• *REVIEW* •

Helicobacter pylori-infected animal models are extremely suitable for the investigation of gastric carcinogenesis

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Abstract

Although various animal models have been developed to clarify gastric carcinogenesis, apparent mechanism of gastric cancer was not clarified in recent years. Since the recognition of the pathogenicity of *Helicobacter pylori* (*H pylori*), several animal models with *H pylori* infection have been developed to confirm the association between *H pylori* and gastric cancer. Nonhuman primate and rodent models were suitable for this study. Japanese monkey model revealed atrophic gastritis and p53 mutation after long-term infection of *H pylori*. Mongolian gerbil model showed the development of gastric carcinoma with *H pylori* infection alone, as well as with combination of chemical carcinogens, such as *N*-methyl-*N*-nitrosourea and *N*-methyl-*N*-nitro-*N*'-nitrosoguanidine. The histopathological changes of these animal models after *H pylori* inoculation are closely similar to those in human beings with *H pylori* infection. Eradication therapy attenuated the development of gastric cancer in *H pylori*-infected Mongolian gerbil. Although several features of animal models differ from those seen in human beings, these experimental models provide a starting point for further studies to clarify the mechanism of gastric carcinogenesis as a result of *H pylori* infection and assist the planning of eradication therapy to prevent gastric carcinoma.

Key words: *Helicobacter pylori*; Gastric carcinoma; Animal model; Japanese monkey; Mongolian gerbil

INTRODUCTION

Gastric cancer is one of the main causes of cancer-related mortality, especially, in East Asia. To clarify the mechanism of gastric cancer development, many experimental models have been used. However, almost all experimental animals, that showed spontaneous gastric cancer were very rare[1]; therefore several animal models were established using chemical carcinogens, such as *N*-methyl-*N*-nitrosourea (MNU)[2,3] and *N*-methyl-*N*-nitro-*N*'-nitrosoguanidine (MNNG)[4,5], which showed a high rate of gastric cancer development, especially in the antrum.

Since Warren and Marshall[6] revealed the microorganism which inhabits the stomach, *Helicobacter pylori* (*H pylori*) was considered as the major factor of many kind of gastroduodenal diseases, such as acute gastritis[7-9], chronic atrophic gastritis[9,10], intestinal metaplasia[11], peptic ulcer[12,13], mucosal associated lymphoid tissue lymphoma[14], gastric cancer[15-18], and others[19,20].

Previously, a large number of epidemiological studies indicated that *H pylori* infection has a close relation with gastric cancer[15-18]. Therefore, the International Agency for Research on Cancer (IARC) conference of the World Health Organization (WHO) defined *H pylori* as a definite carcinogen (Group I) to the human stomach based on three prospective case-control studies[15-17] reported in 1991[21].

However, the mechanisms by which *H pylori* infection develop gastric cancer are not defined in detail. In further studies, attempts have been made to reveal the possible mechanisms by which *H pylori* contributes to the development of gastric carcinoma and many researchers have developed animal models of infection using *Helicobacter* species.

Previously, a large number of animal experimental models have been developed to define the association between *H pylori* infection and gastroduodenal disease, such as piglet[22], beagle dog[23], mice[24], rhesus monkey[25], Japanese monkey (*Macaca fuscata*)[9,26,27], Mongolian gerbil[28], and others. In the beginning of the development of experimental models, only a few models had long periods of infection.

We have reported the results of a 5-year study on *H pylori* infection using Japanese monkeys (*Macaca fuscata*)[27]

and have obtained the findings that advance gastric mucosal atrophy, increase proliferation and mutation of p53 in gastric epithelial cells[29,30].

Several experiments, which demonstrated that chronic H pylori infection models of Mongolian gerbils developed gastric carcinoma, were conducted[31-33]. In these experiments, the animals were mainly divided into two groups: one group was infected with H pylori alone and the group was given a known carcinogen such as MNU and MNNG in addition to persistent H pylori infection. The results of these experiments revealed that animals in different groups developed different histopathological types of gastric carcinoma. These results will be very useful to elucidate the mechanism of gastric carcinogenesis due to H pylori infection.

GASTRIC CANCER AND JAPANESE MONKEY

The nonhuman primate animals are useful to clarify the relationship between H pylori and gastric diseases. Their stomachs are similar to those of human beings anatomically, physiologically, and dietary, compared with rodent animals. They have 10-20 years of long life span, which enables long-term follow-up with endoscopy and repeated histological examinations of the stomach using biopsy or endoscopic resected specimens. Several primate animals have been reported to be successful in experimental transmission of H pylori in chimpanzees (Pan troglodytes)[34], and species of macaques: rhesus monkey (M. mulatta)[25], cynomolgus monkey (M. fascicularis)[25], and Japanese monkey (M. fuscata)[9,26,27]. In these animals, some kinds of Macaque species are available for a wide variety of research field. We have established the Japanese monkey model with H pylori infection. This experimental model is very useful and a promising nonhuman primate model[9,26,27].

The methods of development of this monkey model are described briefly. The bacterial strains used were H pylori MCO 88155, MCO 88099, MCO 88142, and MCO 88156, isolated from two patients with duodenal ulcers and two with gastric ulcers. The colonies were suspended in 5 mL of sterile saline, and the bacterial concentration was adjusted to 10^9 CFU/mL. These were resuspended in 8 mL of sterile saline, and 5 mL of the final resuspension was used in each monkey. The animals were given ampicillin orally to eradicate spiral bacteria other than H pylori. After treatment with ampicillin, spiral bacteria were not found in any of the stomachs. The monkeys were sprayed with 5 mL of a mixed suspension of four bacterial strains endoscopically around their antrum. The gastric mucosa was examined endoscopically, and endoscopic mucosal resection was performed repeatedly during 6 years of observation.

One week after inoculation, all infected monkeys showed endoscopic acute gastritis accompanied by marked erythema and edema. These findings were consistent with the acute gastric mucosal lesion observed in the human stomach. Infection of H pylori was recognized by culture, the rapid urease test, histology,

and the elevation of H pylori-specific IgG in plasma. In the early phase of infection, infiltration of monocytes and polymorphonuclear leukocytes were marked in the edematous lamina propria and superficial erosions were evident. After 3 mo of inoculation, infiltration of mononuclear cells and plasma cells were predominant in the lamina propria layer. However, no superficial erosions and atrophic changes were observed.

In the infected group, the gastritis score which was evaluated by a scoring system based on the method of Rauws et al[35]. were markedly increased in the antral mucosa 1 wk after inoculation (P<0.001). The score then gradually decreased throughout the whole investigation period, but remained significantly higher (P<0.01) than that of the control group.

Six months after inoculation, the pyloric glandular height was apparently lower in the infected animals than in controls. Furthermore, the atrophic change advanced gradually throughout the 5-year observation period[27]. Endoscopically, according to the endoscopic-atrophic-border scale described by Kimura and Takemoto[36], gastric atrophy also gradually advanced for more than 3 years. These findings indicated evidently that H pylori infection caused atrophic gastritis in the Japanese monkey model. Cell proliferation activity, which was revealed with immunohistochemical detection of Ki-67 in the antral mucosa of infected animals, was significantly accelerated throughout the entire observation period (Figure 1). Immunohistochemical detection of p53 and point mutation of p53 was exhibited in the gastric mucosa[29,30] of this model. Genetic alterations in exons 5-8 of the p53 gene were uncommon in the H pylori-uninfected monkeys, whereas a higher prevalence of missense mutations in the p53 gene appeared in association with H pylori infection (Table 1). The number of mutations in the p53 gene increased as the gastric atrophy score increased, which depends on the duration of H pylori infection[30]. These findings of Japanese monkey model may explain the potential mechanism for the causal role of H pylori in the chain of events leading to gastric carcinoma. This monkey model facilitates investigation of the correlation between the long-term sequence of H pylori infection and gradual gastric mucosal change. Although many pathophysiological changes were seen in H pylori-infected gastric mucosa, this Japanese monkey model did not show the development of gastric carcinoma. In their long life span, which is similar to human beings, further continuous infection may be needed to the more dramatic histological change.

DEVELOPMENT OF THE RODENT MODEL

Several rodent models were established for examining the etiological feature of Helicobacter species infection, such as mice[24,37], rat[38], and Mongolian gerbil[28]. Compared with nonhuman primate models, rodent models are treated easily, and are economical.

Marchetti et al[39]. reported the several clinical isolates colonized the stomach of SPF conditioned mice (CD1 mice) and Balb/c mice; however, colonization was very
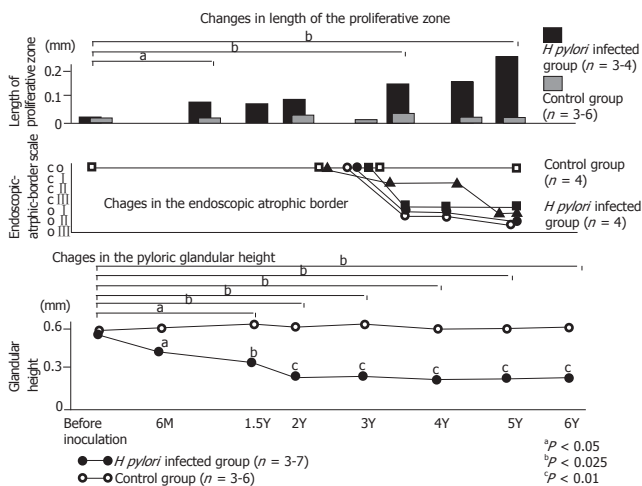
Figure 1 Gastric mucosal alteration of Japanese monkey model with *H pylori* infection. Upper graph showed the gradual increase of the proliferative zone of *H pylori*-infected Japanese monkey model. Middle graph showed the alteration of endoscopic-atrophic-border scale of this model. Macroscopically, gastric atrophy advanced for more than 3 yr. Lower graph showed the alteration of the pyloric glandular height. Six months after inoculation, the pyloric glandular height was apparently lower in the infected animals than in controls. Furthermore, the atrophic change advanced gradually throughout the 6-yr observation period.

