasse E, Perreault P, Bui BT, Dufresne MP, Soulez G. Arterial embolotherapy for upper gastrointestinal hemorrhage: outcome assessment. *J Vasc Interv Radiol* 2001; **12**: 195-200
20 **Carsen GM**, Casarella WJ, Spiegel RM. Transcatheter embolization for treatment of Mallory-Weiss tears of the esophagogastric junction. *Radiology* 1978; **128**: 309-313
21 **Imaizumi H**, Mitsuhashi T, Hirata M, Aizaki T, Nishimaki H, Soma K, Ohwada T, Saigenji K. A giant intramural gastric hematoma successfully treated by transcatheter arterial embolization. *Intern Med* 2000; **39**: 231-234

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CASE REPORT

Successful endoscopic removal of a giant upper esophageal inflammatory fibrous polyp

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Abstract

Giant esophageal inflammatory fibrous polyp (especially > 17 cm in size) is seen rarely. Endoscopic removal has been reported rarely because the procedure is technically demanding and the hemostasis is difficult to ascertain. Here, we describe a case of a giant upper esophageal inflammatory fibrous polyp that was resected successfully by endoscopy.

Key words: Digestive system endoscopic surgery; Polyps; Endosonography; Esophageal neoplasms; Hemostasis; Endoscopic; Middle aged

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INTRODUCTION

Giant esophageal inflammatory fibrous polyp (especially > 17 cm in size) is seen rarely[1]. Surgical excision is usually advised[2-5]. Endoscopic removal has been reported rarely[6] because the procedure is technically demanding and hemostasis is difficult to ascertain. Here, we described a giant upper esophageal inflammatory fibrous polyp that was resected successfully by endoscopy.

CASE REPORT

A 50-year-old man presented to our hospital with a 6-mo history of progressive difficulty in swallowing, initially, solid and then liquid food for the past 2 mo. Physical examination and blood tests, including coagulation parameters, were normal. Barium swallow revealed a 17-cm polypoid filling defect inside the esophagus that moved with deglutition (Figure 1). Contrast thoracic computed tomography (CT) revealed a localized isodense upper esophageal lesion, which was free from the lower part of the esophagus. The esophageal wall was intact and there was no evidence of infiltration into adjacent organs or any mediastinal lymphadenopathy. Gastroscopy showed a giant polyp with smooth overlying mucosa, extending from 18 to 35 cm (measured from the incisors), and endoscopic ultrasonography (EUS) revealed well-distributed, low-echo-level lesions at the root of the polyp, excluding echoless lumen-like structures (no large blood vessels inside the lesions) (Figure 1). Therefore, the esophageal polyp was a benign lesion. Endoscopic removal of the polyp with an electrosurgical snare was attempted, using the ICC 200 (ERBE, Tubingen, Germany). The procedure was performed under general anesthesia with elective intubation and airway protection. Equipment for hemostasis, including hemoclips, Coagrasper, injection needle and adrenaline for injection was prepared before the procedure.

Firstly, we inserted a detachable loop of appropriate stiffness and size through the biopsy channel. Then, the detachable loop was placed from the base to the neck of the esophageal polyp. The loop was closed gradually and the polyp was lifted away from the esophageal wall. Polypectomy was performed and air was inflated to distend the esophagus. The setting of the electrosurgical units was as follows: COAG mode (effect 350 W) was first applied for 2 s, so that the small blood vessels over

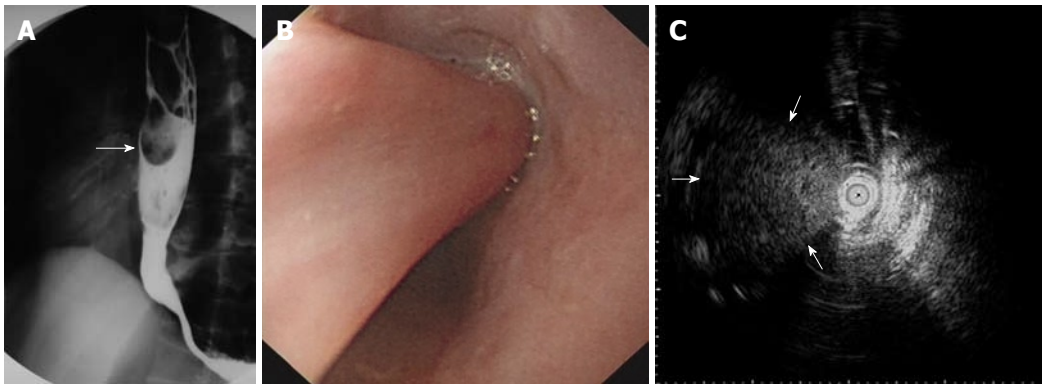

Figure 1 Imaging of esophageal polyp. A: Barium swallow showed a giant esophageal polyp (arrow), which was free from the middle and lower esophageal wall; B: Gastroscopy showed the polyp with smooth overlying mucosa (at 18 cm from the incisors); C: EUS revealed well-distributed, low-echo-level lesions at root of the polyp (white arrows), excluding echoless lumen-like structures.

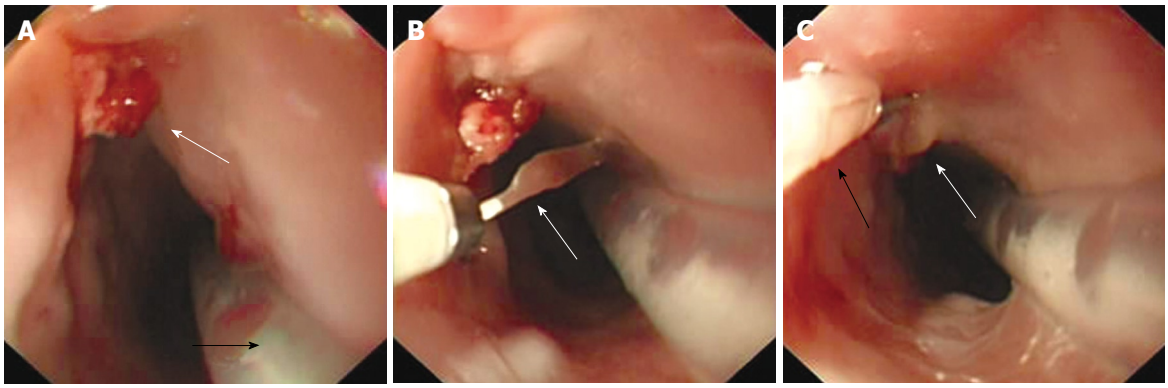

Figure 2 Esophageal polyp removal. A: Residual polyp (white arrow) after polypectomy at the left wall of the esophagus (18 cm from incisor). A nasogastric tube (black arrow) used for suction (size 16 Fr) was placed over the right wall of the esophagus; B: Hemoclips (white arrow) were applied to the residual polyp; C: After application of the hemoclips (black arrow) to the residual polyp (white arrow).

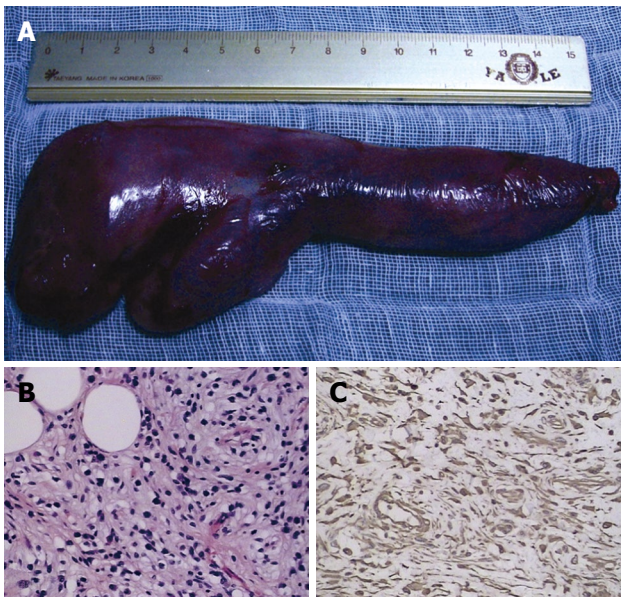

Figure 3 Polypectomy specimen and its pathology. A: The resected esophageal lesion measured 17 cm in length, and the neck, body and tail of the lesion measured 1, 3 and 5 cm, respectively; B: HE staining (original magnification, × 200) showed the presence of lots of fibroblasts, with some acidophilic cells, plasma cells and adipose cells; C: Immunostaining (original magnification, × 200) for vimentin showed strong staining of the cells.

the mucosa were coagulated. Then, we employed the endo-cut mode (effect 380 W) to CUT. Near the end of polypectomy, COAG mode was resumed to minimize bleeding from the central blood vessel. Adequate air insufflation and distension of the esophagus minimized the risk of burning and esophageal perforation. Only minor bleeding was noted at the polypectomy site. The hemostasis was achieved securely with 1:10 000 adrenaline injection and application of two hemoclips over the polypectomy site (Figure 2).