Table 1 Duration of *H pylori* infection and number of point mutations in exon 5-8 of the p53 gene

Monkey	Duration of *H pylori* infection (yr)	Number of nucleotide (amino acid) substitutions in p53				Atrophy score[1]	Intensity of p53 immunostaining[2]
		Ex 5	Ex 6	Ex 7	Ex 8		
A	1.5	0 (0)	2 (1)	0 (0)	1 (1)	2	-
B	2	4 (0)	2 (2)	5 (4)	4 (3)	3	-
C	3	4 (2)	1 (0)	2 (2)	2 (2)	4	+
D	3	2 (1)	1 (0)	1 (1)	4 (2)	5	+
E	3.5	5 (2)	2 (1)	5 (4)	6 (4)	4	+
F	4.5	5 (1)	3 (1)	4 (3)	5 (4)	6	-
G	5	4 (1)	3 (1)	2 (1)	8 (6)	8	+
H	7.5	8 (5)	2 (1)	5 (4)	8 (4)	12	++

[1], The atrophy score was calculated as the sum of the histological evaluations of five gastric specimens according to Updated Sydney System. [2], The intensity of p53 immunostaining was classified into four grades: -, no staining; +, mild staining; ++, moderate staining; +++, intense staining.

low. Lee *et al*[40]. reported the quite good colonization by using the Sydney strain of *H pylori* (strain SS1), which is cagA and vacA positive.

These rodent models showed meager development of spontaneous gastric cancer. Cui *et al*[41]. reported the development of spontaneous gastric carcinoma, classified as malignant enterochromaffin-like (ECL) carcinomas in female cotton rats (*Sigmodon hispidus*). Previously, chemical carcinogens such as MNNG and MNU have been often used in the rodent species for the investigation of experimental gastric cancer[2-5].

From the recognition of *Helicobacter* species' pathogenicity, Fox *et al*[42]. reported a possible carcinogenic role for *Helicobacter* species in the gastric mucosa after oral administration of MNNG in ferrets infected with *Helicobacter mustelae*. Nine out of the ten ferrets, which were given a dose of 50 mg/kg MNNG orally, developed adenocarcinoma. This was the first experimental study using carcinogens combined with infection with a *Helicobacter* species. Although spontaneous gastric adenocarcinomas have been reported[43] in aged ferrets with *H mustelae* even in the absence of carcinogen exposure, an important additional problem is that the bacterium used was not *H pylori* but *H mustelae*. Fox *et al*[44]. also described the development of gastric adenocarcinoma, which was led from severe gastritis in C57BL/6 mice with *Helicobacter felis* inoculation. In their report, p53+/- mice showed significant low prevalence of fundic lymphoplasmacytic infiltration and submucosal lymphoid follicle formation than those in C57BL/6 mice. They indicated two distinct roles of p53, one of them displayed the gastric cancer risk. However, deletion of one *p53* allele results in a down-regulated Th1 response to *Helicobacter* infection, which may indirectly protect against the development of gastric

cancer associated with chronic inflammation.

The differences between *H felis* in mice and *H pylori* in human beings are the lack of induction of neutrophil and cag pathogenicity island, which are recognized as main pathogens of *H pylori*. Kim *et al*[45]. reported that C57BL/6 mice infected with *H pylori* (SS1 strain) showed no evidence of gastric adenoma, dysplasia, and carcinoma during 80 wk of infection. They explained this result by the balance that exists between cell proliferation and apoptosis.

Transgenic hypergastrinemic (INS-GAS) mouse model have also been useful for the investigation of gastric carcinogenesis. Fox *et al*[46]. reported that male INS-GAS mice infected with *H pylori* developed atrophy, intestinal metaplasia, and dysplasia and adenocarcinoma. This murine model with *H pylori cagE* mutant showed the deletion of development of cancer. In contrast, none of the female mice with *H pylori* infection developed adenocarcinoma. However, IL-1 levels showed no significant difference between males and females. Fox *et al*. concluded that the INS-GAS model is effective for investigating discrete host-microbial interactions that culminate in gastric cancer within the context of biologic conditions induced by *H pylori*.

DEVELOPMENT OF MONGOLIAN GERBIL MODEL

Yokota *et al*[28]. developed the experimental Mongolian gerbil model with *H pylori* infection, in which only a mild inflammatory infiltration in the gastric mucosa was seen during two months of their observation. Hirayama *et al*[47]. described that ulcers and intestinal metaplasia were produced 6 mo after inoculation with *H pylori* in Mongolian gerbils.

In our laboratory, 5-wk-old male Mongolian gerbils weighing 30-40 g (Seiwa Experimental Animals Co. Ltd., Fukuoka, Japan) were used[48]. *H pylori* ATCC-43504 possessing the cagA gene and expressing vacuolating cytotoxin was used. A 4-d culture on blood agar at 37 ℃ under microaerophilic conditions was harvested and incubated in brucella broth (DIFCO Laboratories, Detroit,
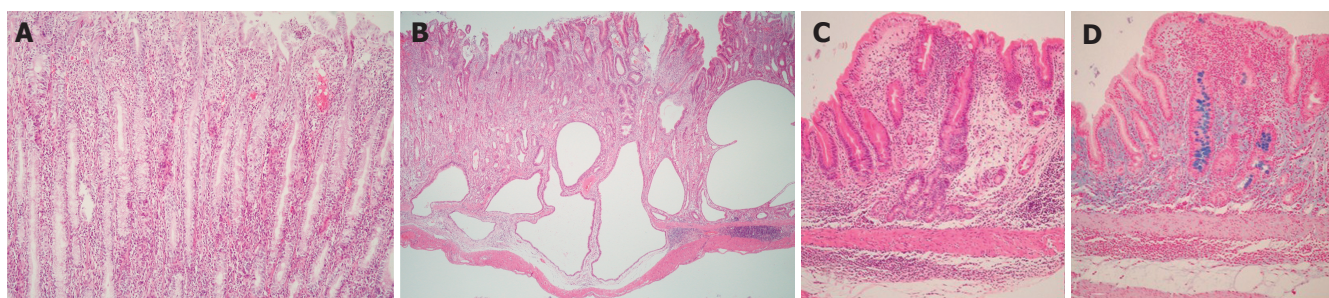
Figure 2 Microscopic views of the gastric body of Mongolian gerbils at 18 mo after *H pylori* inoculation. **A:** Severe infiltration of polymorphonuclear and mononuclear cells were seen in the lamina propria. (HE stain, x100); **B:** Some glands have extended into the submucosa but not into the proper muscularis layer. Severe infiltration of mononuclear cells in the submucosa (HE stain, x10); **C:** Intestinal metaplasia is seen scattering in gastric mucosa (HE stain, x10); **D:** Intestinal metaplasia (Alcian blue stain (pH 2.5); original magnification, x10).

MI, USA) with 10% horse serum for 24 h. Inoculum size was adjusted with sterile saline to produce the optical density of McFarland 4 at 540 nm. Mongolian gerbils were housed five per cage, starved for 24 h, and then fed with chow (Oriental Yeast Co., Tokyo, Japan) and water *ad libitum* beginning 12 h after *H pylori* inoculation. On the day of infection, the Mongolian gerbils were challenged orally with vehicle or 10^9 CFU *H pylori* in 1.0 mL of brucella broth with 10% horse serum. The spiral bacteria were observed in the mucus and gastric pits of all inoculated animals from 1 mo after inoculation throughout the whole observation period. However, nearly half of the animals had barely detectable *H pylori* in the stomach by bacterial culture. The bacterial counts from the stomachs of gerbils 1 and 6 mo after *H pylori* inoculation were 25 and 410 CFU/10 mg of gastric tissue, respectively[48]. These levels of colonized bacteria were nearly 1/10 to 1/100 than those of human being and monkey.

Mongolian gerbils with *H pylori* infection showed irregularly thickened gastric walls and spotty hemorrhages and erosions macroscopically, 1 year after inoculation. A severe infiltration of polymorphonuclear and mononuclear cells was seen in the lamina propria and mononuclear cells infiltration with lymphoid follicle in the submucosa, 1 mo after *H pylori* inoculation (Figure 2A). Erosion of the gastric mucosa appeared soon after inoculation, whereas gastric ulcers, gastritis cystica profunda (Figure 2B), and atrophy with goblet cell metaplasia (Figures 2C and D) occurred between 3 and 6 mo after inoculation[48,49]. Moreover, Suzuki *et al*[50]. reported that *H pylori* inoculation induced neutrophil followed by an increase in the level of lipid peroxidation and activated glutathione (antioxidant) turnover. These sequential changes of histological changes in gastric mucosa were quite similar to those observed in human beings. Therefore, Mongolian gerbil model may be useful to study the relationship between *H pylori* infection and gastric lesions, which include gastric malignancy.

GASTRIC CANCER AND MONGOLIAN GERBIL MODEL

Mongolian gerbils have also been induced by the development of gastric carcinoma with chemical carcinogen alone[51]. In addition, the results of several experimental studies have confirmed that administration of MNNG or MNU to Mongolian gerbils with chronic *H pylori* infection enhanced the development of different histopathological types of gastric carcinoma (Table 2)[31-33].

Sugiyama *et al*[31]. reported the development of carcinoma in the Mongolian gerbils evaluated at 40 wk after an experiment in which 7-wk-old animals were inoculated with *H pylori* (ATCC43504) and given 10 or 30 ppm MNU before or after inoculation. In this report, only the groups of the animals, which were administered with both *H pylori* and MNU, developed gastric cancers; more specifically, they developed different types of adenocarcinoma, such as well-differentiated, poorly differentiated, and signet ring cell carcinoma. These interesting experimental results support the results so far obtained in largescale epidemiological investigations[52].