In addition, a 16-Fr nasogastric tube was placed to remove all the blood and gastric fluid during the procedure (Figure 2). If the esophageal polyp failed to be removed or there was any complication, an on-site thoracic surgeon assessed the patient urgently for operation.

The resected esophageal polyp specimen (Figure 3) was retrieved with the snare and sent to the pathology laboratory for pathological examination. Hematoxylin and eosin (HE) staining of the removed esophageal specimen showed the presence of lots of fibroblasts, with some adipocytes, plasma cells and acidophilic cells (Figure 3). Immunostaining revealed CD 117 (-), CD 34 (-), S-100 (-), desmin (-), actin (-), cytokeratin (-), HMB45 (-) and vimentin (+) (Figure 3). The histological diagnosis was

inflammatory fibrous polyp. The patient remained well and was discharged 1 wk after the procedure. Follow-up gastroscopy 4 wk later was normal.

DISCUSSION

Inflammatory fibrous polyp, also known as inflammatory pseudotumor, is a benign intraluminal tumor that consists of a mixture of inflamed fibrous, granulation tissue and lipomatous elements, which is covered by normal squamous epithelium[7]. Giant esophageal inflammatory fibrous polyp (especially > 17 cm in size) is seen rarely[1,8]. The presenting symptoms of dysphagia and sensation of a mass in esophageal inflammatory fibrous polyp are the same as in other esophageal tumors (such as leiomyoma and gastrointestinal stromal tumor), unless there is development of regurgitation of the polyp through the mouth or asphyxiation[9]. Some cases may stay asymptomatic or with heartburn for a long time[10,11]. Inflammatory fibroid polyps should be considered in the differential diagnosis of submucosal and polypoid esophageal masses, although distinctive radiographic features are not found[12]. Usually, the diagnosis is made by imaging or endoscopic studies. Barium-enhanced contrast of the esophagus usually shows a sausage-shaped mass with multiple filling defects, which originates in the cervical esophagus and extends to the lower esophagus[10]. Endoscopy usually shows an intraluminal mass that is mobile and covered with normal mucosa. Careful examination of the upper esophageal sphincter may reveal the stalk of the pedunculated mass. It is not difficult to distinguish esophageal inflammatory fibrous polyp from leiomyoma, which is usually relatively flat, and non-pedunculated intramural lesions in the middle and lower third of the esophagus[13]. EUS has been reported as a method to demonstrate the submucosal origin of analogical polyps[14,15].

As a result of its special origin, there may be uncontrollable bleeding during endoscopic resection. Endoscopic removal of giant upper esophageal lesions requires thorough assessment before the procedure. Therefore, multiple modalities (barium, CT, EUS and gastroscopy) are important to delineate the nature and origin of the lesion. EUS provides information on the diameter of the polyp as well as its vascularity and insertion point[6]. Special precautions during hemostasis and elective airway protection are necessary to prevent bleeding and aspiration. The placement of the nasogastric tube, adequate insufflation and distension of the esophagus can minimize the risk of burning and esophageal perforation. The site of snare polypectomy should be kept away from the base of stalk to prevent esophageal perforation. Advances in the techniques of endoscopic treatment and the improvement of endoscopic accessories make endoscopic removal of giant esophageal polyps feasible. Post-polypectomy hemostasis can be achieved with adrenaline injection and hemoclips. If hemostasis fails, one can use Coagrasper to coagulate even a small area of bleeding.

In conclusion, with thorough assessment with multiple imaging modalities, and the availability of good endoscopic accessories, giant upper esophageal inflammatory fibrous polyp can be resected by endoscopy safely and successfully.