The group inoculated with *H pylori* after being given MNU showed a distinctive initiation-promotion effect, whereas the group to which MNU was given after inoculation with *H pylori* appeared to demonstrate the simultaneous action of these two factors, with *H pylori* acting as a coinitiator. No gastric carcinoma was found within 40 wk of *H pylori* infection alone.

Tokieda *et al*[32]. conducted a study in which 5-wk-old Mongolian gerbils were inoculated with *H pylori* (ATCC43504) and orally given MNNG at 50 g/mL for 20 wk for comparison against animals administered with MNNG alone. At the 52nd wk after initiation, the group treated with MNNG and *H pylori* developed gastric carcinoma at a significantly higher frequency than the group treated with MNNG alone. In addition, cell proliferation was revealed to be markedly accelerated in those animals infected with *H pylori* with evaluation using a labeling index of 5-bromo-2'-deoxyuridine. This result suggests the possibility of explaining the link between *H pylori* infection and early events in gastric carcinogenesis. One of the interests in this study is that administration of MNNG reduced the infection rate of *H pylori* with the lapse of time, due to the likelihood of MNNG showing low-level (200 µg/mL) antibacterial activity against *H pylori*[32]. It is also of interest that *H pylori*-free animals did not develop gastric carcinoma even with

Table 2 Gastric carcinogenesis in *Helicobacter pylori*-infected Mongolian gerbils

Author	Year	Strain	Study design (ppm)	Incidence of cancer (%)	Duration of experiment (wk)
Sugiyama *et al.*	1998	ATCC43504	HP→MNU (10)	7/19 (36.8)	40
			HP alone	0/20 (0)	40
			MNU (30) → HP	6/18 (33.3)	40
			MNU alone	0/74 (0)	40
Tokieda *et al.*	1999	ATCC43504	HP → MNNG (50)	5/17 (29.4)	52
			persistent HP positive	5/8 (62.5)	52
			HP eradicated	0/9 (0)	52
			Br → MNNG (50)	3/22 (13.6)	50
Shimizu *et al.*	1999	ATCC43504	MNNG (300) → HP	12/27 (44.4)	50
			MNNG (300) → Br	1/19 (5.3)	50
			MNNG (60) → HP	6/25 (24.0)	50
			MNNG (60) → Br	0/20 (0)	50
			HP → MNNG (100)	4/27 (14.8)	50
			Br → MNNG (100)	3/18 (16.7)	50
			HP → MNNG (20)	15/25 (60)	50
			Br → MNNG (20)	1/20 (5)	50
			HP alone	0/20 (0)	50

HP, *Helicobacter pylori*; MNU, *N*-methyl-*N*-nitrosourea; MNNG, *N*-methyl-*N*'-nitroso-*N*-nitrosoguanidine; Br, Brucella broth.

Table 3 Gastric carcinogenesis in *Helicobacter pylori*-infected Mongolian gerbils

Author	Year	Strain	*cag*A gene	Vacuolating cytotoxin	Incidence of cancer (%)	Duration of experiment (wk)	Histological type of carcinoma
Watanabe *et al.*	1998	TN2GF4[1]	+	+	10/27 (37)	62	Well differentiated adenocarinoma
Honda *et al.*	1998	ATCC43504[2]	+	+	2/5 (40)	72	Well differentiated adenocarinoma
Hirayama *et al.*	1999	ATCC43504[2]	+	+	1/56 (1.8)	64	Poorly differentiated adenocarinoma
Ogura *et al.*	2000	TN2[2]	+	+	1/23 (4)	62	Well differentiated adenocarinoma
Zheng *et al.*	2004	ATCC43504[2]	+	+	3/17 (18)	84	Well differentiated adenocarinoma
		H pylori 161[3]	+	+			

[1]*H pylori* isolated from patient with gastric ulcer; [2]Type of strains; [3]*H pylori* isolated from patient with gastric adenocarcinoma.

MNNG administration. This result indicated a stronger carcinogenic role of *H pylori* infection. Although it has been reported by Sugiyama[31] that *H pylori* can persistently colonize the stomach of MNUtreated Mongolian gerbils, it is interesting that the two studies[32,33] report that MNNG administration eradicates *H pylori* infection, resulting in a reduction of its carcinogenic effects in the stomach. In *H pylori*-infected Mongolian gerbils with MNNG administration, duodenogastric reflux due to surgical procedure might attenuate the effect of *H pylori* on gastric tumorigenesis[53]. Because of our study, which indicated that bile reflux might lead to *H pylori* eradication[54], their results may probably depend on the *H pylori* eradication.

CARCINOGENICITY OF *H PYLORI* INFECTION ALONE

Although these studies showed marked increase of the chemical carcinogenic risk in the Mongolian gerbils, direct relationship between *H pylori* and gastric carcinogenesis was not indicated.

Two experimental studies attempted to confirm prior epidemiological studies that have demonstrated an association between *H pylori* infection and gastric carcinogenesis in human beings using Mongolian gerbils chronically infected with this bacterium (Table 3)[55,56]. Both studies confirmed gastric carcinogenesis resulting from *H pylori* infection alone, and were the first papers to fulfill Koch's postulates concerning *H pylori* infection and gastric carcinoma.

Watanabe *et al*[55]. used *H pylori* isolated from patients with gastric ulcer (TN2GF4), and Honda *et al*[56]. used ATCC43504 type strain, both of which were inoculated into 5-wk-old SPF Mongolian gerbils. The results showed that 37% (10 out of 27) of the animals in the former study developed well-differentiated adenocarcinoma at 62 wk after inoculation, whereas 40% (2 out of 5) of the animals in the latter study developed well-differentiated adenocarcinoma at 72 wk after inoculation (Figure 3). Both of these strains contained *cag*A and produced vacuolating cytotoxins. Sequential histopathological changes leading to carcinogenesis of the gastric mucosa were found to be common to the two studies, and very closely resembled the histopathological changes in human gastric mucosa caused by *H pylori* infection.

Hirayama *et al*[57]. reported that poorly differentiated adenocarcinoma and carcinoid were developed in
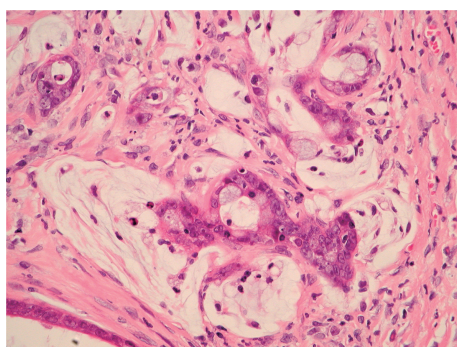
Figure 3 Microscopic views of the gastric mucosa of Mongolian gerbils at 18 mo after *H pylori* inoculation. Well-differentiated adenocarcinoma has extended into the muscular layer. Atypical glands and nuclei and abnormal mitosis are evident (HE stain, x40).
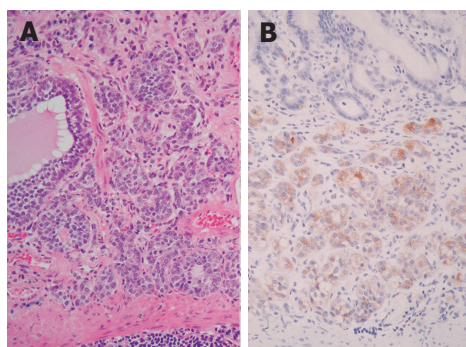

Figure 4 Gastric carcinoid in the stomach of a Mongolian gerbils colonized for 24 mo by *H pylori*. Microscopic view showing intramucosal carcinoid tumor (**A:** HE stain, x20; **B:** immunohistochemistry of chromogranin A, x20').

Table 4 Gastric carcinogenesis in *Helicobacter pylori*-infected Mongolian gerbils

Histropathological findings	Mo			
	6	12	18	24
Gastritis	5/5	4/4	5/5	10/10
Gastric ulcer	4/5	3/4	5/5	5/10
Atrophy	4/5	4/4	5/5	10/10
Intestinal metaplasia	2/5	3/4	5/5	10/10
Dysplasia	0/5	2/4	4/5	10/10
Gastric cancer	0/5	0/4	2/5	5/10
Gastric carcinoid	0/5	0/4	0/5	5/10

Data represent positive case/control Uninfected control. animals (n = 5 each) showed no abnormal findings.

Table 5 Relation between p53 and *H pylori* and gastric mucosal change (tentative opinion)

Condition	Human	Japanese monkey	Mongolian gerbil
H pylori and p53 overexpression (histology)	++	++	+[1]
H pylori and p53 point mutation	++	++	-
H pylori and atrophic gastritis	++	++	++
H pylori and intestinal metaplasia	++	-	++
H pylori and gastric cancer	+[2]	-	++

[1]The p53 overexpression was observed only in gastric cancer; [2]Not proven by interventional study; ++, strong evidence; +, weak evidence; -, no evidence

PREVENTION OF GASTRIC CARCINOMA BY ERADICATION OF *H PYLORI*

Shimizu *et al*[61]. reported that the incidence of adenocarcinomas in MNU-administered Mongolian gerbils with *H pylori* infection (15 out of 23) was significantly higher than in MNU-administered Mongolian gerbils that underwent *H pylori* eradication (5 out of 24). Their results suggest that *H pylori* eradication may prevent gastric carcinogenesis, and Mongolian gerbil's models have also been useful to study the prevention of gastric carcinogenesis in human beings.

DIFFERENCES BETWEEN ANIMAL MODELS AND HUMAN BEINGS

Although the Japanese monkey model and Mongolian gerbil model showed the similar change of human stomach that was infected with *H pylori*, several features of animal models differ from those seen in human beings. In Japanese monkey model intestinal metaplasia was not seen during the whole observation period. Severe gastritis and lymphoid follicular hyperplasia in the submucosal layer and gastritis cystica profunda, which are seen in Mongolian gerbils, are not observed in human gastric mucosa.

Table 5 shows our tentative opinion on p53 and *H pylori* infection in animal model and human beings. Although no gastric carcinoma developed in Japanese monkey model, Mongolian gerbil model showed gastric carcinoma resulting from *H pylori* infection alone. In human and Japanese monkey, both p53 immunostaining[29,62-64] and point mutations[30,65] were observed in *H pylori* infection. In

Mongolian gerbils model with *H pylori* (ATCC43504 type strain) infection alone. Zheng *et al*[58]. reported that Mongolian gerbils models, which were infected with *H pylori* (ATCC43504) and *H pylori* 161 (isolated from a Chinese patient with gastric adenocarcinoma) showed the development of well-differentiated adenocarcinoma (Table 3). Ogura *et al*[59]. reported the development of well-differentiated gastric cancer in wild type (TN2) and isogenic mutant of *vacA* (TN2Δ*vacA*) of Mongolian gerbil.

Mongolian gerbils' model also showed the development of gastric carcinoid[57,59,60]. In our laboratory, ECL cell tumors with marked atrophic gastritis and with hypergastrinemia were observed in the fundic gland area of infected Mongolian gerbils, 24 mo after inoculation (Figure 4); in contrast, adenocarcinoma developed in pyloric gland area. Histopathological findings of the entire observation period in Mongolian gerbils after *H pylori* inoculation are summarized in Table 4.

Ogura *et al*[59]. discussed the virulence factors of *H pylori* in Mongolian gerbils. Experimental gastric cancer derived in Mongolian gerbils with wild type of *H pylori* and *vacA* mutant infection, whereas *cagE* mutant induced far milder change of gastritis and induced no gastric cancer, which indicates the essential role of cagPAI in the gastric diseases with *H pylori* infection.

Mongolian gerbil, the p53 immunostaining was detected in gastric cancer but not in atrophic gastritis; moreover, there were no p53 mutations in exons 5 to 8 in infected gastric mucosa[66].

Suzuki *et al*[67]. reported that Mongolian gerbil model showed significant attenuation of apoptosis and promotion of cell proliferation than those seen in mice model with *H pylori* inoculation. Crabtree *et al*[68]. also described the differences of mucosal cytokine response between Mongolian gerbils and mice, and gender differences in the magnitude of cytokine response to *H pylori*. The differences of features between the species suggested that the pathogens of gastric diseases does not associate only with *H pylori* and may reflect in part other host factors.

Sonic hedgehog (Shh) is an important endometrial morphogenetic signal during the development of the vertebrate gut. Shh controls gastrointestinal patterning in general and gastric gland formation in particular. Suzuki *et al*[69]. reported that the long-term colonization of *H pylori* led to attenuation of Shh expression. Loss of Shh expression correlated with the loss of parietal cells, disturbed maturation of the mucous neck cell-zymogenic cell lineage. van den Brink *et al*[70]. described the loss of Shh expression in the intestinal metaplasia of the human stomach. Loss of Shh expression not only in intestinal metaplasia, but also in the tissue of *H pylori*-induced fundic gland atrophy is important for considering the possible link to preneoplastic lesion formation[69].

QUITE A NEW CONCEPT OF GASTRIC CANCER ORIGIN

In 2004, Houghton *et al*[71]. reported the innovative idea of gastric cancer origin with the usage of *H. felis*/ C57BL/6 mouse model. Previously, tissue stem cells have been recognized as the origin of carcinoma. However, their study showed that bone marrow-derived cells (BMDCs) might also represent a potential source of malignancy. Female C57BL/6 mice after undergoing lethal irradiation were transplanted with bone marrow from male C57BL/*6JGtrosa26* (ROSA26), which was labeled with X-galactosidase or green fluorescent protein. In this model, gastric mucosal apoptosis increased at 6-8 wk after *H. felis* inoculation. After 52 wk of inoculation, beta-galactosidase (gal) and trefoil factor2 (TFF2) positive cells increased gradually, then 90% of the gastric mucosa at the squamocolumnar junction was replaced with cells derived from the donor marrow. One year after infection, intramucosal carcinoma or high-grade gastrointestinal intraepithelial neoplasia were seen in these mice. No evidence of BMDC engraftment was seen in *H. felis* uninfected mice. Authors indicated that BMDC originated the epithelial cancer and the necessity of *Helicobacter* infection in this process.

CONCLUSION

In various experimental models, nonhuman primate and rodent models showed the variable evidences, which clarified the association between *H pylori* infection and gastric cancer.

Experiments developed using Mongolian gerbils have demonstrated that *H pylori* infection is clearly responsible for gastric carcinogenesis, and provide important confirmation of the statements issued by IARC/WHO. It will be of critical importance to extrapolate the sequential histopathological changes found in the Mongolian gerbil to lesions in the human gastric mucosa, since this model is proven to provide important pointers for the study of the mechanism of gastric carcinogenesis as a result of *H pylori* infection. While Koch's postulates for *H pylori* and gastric carcinoma have now been fulfilled, an important question to be addressed is why the Mongolian gerbil is the only species in which carcinogenesis has been experimentally induced by infection with *H pylori*.

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• *GASTRIC CANCER* •

Effect of p27^{KIP1} on cell cycle and apoptosis in gastric cancer cells

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Abstract

AIM: To elucidate the effect of p27^{KIP1} on cell cycle and apoptosis regulation in gastric carcinoma cells.

METHODS: The whole length of p27^{KIP1} cDNA was transfected into human gastric cancer cell line SCG7901 by lipofectamine. Expression of p27^{KIP1} protein or mRNA was analyzed by Western blot and RNA dot blotting, respectively. Effect of p27^{KIP1} on cell growth was observed by MTT assay and anchorage-independent growth in soft agar. Tumorigenicity in nude mice was used to assess the *in vivo* biological effect of p27^{KIP1}. Flow cytometry, TUNEL, and electron microscopy were used to assess the effect of p27^{KIP1} on cell cycle and apoptosis.

RESULTS: Expression of p27^{KIP1} protein or mRNA increased evidently in SCG7901 cells transfected with p27^{KIP1}. The cell growth was reduced by 31% at 48 h after induction with zinc determined by cell viability assay. The alteration of cell malignant phenotype was evidently indicated by the loss of anchorage-independent growth ability in soft agar. The tumorigenicity in nude mice was reduced evidently (0.55±0.14 cm *vs* 1.36±0.13 cm, $P<0.01$). p27^{KIP1} overexpression caused cell arrest with 36% increase (from 33.7% to 69.3%, $P<0.01$) in G1 population. Prolonged p27^{KIP1} expression induced apoptotic cell death reflected by pre-G1 peak in the histogram of FACS, which was also confirmed by TUNEL assay and electron microscopy.

CONCLUSION: p27^{KIP1} can prolong cell cycle in G1 phase and lead to apoptosis. p27^{KIP1} may be a good candidate for cancer gene therapy.

INTRODUCTION

The number of cells is regulated by a balance between proliferation, growth arrest and programmed cell death (apoptosis, PCD). Disorders of the cell cycle and apoptosis are closely related to the progression and aggressiveness of cancers[1]. p27^{KIP1}, a member of the Cip/Kip family of cyclin-dependent kinase inhibitors (CDKI), was first identified as a negative cell cycle regulator in transforming growth factor β-treated cells and in G1 phase quiescent cells by cell contact inhibition[2,3]. p27^{KIP1} binds to a wide variety of cyclin/CDK complexes including CDK2 and CDK4[4], inhibits kinase activity[5] and blocks cell cycle[6-8]. The overexpression of p27^{KIP1} protein in mammalian cells induces G1 arrest of the cell cycle and apoptosis[9,10]. Decreased p27^{KIP1}, which is positively correlated with a deceased rate of apoptosis, may be not only an indicator of tumor aggressiveness, but also an important prognostic marker in gastric carcinoma[11].

In this study, an inducible expression system was used to induce overexpression of p27^{KIP1} in human gastric cancer cells. Then whether transfection of SCG7901 with p27^{KIP1} gene could alter the cell and molecular biology as well as the spontaneous rate of apoptosis was studied.

MATERIALS AND METHODS

Cell culture

Human gastric cancer cell line SCG7901 was provided by the Department of Pathology, Fourth Military Medical University (Xi'an, China). SCG7901 cells were cultured in Eagle's medium containing phenol red supplemented with 50 mL/L fetal bovine serum (FBS) in a humidified atmosphere of 950 mL/L air and 50 mL/L CO_2 at 37℃. All the media were supplemented with 2 mmol/L L-glutamine, 100 mg/L penicillin and 100 kU/L streptomycin. Culture medium and supplements were obtained from Gibco BRL.

Plasmid construction and DNA transfection

pGEM T-Easy-KIP1 vector containing human full-length

cDNA of p27^KIP1 was used. Complementary DNA of p27^KIP1 cleaved from the pGEM T-Easy-KIP1 plasmid by EcoRI was subcloned into the inducible vector neo-control pMD-neo vector to generate pMD-KIP1 containing 0.6 kb p27^KIP1 cDNA and controlled expression of protein upon addition of 100 mmol/L zinc as an external inducer. Clonfectin (Clontech, USA) was used to transfect p27^KIP1 inducible expression vectors into SGC7901 cells. Logarithmically growing cells were transfected with 1 μg of plasmids and 2 μL of clonfectin reagent according to the manufacturer's instructions. For stable transfection, the transfected SGC7901 cells were selected in medium plus 0.5 g/L G418 (Life Technologies, USA) for 2 wk, then the G418 concentration was reduced to 0.2 g/L. Stable SGC7901 cell line transfected with pMD-KIP1 vector was treated by continuous exposure to 100 mmol/L ZnSO₄.