REFERENCES

1 **Solito B**, Anselmino M, Tognetti A, Vignati S, Rossi M. Rare case of inflammatory fibrous polyp of the esophagus. *Dis Esophagus* 2002; **15**: 326-329
2 **Ozcelik C**, Onat S, Dursun M, Arslan A. Fibrovascular polyp of the esophagus: diagnostic dilemma. *Interact Cardiovasc Thorac Surg* 2004; **3**: 260-262
3 **Bosch O**, González Campos C, Jurado A, Guijo I, Miro C, Renedo G, Porres JC. Esophageal inflammatory fibroid polyp. Endoscopic and radiologic features. *Dig Dis Sci* 1994; **39**: 2561-2566
4 **Iván L**, Paczona R, Szentpáli K, Jóri J. Peroral endoscopic removal: as a minimally invasive long-term surgical treatment of a regurgitated giant polisegmented fibrovascular polyp of the esophagus. *Eur Arch Otorhinolaryngol* 2009; **266**: 1031-1034
5 **Kanaan S**, DeMeester TR. Fibrovascular polyp of the esophagus requiring esophagectomy. *Dis Esophagus* 2007; **20**: 453-454
6 **McLean JN**, DelGaudio JM. Endoscopic resection of a giant esophageal polyp: case report and review of the literature. *Am J Otolaryngol* 2007; **28**: 115-117
7 **LiVolsi VA**, Perzin KH. Inflammatory pseudotumors (inflammatory fibrous polyps) of the esophagus. A clinicopathologic study. *Am J Dig Dis* 1975; **20**: 475-481
8 **Minutolo V**, Rinzivillo C, Li Destri G, Scilletta B, Cammisuli F, Puleo S. Giant esophageal polyp: a rare and benign neoplasm. *Chir Ital* 1999; **51**: 313-316
9 **Pham AM**, Rees CJ, Belafsky PC. Endoscopic removal of a giant fibrovascular polyp of the esophagus. *Ann Otol Rhinol Laryngol* 2008; **117**: 587-590
10 **Ginai AZ**, Halfhide BC, Dees J, Zondervan PE, Klooswijk AI, Knegt PP. Giant esophageal polyp: a clinical and radiological entity with variable histology. *Eur Radiol* 1998; **8**: 264-269
11 **Yamane T**, Uchiyama K, Ishii T, Ishii H, Takizawa R, Omura M, Fujise K, Tajiri H. Case of inflammatory fibroid polyp of the esophagogastric junction. *Dig Endosc* 2009; **21**: 97-100
12 **Simmons MZ**, Cho KC, Houghton JM, Levine CD, Javors BR. Inflammatory fibroid polyp of the esophagus in an HIV-infected individual: case study. *Dysphagia* 1995; **10**: 59-61
13 **Levine MS**. Benign tumors of the esophagus: radiologic evaluation. *Semin Thorac Cardiovasc Surg* 2003; **15**: 9-19
14 **Lawrence SP**, Larsen BR, Stacy CC, McNally PR. Echoendosonographic and histologic correlation of a fibrovascular polyp of the esophagus. *Gastrointest Endosc* 1994; **40**: 81-84
15 **Costa PM**, Marques A, Távora, Oliveira E, Diaz M. Inflammatory fibroid polyp of the esophagus. *Dis Esophagus* 2000; **13**: 75-79

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CASE REPORT

Pedunculated hepatocellular carcinoma and splenic metastasis

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Abstract

Only a few cases of pedunculated hepatocellular carcinoma (P-HCC) have been reported in the literature. The common sites of extrahepatic metastases in patients with HCC are the lungs, regional lymph nodes, kidney, bone marrow and adrenals. Metastasis to spleen is mostly *via* hematogenous metastasis, direct metastasis to spleen was very rare. We report a case of P-HCC presenting as a left upper abdominal lesions which involved the spleen that was actually a P-HCC with splenic metastasis. This case is unique as P-HCC directly involved the spleen which is not *via* hematogenous metastasis.

Key words: Pedunculated hepatocellular carcinoma; Splenic metastasis; Hematogenous metastasis; Direct metastasis; Splenectomy

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INTRODUCTION

The pedunculated hepatocellular carcinoma (P-HCC) which protrudes from its pedicel or presents as epibiotic mass almost making no invasion into the liver, is a rare exception to the gross type[1,2]. To date, only a few cases have been reported[2-4]. The common sites of extrahepatic metastases in patients with hepatocellular carcinoma (HCC) are the lungs, regional lymph nodes, kidney, bone marrow and adrenals, which is *via* hematogenous metastasis. The P-HCC directly invading the spleen not *via* hematogenous metastasis is extremely rare. In this report, we describe a case of P-HCC which directly involved the spleen.

CASE REPORT

A 68-year-old man with HBV-related cirrhosis was admitted to our hospital because of left flank pain and loss of weight for a forty-day duration. A mass lesion could be touched in left upper abdomen. AFP level was 166.02 ng/mL, CA125, CA199 and CEA were negative. HBVDNA level was 1.51×10^4 copies/mL. Sonographic and CT scan showed a 17 cm × 14 cm × 10 cm tumor between left hepatic lobe and spleen, which also involved the upper pole of spleen and almost made no invasion into the liver (Figure 1). Celiac and hepatic arteriography displayed mass lesions taking blood from left hepatic artery, splenic artery and left inferior phrenic artery, and transarterial chemoembolization was performed (Figure 2). Image-guided biopsy of tumor was consistent with HCC.