Western blot analysis of transgene expression

Monolayers of the cells were rinsed with PBS and lysed with SDS-PAGE loading buffer (50 mmol/L Tris-HCl pH 6.8, 100 mmol/L dithiothreitol, 2 g/L SDS). Mock and pMD-neo transfected cells served as controls. Samples were analyzed by SDS-PAGE and transferred into Hybond-C super membranes. The membranes were blocked with 50 mL/L skim-milk and 1 g/L Tween-20, then probed with primary antibody overnight according to the manufacturer's instructions, washed in PBS, 2g/L Tween-20 and then incubated with appropriate horseradish-peroxidase (HRP) conjugated second antibody. After being washed, the membranes were developed by DAB reagents according to the manufacturer's guide (Dako Co., USA). p27^KIP1 (Santa Cruz Biotechnology, USA) was detected after HRP-conjugated anti-rabbit IgG (Fc) was used. The level of β-actin was used as a control for equal loading of protein.

Total RNA extraction and RNA dot blotting

Total RNA was extracted from SGC7901 cells using TRIzol (Life Technologies, USA) following a standard acid-guanidium-phenolchloroform method. The digoxingenin-labeled specific DNA probe was obtained as follows. The plasmid containing p27^KIP1 cDNA was used as template, a pair of primers (-5 ℃-ggggtaccatgtcaaacgtgcgagt-3 ℃ and -5 ℃-gctcaacgtttgacgtcttc-3 ℃) was used to amplify the sequence. The PCR system containing Dig-11-dUTP and four normal dNTPs and Taq polymerase were used. After a standard PCR procedure (94 ℃ to 30 s, 60 ℃ for 30 s, 72 ℃ for 1 min, 30 cycles), the PCR product was purified by 1% agarose electrophoresis and DNA extraction. The labeling efficiency was further determined by Dot blot of serially diluted plasmid. Finally, the digoxingenin-labeled DNA probe was used in the experiment. RNA Dot blot was performed according to the Dig system user's guide for filter hybridization (Boehringer Mannheim, Germany). Briefly, RNA sample was diluted in RNA dilution buffer (a mixture of DEPC-treated H₂O, 20×SSC, and formaldehyde at 5:3:2). The membrane was marked slightly with a pencil to identify each dilution before spotting. One microliter of the RNA sample was spotted onto a dry

nylon membrane using a micropipettor. Then the RNA was fixed to the membrane by baking in an oven at 120 ℃ for 30 min. Hybridization and the following procedures were similar to *in situ* hybridization. Hybridization mixture contained 5×SSC, 500 g/L deionized formamide, 1 g/L sodium-lauroyl sarcosine, 0.2 g/L SDS, 20 g/L blocking reagent and a 25 ng/mL DNA probe. The level of GADPH mRNA was used as a control for equal loading of RNA.

MTT and colony assay

SGC7901/KIP1 and SGC7901/pMD cells ($1×10^4$) were seeded in 96-well plates and continuously exposed to 100 mmoL/L ZnSO₄. After treatment, 50 μL of 5 g/L 3-(4,4-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) in PBS was added to each well, incubated for 4 h at 37 ℃ and the formed formazan crystals were dissolved in 50 μL of dimethyl sulfoxide. The absorbance was recorded at 490 nm on a microplate reader (BioRad). Cell proliferation was expressed as IC50 for cells. Soft agar was prepared with the 6 g/L bottom agar layer and the 4 g/L top agar layer. The number of cells plated for each clone was 2 000. Plates were incubated at 37 ℃ with 50 mL/L CO₂ for 2 wk and the colonies were counted and photographed under a phase contrast microscope (Nikon, Tokyo, Japan).

Tumorigenicity tests in nude mice

p27^KIP1-transfected SGC7901/KIP1 cells were injected into the right flank of a nude mouse at $2.5×10^6$. pMD-neo-transfected SGC7901/pMD cells served as control. Each group had five nude mice. From the second day, each nude mouse received 100 mmol/L ZnSO₄ at a dose of 0.2 mL daily by subcutaneous injection for over 4 wk. The time of tumorigenicity and the tumor volume were measured.

Cell cycle analysis

SGC7901 cells including adherent cells (with trypsin-EDTA) and nonadherent cells were harvested. All cells were washed with PBS, resuspended and incubated in 700 mL/L ethanol for at least 12 h at 4 ℃ to permeabilize the plasma membrane. Cells were centrifuged at 1 000 r/min, resuspended in 100 mg/L RNase and 10 mg/L propidium iodide, incubated for 15 min at 25 ℃ in the dark. Single color fluorescent flow cytometry was performed with a FACScalibur flow cytometer (Becton Dickinson, USA). The histograms were analyzed with Multiplus Software Π.

Electron microscopy

SGC7901/pMD cells were gently washed with serum-free medium and fixed with 25 g/L glutaraldehyde in 0.1 mol/L sodium cacodylate buffer. These cells were scraped from the surface of the dishes and pelleted by spinning for 5 min at 10 000 *g*. The cells were osmicated with 10 g/L osmium tetroxide. The block was stained, dehydrated in graded ethanol, infiltrated with propylene oxide, embedded with EMBED overnight and cured in a 60 ℃ oven for 48 h. Silver sections were cut with an

Ultracut E microtome, collected on a formvar and carbon-coated grid, stained with uranyl acetate and Reynold's lead citrate, and viewed under a JEOL 100 CX electron microscope.

Apoptosis analyzed by TUNEL assay

SGC7901/KIP1 and SGC7901/pMD cells were harvested for TUNEL staining. The proportion of cells showing DNA fragmentation was measured by incorporation of fluorescein (FITC)-12-dUTP into DNA using terminal de oxynucleotidyltransferase (TdT). The kit was bought from Boehringer Mannheim (*In Situ Cell Death Detection*). Cells were fixed by 40 g/L paraformaldehyde in PBS overnight at 4 ℃. The samples were washed thrice with PBS and permeabilized by 2 g/L Triton X-100 in PBS for 15 min on ice. After being washed twice, cells were equilibrated at room temperature for 15-30 min in a equilibration buffer with 30 g/L BSA and 200 mL/L normal bovine serum in PBS at pH 7.4, then the slides were covered with the TUNEL mixture (calf thymus TdT, FITC-12-dUTP, and cobalt chloride in 1×reaction buffer) for 1-2 h at 37 ℃ in the dark. The tailing reaction was terminated by 2× standard saline citrate (SSC). The samples were washed thrice with PBS and analyzed by fluorescence microscopy. Routine HE staining was also conducted. Negative control was performed by omitting TdT. Quantitative analysis of apoptosis was represented by apoptotic index (AI) as described previously. AI referred to percentages from at least 1 000 counts apoptotic and non-apoptotic cells.

Statistical analysis

Fisher's exact test was performed. $P < 0.05$ was considered statistically significant.

RESULTS

Inducible expression of p27^KIP1 in SGC7901 cells

Western blot analysis demonstrated $p27^{KIP1}$ expression 0, 4, 24, 48, and 72 h after the addition of $ZnSO_4$. Mock and pMD-neo vector-transfected cells as a control contained no detectable level of $p27^{KIP1}$ in the presence of 100 mmol/L $ZnSO_4$. However, $p27^{KIP1}$ expression was increased at 4 h and further increased to a steady level at 24 h after the addition of $ZnSO_4$ as determined by the Western blot analysis (Figure 1). In RNA dot blotting survey of the expression, $p27^{KIP1}$ mRNA was very low in untransfected or noninduced SGC7901 cells. In contrast, mRNA for $p27^{KIP1}$ was expressed at a high level 4 and 24 h after the addition of $ZnSO_4$ (Figure 2). This expression pattern was rather consistent with the expression of protein.

Inhibition of tumor cell growth by exogenous p27^KIP1

Exogenous $p27^{KIP1}$ inhibited cell growth significantly. SGC7901 cell viability was determined by MTT assay (Figure 3). The cell viability rate decreased from 88% to 57% 48 h after $p27^{KIP1}$ induction with zinc treatment ($P < 0.01$). Another biological feature of malignant cells was anchorage-independent growth capability. The neo-
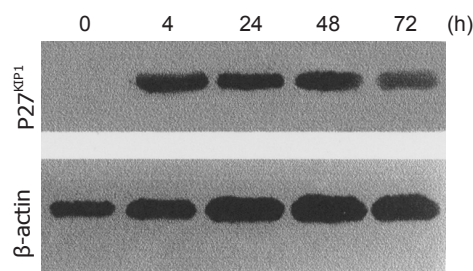

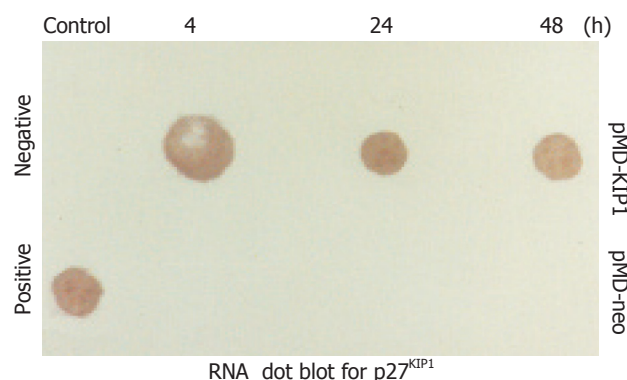**Figure 1** Induction of p27^KIP1 expression by Western blotting analysis.

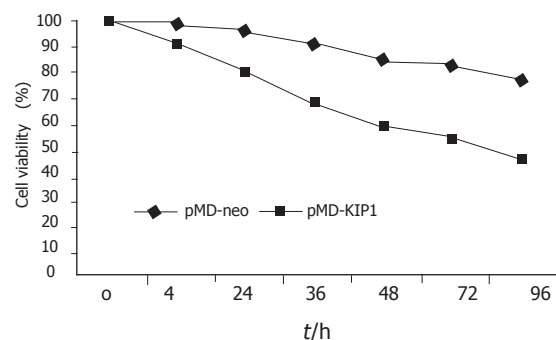**Figure 2** Expression of p27^KIP1 in mRNA level by RNA dot blot analysis.

Figure 3 Growth course of SGC7901 cells.

control SGC7901/pMD and SGC7901 cells formed large colonies in each well after 2 wk, and the rate of colony formation was increased by 80% and 60%, respectively. SGC7901/KIP1 cells showed markedly lower ability to form colony with a rate of 30% ($P < 0.01$). Moreover, tumors were generated in nude mice on the 7th d from SGC7901/pMD cells, but SGC7901/KIP1 cells generated tumor on the 12th d and grew slowly. The tumor diameter generated from SGC7901/KIP1 cells was smaller than that from SGC7901/pMD cells (0.55 ± 0.14 *vs* 1.36 ± 0.13 cm, $P < 0.01$, Figure 4). These data indicated that the exogenous $p27^{KIP1}$ had a cytostatic effect on cell growth.

Overexpression of p27^KIP1 prolonged cell cycle in G₁ phase

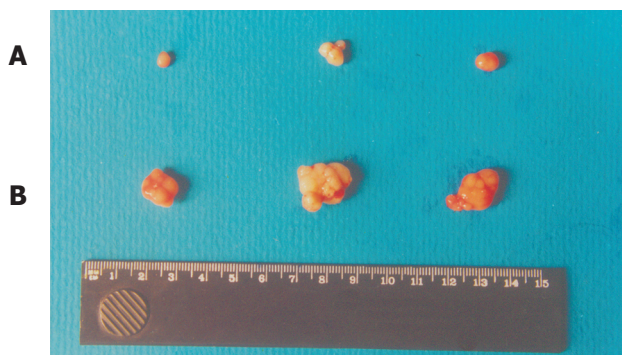$p27^{KIP1}$ was identified as a negative cell cycle regulator

Figure 4 Tumorigenicity in nude mice. **A:** SGC7901/KIP1 group; **B:** SGC7901 group.

in transforming growth factor β-treated cells and in G_1 phase quiescent cells by cell contact inhibition. The overexpression of $p27^{KIP1}$ protein in mammalian cells induced G_1 arrest of the cell cycle. In order to investigate whether $p27^{KIP1}$ had the ability to induce G_1 arrest in SGC7901 cells, cell cycle analysis was performed on transfected SGC7901 cells. Flow cytometry analysis showed that overexpression of $p27^{KIP1}$ increased remarkably the proportion of SGC7901 cells with G_1 phase DNA content increased from 33.7% to 69.3% ($P = 0.000$) and decreased with S phase DNA content at 48 h after induction (Figure 5). There was no significant change in the proportion of cells with G_2/M phase DNA content.

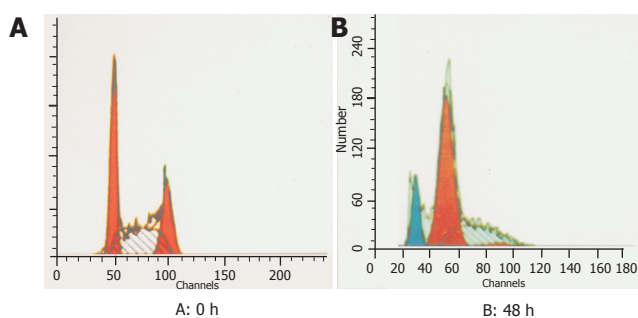### Overexpression of $p27^{KIP1}$-induced apoptosis in SGC7901 cells

Figure 5 Flow cytometric analysis of SGC7901 cells at 0 h (**A**) and 48 h (**B**).

Morphological changes SGC7901/KIP1 cells-transfected $p27^{KIP1}$ altered their morphology and induced DNA strand breaks in a manner consistent with apoptosis. The changes were indeed induced by apoptosis rather than necrosis, which was confirmed by electron microscopy. SGC7901/KIP1 cells showed compacted nuclear chromatin with fine granular masses marginated against the nuclear-enveloped and condensed cytoplasm, the nuclear outline was convoluted and the organelles were preserved (Figure 6).

TUNEL assay To determine whether overexpression of $p27^{KIP1}$ was able to induce apoptosis in SGC7901 cells, TUNEL assay was performed. Compared to the pMD-neo-transfected SGC7901/pMD cells, $p27^{KIP1}$-
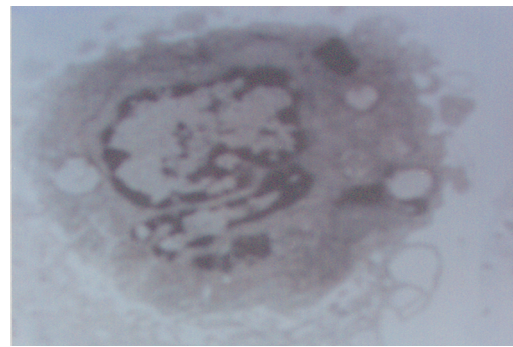

Figure 6 Ultrastructural changes associated with apoptosis in SGC7901/KIP1 cells (TEM×7 500).

overexpressed SGC7901/KIP1 cells showed obvious morphological changes. Some apoptotic cells appeared round and condensed cytoplasm as well as highly fragmented chromatin. TUNEL-positive cells were found with Kelly or green fluorescence in nucleoli. Cells without apoptosis observed by fluorescence microscopy had no fluorescence. Apoptotic index significantly increased in $p27^{KIP1}$-transfected SGC7901/KIP1 cells 24 and 48 h after being exposed to 100 mmol/L $ZnSO_4$ (3.0 *vs* 21.2, 3.6 *vs* 26.3, $P<0.05$).

Flow cytometry In order to determine the effect of $p27^{KIP1}$ overexpression on apoptosis in SGC7901 cells, cells were exposed to 100 mmol/L $ZnSO_4$ for 48 h and apoptotic damage of DNA was detected according to the sub-G_1 peak on a flow cytometer. Overexpression of $p27^{KIP1}$ showed significantly higher apoptotic peak than normal control as the sub-G_1 peak indicated. The apoptotic cells accounted for about 14.7% (Figure 5).

Taken together, $p27^{KIP1}$ overexpression could inhibit proliferation of SGC7901 cells and subsequently induce apoptosis.

DISCUSSION

Gastric cancer is the second most common fatal malignancy in the world[12-14] and is still the leading cause of cancer-related deaths in China. The development of gastric cancer is a multi-factorial process[15]. A balance between proliferation and apoptosis of tumor cells is important for tumor growth. In gastric cancer, proliferative activity and apoptosis of cancer cells are associated with tumor growth[16]. Cyclins, cyclin-dependent kinase (CDK) and their inhibitors regulate cell growth, differentiation, survival and death. Cell cycle which progresses sequentially through G_1, DNA synthesis (S), G_2, and mitosis (M) phases, regulates DNA replication and chromosomal segregation into the daughter cells. Gene abnormalities and

aberrant expression of cell cycle regulators play a pivotal role in the pathogenesis of gastrointestinal tumors[17], of which CDKIs are major regulators.

p27[KIP1] is a member of the Cip/Kip family of CDKIs that regulate cell cycle progression, thus inhibiting various cycle-CDK complexes. Physiologically, p27[KIP1] is believed to primarily regulate progression of cells from late G_1 into S phase by interacting with cyclin E-CDK2 complexes[9]. p27[KIP1] has been implicated as a mediator of growth arrest due to TGF-β, cAMP and other extracellular factors. Moreover, elevated expression of p27[KIP1] protein leads to G_1 arrest in many cell types and promotes neuronal differentiation in mouse neuroblastoma cells, while inhibition of p27[KIP1] expression through the use of antisense technology prevents G_1 arrest and/or suppresses entry of fibroblasts into a state of quiescence in response to mitogen depletion.

Interestingly, in many types of tumors such as gastric[18,19], prostate[20,21] and breast carcinoma, the expression of p27[KIP1] gene is downregulated[22,23]. Loss of p27[KIP1] expression may result in tumor development and/or progression. However, this loss of expression does not appear from the result of gene mutations. It is believed that the aberrant expression of p27[KIP1] plays a pivotal role in the pathogenesis of carcinoma. Malignant human renal tumor cell transfection with p27[KIP1] gene leads to inhibition of proliferation and cell cycle arrest in G_1[24]. Ectopic overexpression of p27[KIP1] is associated with a striking decrease in aneuploid cells, loss of anchorage-independent growth in soft agar and failure to induce tumor development in a xenograft model[25]. In this report, we have also demonstrated that p27[KIP1] overexpression could inhibit proliferation of SGC7901 cells. The cell growth was reduced by 31% at 48 h, after the induction with zinc as determined by cell viability assay. The alteration of cell malignant phenotype was evidently indicated by the loss of anchorage-independent growth ability in soft agar. The tumorigenicity in nude mice was reduced evidently ($P<0.01$). p27[KIP1] overexpression increased cell arrest from 33.7% to 69.3% ($P<0.01$) in G_1 population.

Recent evidence demonstrated that high levels of p27[KIP1] protein induced by adenovirus vector leads to growth arrest as well as enhancement of apoptosis in several cell lines from different species and tissues of various origins[11,25]. Decreased p27[KIP1], which is positively correlated with a decreased rate of apoptosis, may be not only an indicator of tumor aggressiveness, but also an important prognostic marker in gastric carcinoma[16]. In this study, we have demonstrated that overexpression of p27[KIP1] not only prolonged cell cycle in G_1 phase of SGC7901 cells but also subsequently induced apoptosis as reflected by pre-G_1 peak in the histogram of FACS, TUNEL assay and electron microscopy. This is the first report in which a significant correlation between p27[KIP1] expression and cell cycle arrest and apoptosis has been demonstrated in SGC7901 cell line.