At operation, mild cirrhosis was found in the liver, a large tumor lied in the left upper abdomen between left hepatic lobe and spleen. The upper pole of spleen was involved, almost making no invasion into the liver, gastrointestine and pancreas (Figure 3). He underwent spleen, tumor and partial left hepatic lobe resection in January 2008. The loss of blood was 1000 mL in total. HCC and splenic metastasis were confirmed by pathological examination (Figure 4). The postoperative clinical course was uneventful, with a negative follow-up for clinical and radiological investigation at 17 mo after surgery.

DISCUSSION

The P-HCC has been reported to occur in 0.24%-3.0% of all HCC patients[5]. Hematogenous metastasis to spleen

Table 1 Previous cases reported in literature with HCC and splenic metastasis

Authors	Age (yr)/ sex	Metastasis type	Clinical manifestations	Intrahepatic metastasis at time of splenic metastasis	Metastasis to other organs
Filik *et al*[6]	62/W	H	Severe ascites and abdominal pain	Multiple HCC	None
	47/M	H	Right flank of pain	HCC	None
Hanada *et al*[7]	59/M	H	Asymptomatic	Multiple HCC	Adrenal gland
	69/W	H	Not described	None	None
	67/M	H	Not described	None	Lung
Yamamoto *et al*[11]	68/W	H	Abdominal fullness	Single HCC	None
	61/M	H	Swelling cervical lymph nodes	HCC	Cervical lymph nodes
Fujimoto *et al*[12,13]	62/M	H	LUQ mass	None	None
	62/M	H	Spontaneous rupture of spleen	HCC	None
Horie *et al*[14]	62/W	H	Spontaneous rupture of spleen	HCC	None
Katoh *et al*[17]	55/M	H	Not described	HCC	None
Iwaki *et al*[8]	60/M	H	Asymptomatic	Multiple HCC	Lung and jejunal
Sumiya *et al*[16]	78/M	H	Spontaneous rupture of spleen	None	Lung
Hayashi *et al*[15]	76/M	H	Asymptomatic	None	None
Hama *et al*[9]	61/M	H	Asymptomatic	Multiple HCC	Lung
Nakamura *et al*[10]	54/M	H	Asymptomatic	Multiple HCC	Lung

HCC: Hepatocellular carcinoma; H: Hematogenous metastasis; LUQ: Left upper quadrant.

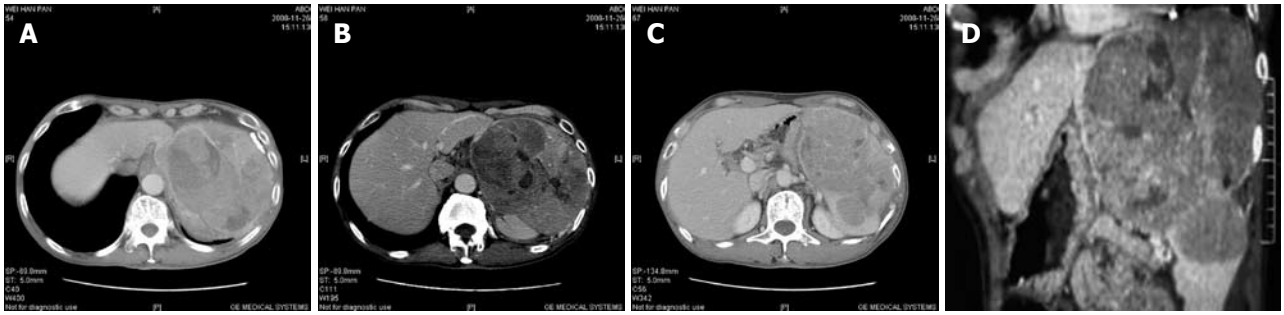

Figure 1 CT scan in abdomen showing a mass tumor between left hepatic lobe and spleen directly involving the upper pole of spleen and almost making no invasion into the liver (A-D).

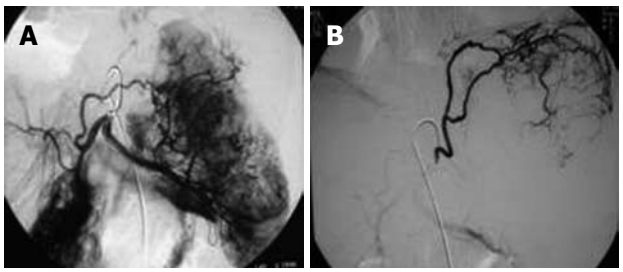

Figure 2 Celiac and hepatic arteriography confirmed the mass lesions taking blood from left hepatic artery and splenic artery (A) and inferior phrenic artery (B).