Mice lacking p27[KIP1] are abnormally large and display multiple organ hyperplasia indicative of tissue overgrowth[26,27]. Thus, it is possible that p27[KIP1] plays an important role in maintaining organ size through the induction of apoptosis. However, the mechanism of tumor growth suppression appears to include p27[KIP1] but also various other properties such as p53. p53 serves as a major G_1 checkpoint regulator through the induction of p21 but not of p27[KIP1] and p57, while it induces apoptosis. The p53-mediated CDK inhibitor p21 does not lead to significant apoptosis though both p21 and p27[KIP1] induce G_1 arrest through their potential abilities to inhibit CDK activity. These findings suggest that the inhibition of CDK activity may not be sufficient to induce apoptotic cell death. A possible explanation of these differences between p27[KIP1] and p53-mediated p21 function is that p27[KIP1] may have other functions in addition to its notable function as a CDKI. It can also be speculated that p27[KIP1], which has the potential to induce growth arrest and apoptosis, may play a more important role in tumor growth suppression than p53-mediated p21. Further understanding of the mechanism involved in this process can improve chemotherapeutic strategies aimed at achieving high level p27[KIP1] expression and gene therapy.

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• *BASIC RESEARCH* •

Vitamin B12 deficiency and gastric histopathology in older patients

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Abstract

AIM: To compare upper gastric endoscopic and histopathologic findings in older adults in the presence and absence of B12 deficiency.

METHODS: A prospective analysis of upper gastric endoscopic and gastric histopathologic findings from 30 newly identified B12-deficient patients (11 males, 19 females) and 16 controls with normal B12 status (6 males, 10 females) was performed. For all subjects, the indication for upper endoscopy and gastric biopsy were unrelated to B12 status. A single pathologist, blinded to B12 status, processed and interpreted the biopsy samples. Endoscopic and histopathologic findings were correlated with age, gender, hematocrit (Hct), MCV and B12 status.

RESULTS: The B12-deficient group had significantly lower mean serum B12 levels compared to the controls ($P < 0.00005$) while their mean Hct, MCV and serum albumin levels were similar. Iron deficiency (ferritin-based) was present in 21% of B12-deficient patients and intrinsic factor antibodies were present in 29% (5/17) of B12-deficient patients. The endoscopic findings revealed significantly different rates of gastritis and atrophy between the B12-deficient and control groups ($P = 0.017$). B12-deficient patients had significantly less superficial gastritis (62% *vs* 94%) and significantly more atrophic gastritis (28% *vs* 0%) as compared to the controls ($P = 0.039$). Intestinal metaplasia was similar in both groups. *Helicobacter pylori* infection rates were similar in the B12-deficient patients and controls (40% *vs* 31%).

CONCLUSION: Significantly different endoscopic findings and types of gastritis could often be observed in the presence and absence of B12 deficiency. Atrophy, based on endoscopy, and atrophic gastritis, based on histopathology, suggest the presence of B12 deficiency. Gastric histopathology is not influenced by the age, gender, Hct or MCV of the patients.

INTRODUCTION

Vitamin B12 deficiency is a common but under-recognized disorder with a prevalence ranging from 3% to 40% in the adult population[1-4]. In a previous study, we noted a prevalence of between 15% and 25% in the community and nursing home hospitalized older subjects[3]. B12 deficiency often goes undetected, with manifestations that range from asymptomatic to a wide spectrum of hematologic and/or neuropsychiatric features. For the purpose of this discussion, the term B12 will be used interchangeably with cobalamin.

B12 deficiency affects proliferating epithelium at all the sites[5]. Although the histopathological effects of B12 deficiency relating to the blood and nervous system are well described, histopathological changes in the stomach associated with cobalamin deficiency in older subjects have

received less emphasis in the literature. Presently, there exist some confusions whether gastric changes are a cause or an effect of deficiency[6]. It is worth stating that the stomach plays a major role both in the absorption of B12 and the pathogenesis of cobalamin deficiency[5,7]. Among the etiologies of cobalamin deficiency, pernicious anemia (PA), once believed to be the most common cause[6,9], actually accounts for only a small fraction of cases of B12 deficiency[2,3,8]. It is well recognized that PA, a type A gastritis, is characterized by fundic atrophic gastritis. There is a lack of knowledge about the examination of gastric endoscopic findings and histopathologic changes associated with vitamin B12 deficiency in older adults. We, therefore, have attempted to evaluate the endoscopic and histopathologic changes in older adults with newly diagnosed B12 deficiency and to compare the findings in adults of the same age group and normal B12 status.

MATERIALS AND METHODS

The study was performed by the staff from the divisions of geriatrics, gastroenterology, medicine, pathology and biomedical research at Our Lady of Mercy Medical Center, University Hospital of New York Medical College, Valhalla, NY, USA. A prospective analysis of upper gastric endoscopic and gastric histopathologic findings was performed on 30 adults who were above the age of 60 with newly diagnosed B12 deficiency. The subjects included men and women, ages ranging from 60 to 99 years. All patients required hospitalization for a cause other than B12 deficiency. The diagnosis of B12 deficiency was made following routine screening or during evaluation for hematological or neurological manifestations. A randomly selected group of 6 male and 10 female patients with normal B12 status, ages ranging from 63 to 93 years, served as controls. The male/female ratio in the B12-deficient group was 11/19, and 6/10 in the control group. Several indicators of nutritional status were assessed in the B12-deficient patients. We observed low folate status in 16%, low albumin status in 16% and low iron status (ferritin based) in 21% of the B12-deficient patients.

The primary indication for endoscopy was unrelated to cobalamin status and based on the usual indications for upper gastric endoscopy on both B12-deficient patients and controls. After obtaining informed consent, upper gastric endoscopy was performed and multiple endoscopic biopsies were obtained from several sites within the gastric fundus and antrum. Gastric biopsies were performed for reasons unrelated to cobalamin status and hence the sites examined and the number of biopsies taken varied between individuals. Endoscopic findings included a normal appearance of the stomach, atrophy and gastritis. Gastritis on endoscopy was defined as the presence of erosions or hemorrhage. Erosions were seen as breaks in the mucosa manifesting as multiple lesions with white bases that were commonly encircled by a halo of erythema. Atrophy on endoscopy was defined as the disappearance of gastric rugae and a thinning of the gastric mucosal folds with the prominence of the blood vessels seen through the

thin mucosa (paper money appearance)[10]. Erosive gastritis and friable mucosa are suggestive findings of gastritis on endoscopy.

Gastric histopathology entailed the use of formalin-fixed and paraffin-embedded tissues, which were sectioned at five micrometer thickness, mounted on slides and stained with hematoxylin and eosin. All specimens (in both the B12-deficient group and controls) were reviewed by a single histopathologist who was unaware of the individual's B12 status. Biopsy findings were classified as normal (Figure 1A), inflammation (Figures 1B-D), atrophy and intestinal metaplasia (Figures 1B and C). Each specimen was also stained for the presence of *helicobacter*-like organisms using toluidine blue stain with Alcian yellow counterstain (Polyscientific, Bayshore, NY, USA) (Figure 1E). See Table 1 for definitions.

For the purpose of this study, B12 status was defined as normal, borderline and deficient as follows: normal >349 ng/L; borderline 100-349 ng/L; and deficient <100 ng/L. The reference range for cobalamin values in our laboratory is 157-1 059 ng/L. Endoscopic and histopathologic findings were correlated with age, gender, hematocrit

Figure 1 A: Normal histopathologic appearance of the gastric mucosa; **B:** Histopathology of the gastric mucosa, antral type, showing chronic inflammation with metaplasia (low power); **C:** Gastric mucosa, antral type (at higher power than in Figure 1B) showing chronic inflammation with intestinal metaplasia; **D:** Gastric biopsy showing presence of severe chronic active gastritis; **E:** Toluidine blue stain with alcian yellow counterstain of gastric mucosa revealing presence of *H pylori*.

Table 1 Histopathology: Definitions

- Chronic atrophic gastritis (CAG): more extensive inflammation accompanied by glandular atrophy. CAG further subdivided into mild, moderate and severe based on atrophy involving the upper one-third, upper two-thirds and full thickness of the mucosa respectively.
- Chronic superficial gastritis (CSG): inflammation limited to the foveolar region unaccompanied by glandular atrophy. (Figures 1B-1D).
- Gastric atrophy (GA): thinning of the mucosa with an absence of inflammatory changes.
- Hyperplasia: an increase in the number of mucosal epithelial cells.
- Metaplastic changes:
    Intestinal metaplasia characterized by goblet cells, brush border cells, Paneth cells and endocrine cells. (Figures 1B and 1C).
    Pyloric metaplasia of the fundus characterized by mucus secreting glands.

Table 3 Endoscopy findings

B12 deficient group ($n = 30$) (%)		Control group ($n = 16$) (%)	
Normal	11 (36.7)	Normal	5(31.3)
Gastritis	8 (26.7)	Gastritis	11(68.8)
Atrophy	8 (26.7)	Atrophy	0(0)
Polyps	1 (3.3)	Polyps	0(0)
Others [1]	2 (6.7)	Others	0(0)

[1] (includes 1 case of hiatal hernia and 1 case of candidiasis). Endoscopic findings between groups were compared using Fisher's exact test, (P=0.017).