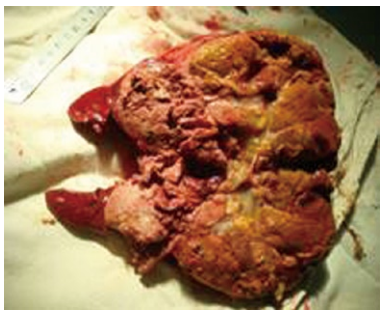

Figure 3 Postoperative photography showing the lesions directly involving the upper pole of spleen.

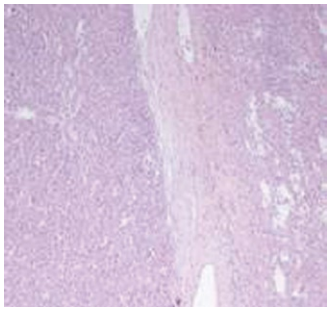

Figure 4 Histopathology showing the splenic metastasis of hepatocellular carcinoma (HE, × 40).

is very rare with a reported prevalence of 0.7%-0.8% in HCC patients[6,7], but it is probably more common than direct metastasis.

Preoperative differential diagnosis between metastatic or primary splenic tumors is difficult. High levels of AFP (> 1210 ng/mL) may contribute to the diagnosis of P-HCC. With improvement in diagnostics such as angiography and CT scan, the preoperative diagnosis is feasible in patients with negative or mild increase of AFP level. In this patient, selective celiac arteriography showed a tumor fed by hepatic artery, splenic artery and left inferior phrenic artery, from which we can judge the blood supply and diagnose the tumor. Image-guided biopsy of tumor was utilized to confirm the presence of HCC when the imaging study could not draw a conclusion.

The intrahepatic metastasis from HCC occurs mostly commonly *via* the portal vein, which is followed by hematogenous metastasis to the lungs and bone, lymph node metastasis, direct metastasis and peritoneal metastasis. Previous cases in the literature with HCC and splenic metastasis are summarized in Table 1[6-17]. Metastasis to spleen occurred hematogenously in previous cases. In the present case, the splenic metastasis occurred directly. The cumulative survival rates of extrahepatic metastasis of HCC were very poor. Such lesions in the case may not represent remote metastases, but they are actually HCC with extended invasion to the spleen. Whether splenic metastasis happens directly or hematogenously should be distinctive and the resection of P-HCC and splenic metastasis can be curative in the former. The distinction between the two is important, as it affects the stage, prognosis and management of the patient. Although the long-term outcome of resection for such splenic metastasis is unknown, direct splenic metastasis of P-HCC can be easily controlled to obtain gross disease clearance and may achieve better long-term survival.

In conclusion, splenic metastases of P-HCC are difficult to distinguish from primary splenic tumors, even with modern imaging studies. The treatment involves resection and surgical exploration, whenever possible.

REFERENCES

1 **Woodall CE**, Scoggins CR, Loehle J, Ravindra KV, McMasters KM, Martin RC. Hepatic imaging characteristics predict overall survival in hepatocellular carcinoma. *Ann Surg Oncol* 2007; **14**: 2824-2830

2 **Tzouliadis L**, Hulin SJ, Shaw I, Rees M. Image of the month--pedunculated hepatocellular carcinoma. *Arch Surg* 2007; **142**: 95

3 **Campos Franco J**, Mallo González N, Barros Alcalde P, Pazos González G, González Quintela A. [Pedunculated hepatocellular carcinoma presenting as a mass in the right iliac fossa] *Rev Esp Enferm Dig* 2008; **100**: 718-719

4 **Yeh CN**, Lee WC, Jeng LB, Chen MF. Pedunculated hepatocellular carcinoma: clinicopathologic study of 18 surgically resected cases. *World J Surg* 2002; **26**: 1133-1138

5 **Horie Y**, Katoh S, Yoshida H, Imaoka T, Suou T, Hirayama C. Pedunculated hepatocellular carcinoma. Report of three cases and review of literature. *Cancer* 1983; **51**: 746-751

6 **Filik L**, Biyikoğlu I, Akdoğan M, Oğuz D, Köklü S, Köksal AS. Two cases with hepatocellular carcinoma and spleen metastasis. *Turk J Gastroenterol* 2003; **14**: 138-140

7 **Hanada K**, Saito A, Nozawa H, Haruyama K, Hayashi N, Yamada M, Katagiri S, Katsuragawa H, Otsubo T, Takasaki K. Histopathologically-diagnosed splenic metastasis in a hepatocellular carcinoma case with adrenal metastasis. *Intern Med* 2004; **43**: 484-489

8 **Iwaki K**, Ohta M, Ishio T, Kai S, Iwashita Y, Shibata K, Himeno K, Seike M, Fujioka T, Kitano S. Metastasis of hepatocellular carcinoma to spleen and small intestine. *J Hepatobiliary Pancreat Surg* 2008; **15**: 213-219

9 **Hama N**, Marubashi S, Dono K, Kobayashi S, Kim C, Asaoka T, Takeda Y, Nagano H, Umeshita K, Monden M, Doki Y, Mori M. [A long-term survival case of hepatocellular carcinoma recurrence after the patient received a living donor liver transplantation treated by combined modality therapy] *Gan To Kagaku Ryoho* 2008; **35**: 2092-2095

10 **Nakamura M**, Nagano H, Wada H, Noda T, Ota H, Damdinsuren B, Marubashi S, Miyamoto A, Takeda Y, Umeshita K, Dono K, Monden M. A case of hepatocellular carcinoma with multiple lung, spleen, and remnant liver metastasis successfully treated by combination chemotherapy with the novel oral DPD-inhibiting chemotherapeutic drug S-1 and interferon-alpha. *J Gastroenterol* 2006; **41**: 1120-1125

11 **Yamamoto R**, Yamamoto S, Fukushima K, Ohmoto K, Hino K, Kojoh K, Ohumi Y, Ideguch S, Hirannao H, Hirokawa M. [Splenic metastasis of hepatocellular carcinoma] *Gan No Rinsho* 1986; **32**: 1486-1490

12 **Fujimoto H**, Murakami K, Ozawa K, Iino M, Kimura M, Fukunaga T, Shinozaki M. [Huge splenic metastasis from hepatocellular carcinoma; a case report] *Rinsho Hoshasen* 1990; **35**: 1439-1442

13 **Fujimoto H**, Murakami K, Nosaka K, Arimizu N. Splenic metastasis of hepatocellular carcinoma. Accumulation of Tc-99m HDP. *Clin Nucl Med* 1992; **17**: 99-100

14 **Horie Y**, Suou T, Hirayama C, Nagasako R. Spontaneous rupture of the spleen secondary to metastatic hepatocellular carcinoma: a report of a case and review of the literature. *Am J Gastroenterol* 1982; **77**: 882-884

15 **Hayashi H**, Tanaka S, Shuto T, Tanaka H, Ichikawa T, Yamamoto T, Takemura S, Hirohashi K, Kubo S. Splenic metastasis of hepatocellular carcinoma. *Osaka City Med J* 2006; **52**: 79-82

16 **Sumiya H**, Takayama H, Arai H, Mori K, Iizuka H, Sagawa T, Onozato Y, Ishihara H, Ogawa T, Abe T. [A survival case of hepatocellular carcinoma with splenic metastasis: metachronous rupture at the liver primary and splenic metastatic lesion] *Nippon Shokakibyo Gakkai Zasshi* 2007; **104**: 1639-1644

17 **Katoh M**, Takeuchi K, Murashima N, Nakajima M, Yamaguchi H, Endo Y, Hara M. Hepatocellular carcinoma with splenic metastasis developing after 16 years of chemotherapy for chronic myelogenous leukemia: a case report. *Jpn J Clin Oncol* 1994; **24**: 111-115

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LETTERS TO THE EDITOR

Vascular tumors and malformations of the colon

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Abstract

The term "hemangioma" refers to the common tumor of infancy that exhibits rapid postnatal growth and slow regression during childhood. It may cause confusion with venous malformations that are often incorrectly called "cavernous hemangioma". Venous malformations comprise abnormally formed channels that are lined by quiescent endothelium. Accurate diagnosis is required for selecting the appropriate treatment.

Key words: Hemangioma; Venous malformations; Surgery; Sclerotherapy; Colon

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TO THE EDITOR

With regard to the article entitled "Large cavernous hemangioma in the cecum treated by laparoscopic ileocecal resection" by Huh *et al*[1] published recently on the *World Journal of Gastroenterology*, there are some pertinent considerations. In 1982, Mulliken and Glowacki[2] classified vascular lesions into vascular tumors (infantile hemangioma, rapidly involuting congenital hemangioma, non-involuting congenital hemangioma, kaposiform hemangioendothelioma and tufted angioma) and vascular malformations (arteriovenous malformation, venous malformation, lymphatic malformation, lymphatic-venous malformation, and capillary malformation). In 1996, the International Society for the Study of Vascular Anomalies approved this classification system to establish a common language for the many different medical specialists who are involved in the management of these lesions. A great variety of vascular anomalies is incorrectly referred to as "hemangiomas" in the medical literature and a significant number of patients receive ineffective and potentially harmful treatment based on misclassification. Hemangiomas are usually not present at birth; they proliferate during the first year of life; and then they involute. They are composed of proliferating endothelial cells. Venous malformations consist of dysplastic vessels and are present on a lifelong basis. Unlike hemangiomas, there is no proliferation phase. They seem to grow because the vessels progressively dilate and they do not present a regression phase[3]. Histological findings in venous malformations consist of large, dilated, blood-filled vessels lined by flattened endothelium as reported in the article.

After close examination of the reported case of "cavernous hemangioma", we support the diagnosis of venous malformation in the cecum. Colonic venous malformations are often mistaken for tumors because of a similar presentation (from a vague blue patch to a soft blue mass as described in the article) and improper nomenclature. The term "cavernous hemangioma" is frequently used to name a venous malformation. These lesions may cause chronic and acute gastrointestinal hemorrhage.

Sclerotherapy of venous malformations is an effective treatment and seems to be the best therapeutic option, although the surgical resection is preferred for venous malformations localized in the colon in case of bleeding. Although these vascular malformations generally are incompletely resectable because of diffuse pelvic and mesenteric involvement, the goal is to abate bleeding by excluding the lesion from the gastrointestinal lumen. The minimally invasive surgical procedure performed by the authors of this article seems to be appropriate since the venous malformation was removed from the lumen. Other procedures like colectomy with mucosectomy and endorectal pull-through should be considered for diffuse venous malformations of the colorectum[4].

REFERENCES

1 **Huh JW**, Cho SH, Lee JH, Kim HR. Large cavernous hemangioma in the cecum treated by laparoscopic ileocecal resection. *World J Gastroenterol* 2009; **15**: 3319-3321

2 **Mulliken JB**, Glowacki J. Hemangiomas and vascular malformations in infants and children: a classification based on endothelial characteristics. *Plast Reconstr Surg* 1982; **69**: 412-422

3 **Frieden I**, Enjolras O, Esterly N. Vascular birthmarks and other abnormalities of blood vessels and lymphatics. In: Schacner LA, Hansen RC, editors. Pediatric dermatology. 3rd ed. Saint Louis, MO: Mosby, 2003: 833-862

4 **Fishman SJ**, Shamberger RC, Fox VL, Burrows PE. Endorectal pull-through abates gastrointestinal hemorrhage from colorectal venous malformations. *J Pediatr Surg* 2000; **35**: 982-984

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Meetings

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Mouse Models of Cancer

January 21-24, 2009
Westin San Diego Hotel, San Diego, CA
Advances in Prostate Cancer Research

February 3-6, 2009
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)
Second AACR Conference
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009
Hyatt Regency Boston, Boston, MA
Translation of the Cancer Genome

February 8-11, 2009
Westin New Orleans Canal Place, New Orleans, LA
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009
Hong Kong Convention and Exhibition Centre, Hong Kong, China
19th Conference of the APASL
http://www.apasl2009hongkong.org/en/home.aspx

February 27-28, 2009
Orlando, Florida
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009
Vienna, Austria
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease
www.easl.ch/vienna2009

March 13-14, 2009
Phoenix, Arizona
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009
Marriott Wardman Park Hotel
Washington, DC
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009
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British Society of Gastroenterology (BSG) Annual Meeting
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April 8-9, 2009
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April 18-22, 2009
Colorado Convention Center, Denver, CO
AACR 100th Annual Meeting 2009

April 22-26, 2009
Copenhagen, Denmark
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)
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Digestive Disease Week 2009

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45th ASCO Annual Meeting
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DDW 2009
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Accelerating Anticancer Agent Development

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Flims, Switzerland
Methods in Clinical Cancer Research (Europe)

June 24-27 2009
Barcelona, Spain
ESMO Conference: 11th World Congress on Gastrointestinal Cancer
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World Conference on Interventional Oncology
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Molecular Biology in Clinical Oncology

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Vail Marriott Mountain Resort, Vail, CO, United States
Methods in Clinical Cancer Research

August 14-16, 2009
Bell Harbor Conference Center, Seattle, Washington, United States
Practical Solutions for Successful Management
http://www.asge.org/index.aspx?id=5040

September 23-26, 2009
Beijing International Convention Center (BICC), Beijing, China
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists (IASGO)
http://iasgo2009.org/en/index.shtml

September 27-30, 2009
Taipei, China
Asian Pacific Digestive Week
http://www.apdwcongress.org/2009/index.shtml

October 7-11, 2009
Boston Park Plaza Hotel and Towers, Boston, MA, United States
Frontiers in Basic Cancer Research

October 13-16, 2009
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009
Versailles, France
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009
Boston, MA, United States
The Liver Meeting

November 15-19, 2009
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

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Instructions to authors

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In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of

balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

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Issue with no volume

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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Conference proceedings

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Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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