Table 2 Baseline patient characteristics

Variable	B12 deficient ($n = 30$)	Controls ($n = 16$)	P-value
Age (yr)	78 ± 11	77 ± 8	0.9090 [1]
Gender (M:F)	11:19	6:10	1.0000 [2]
Serum B12 (pg/L)	183 ± 60	872 ± 459	<0.00005 [1]
Hematocrit (%)	34.9 ±4.8	33.4 ± 3.7	0.3010 [1]
MCV (%)	86.5 ± 7.6	87.4 ± 9.9	0.7321 [1]
Serum albumin (g/dL)	2.9 ± 0.5	2.9 ± 0.7	0.9692 [1]
H pylori infection (+/-)	12 / 18	5 / 11	1.000 [2]
Intrinsic factor Ab (+/-)	5/ 12	-	-

[1]Data presented as the means ± SD or actual numbers. Statistical comparisons used Student t-tests or χ^2-square analysis.

(Hct), MCV and B12 status. Additional tests, such as serum homocysteine and methylmalonic acid levels, which further delineate cobalamin status, were not performed in this study. Neither did we undertake to elucidate the etiology of B12 deficiency, barring tests for intrinsic factor antibodies in 23 patients in the B12-deficient group.

Statistical analysis

Statistical comparisons of sample means between patients and controls were performed using Student's *t*-tests while comparisons of proportions were carried out using Fisher's exact test. Logistic regression analysis was used to examine the effect of aging, gender, and nutritional values on gastric histopathology.

RESULTS

Baseline characteristics of the study samples are shown in Table 2. The results of histopathological and endoscopic findings are shown in Tables 3-8. A statistically significant difference was observed for the endoscopic findings between the B12-deficient patients and controls (P=0.014). The prevalence of normal endoscopic appearance was similar in both groups. However, atrophy was noted only in patients with B12 deficiency, while gastritis was more common in patients with normal B12 status. Polyps, notably of the hyperplastic type, were more frequently detected in the presence of cobalamin deficiency (Table 3). We noted a statistically significant difference in the degree

of gastritis between B12-deficient patients and controls (P=0.039). Atrophic gastritis was more common in individuals with B12 deficiency, while superficial gastritis was the most common finding in controls. The incidence of intestinal metaplasia (in the antrum) was similar in the individuals with or without B12 deficiency. None of the controls had features of atrophy on endoscopy or histopathology. In this study, age and gender had no influence on gastric histopathology. In addition, endoscopic findings and gastric histopathology were not related to Hct levels (Tables 4-7).

Our findings suggested that the rate of *H pylori* infection was similar in patients with B12 deficiency and controls (40% *vs* 31%; Table 8). Twenty-six percent of the B12-deficient patients had undiagnosed PA as demonstrated by the presence of intrinsic factor antibodies. Age appeared to have no effect on the presence or absence of intrinsic factor antibodies that is consistent with the literature. Parietal cell antibodies (not tested in our study), which are less specific, are known to increase with age[8]. The presence of intrinsic factor antibodies also was not related to *H pylori* infection (Table 8). Histopathological features also tended to overlap in both the groups.

DISCUSSION

Physiological changes accompanying the aging gut, in particular the stomach, may be difficult to differentiate from the disease[11]. Studies in older adults have suggested a high prevalence of subnormal cobalamin concentrations, and in some reports, an inverse relationship between age and serum cobalamin concentrations[12,13]. The most common etiology of vitamin B12 deficiency is food-cobalamin malabsorption resulting from gastric dysfunction. This entity is defined as the inability to absorb protein (or food) bound cobalamin, although the ability to absorb free cobalamin remains intact. Food cobalamin malabsorption may be a consequence of the use of acid-lowering agents, such as proton pump inhibitors (PPI) and histamine 2 receptor antagonists[2,14,15]. A small proportion of patients on continuous, long-term PPI therapy may manifest reduced cobalamin levels, however, a resultant distinct B12 deficiency is seldom reported[16]. Our earlier data suggested that the use of H2 antagonists and PPI

Table 4 Histopathology of the gastric fundic region

B12 deficient group (*n* = 27) (%)		Control group (*n* = 4) (%)	
Normal	3 (11.1)	Normal	0 (0)
Inflammation	16 (59.3)	Inflammation	4 (100)
Atrophy	8 (29.6)	Atrophy	0 (0)

Histopathology findings between groups were compared using Fisher's exact test, (*P*=0.541).

Table 5 Histopathology of the gastric antral region

B12 deficient group (*n* = 6) (%)		Control group (*n* = 12) (%)	
Normal	0 (0)	Normal	2 (14.3)
Inflammation	5 (83.3)	Inflammation	6 (42.9)
Hyperplasia	1 (16.7)	Hyperplasia	0 (0)
Intestinal metaplasia	0 (0)	Intestinal metaplasia	4 (28.6)

Histopathology findings between groups were compared using Fisher's exact test, (*P*=0.157).

Table 6 Gastritis types

B12 deficient group (*n* = 29) (%)		Control group (*n* = 16) (%)	
Normal	3 (10.3)	Normal	1 (6.3)
Superficial gastritis	18 (62.1)	Superficial gastritis	15 (93.7)
Atrophic gastritis	8 (27.6)	Atrophic gastritis	0 (0)

Gastritis types between groups were compared using Fisher's exact test, (*P*=0.039). The B12 deficient group is 29 in Table 6 *vs* 30 in Tables 7 and 8 because one biopsy sample could not be classified into a specific category.

Table 7 Metaplasia in B12 deficiency and controls

B12 deficient group (*n* = 30) (%)		Control group (*n* = 14) (%)	
No metaplasia	22 (73.3)	No metaplasia	10 (71.4)
Hyperplasia	1 (3.3)	Hyperplasia	0 (0)
Intestinal metaplasia	7 (23.3)	Intestinal metaplasia	4 (28.5)

Metaplasia between groups was compared using Fisher's exact test, (*P*=1.000).

Table 8 Frequency of *H pylori* infection

B12 deficient group (*n* = 30) (%)		Control group (*n* = 16) (%)	
H pylori positive	12 (40)	*H pylori* positive	5 (31.2)
H pylori negative	18 (60)	*H pylori* negative	11 (68.8)

Fisher's exact test determined that *H pylori* infection rates were similar in B12 deficient and control patients (*P* = 1.000) and that the presence of intrinsic factor antibodies was low and unrelated to *H.pylori* infection in the B12 deficient group (1/7 in *H pylori*+ *vs.* 0/10 in *H pylori*– patients, *P* = 0.338; data not presented).

over two years did not produce cobalamin deficiency[17]. As stated earlier, PA accounts only for a small number of cases of B12 deficiency. PA is characterized by an autoimmune gastric atrophy (GA) mediated by anti-intrinsic factor antibodies. Histomorphologically, PA is characterized by fundic atrophic gastritis (type A) leading to atrophy of the fundus and achlorhydria[18]. While the

fundus and the body of the stomach contain acid-secreting gastric parietal cells and pepsinogen-secreting zymogenic cells, the antrum possesses gastrin-producing G cells. Chronic atrophic gastritis (CAG) is histopathologically classified into two types based on the presence or absence of antral involvement[8,19]. Strickland *et al*[19]. originally divided chronic nonspecific gastritis into type A (associated with PA) and type B (not associated with PA). Others have added type C (chemical gastritis, related to drug therapy or bile reflux)[20,21]. Though these concepts are still valid, the discovery of *H pylori* as an important cause of gastritis signifies that type B gastritis is now too broad a category and may not be as useful clinically. Hence, some authors suggest abandoning the alphabetic terminology[20]. Type A (autoimmune) gastritis involves the fundus and the body of the stomach and spares the antrum, whereas type B (non-autoimmune) gastritis involves the antrum as well as the fundus and body. Type A gastritis is associated with PA and the presence of auto-antibodies to gastric parietal cells and to intrinsic factor, achlorhydria, low serum pepsinogen 1 concentrations and high serum gastrin levels as a result of the lack of negative feedback inhibition by gastric acid and consequent hyperplasia of gastrin-producing cells. Type B gastritis, which is more common, is usually associated with *H pylori* infection, alcoholism and various medications, and is characterized by low serum gastrin concentrations because of the destruction of the gastrin-producing cells associated with antral gastritis[8,22]. Gastric biopsy specimens in PA demonstrate a mononuclear cellular infiltrate in the submucosa consisting of plasma cells, T cells and a large non-T cell population, extending into the lamina propria between the gastric glands[8,23]. The infiltrating plasma cells contain auto-antibodies to parietal cell antigen and to intrinsic factor[8,24]. Extension of the cellular infiltrate into the mucosa is accompanied by degenerative changes in parietal cells and zymogenic cells. With fully established lesions, there is a marked reduction in the number of gastric glands, parietal and zymogenic cells, with replacement by mucus containing cells[8]. More common than PA is a state, usually in the older patient, in which there is diffuse severe atrophic oxyntic gland gastritis with achlorhydria but the residual ability to absorb vitamin B12. To prove the existence of severe oxyntic gland gastritis, biopsy specimens are best taken from the midbody region on the greater curve since the mucosa on the lesser curve is thinner and can be falsely interpreted as atrophic. Severe oxyntic gland atrophy is commonly associated with only mild inflammatory cells infiltrate and a reduced prevalence of *H pylori*. The other features include varying amounts of metaplasia[10]. Hyperplastic or inflammatory polyps are the most common lesions found in endoscopic surveys in PA with a prevalence of 10-40%[10,25].

Characteristic gastric features, both gross and histopathologic, are not well described with B12 deficiency resulting from causes other than PA, particularly in older subjects. For this reason, we have attempted to relate gastric histopathology with B12 status in an attempt to examine whether gastric histopathology is a cause or an

effect of B12 deficiency. Furthermore, B12 deficiency can affect proliferating cells at all sites and so an association between B12 status and gastric histopathology would help in the development of strategies for screening and early diagnosis of B12 deficiency based on endoscopic and histopathologic findings.

Our study demonstrated that a normal endoscopic appearance was not correlated with B12 status but that endoscopic findings of atrophy and gastritis were significantly different between B12-deficient patients and controls ($P = 0.017$). Similar rates of normal and abnormal histopathologic findings were observed in older adults with and without B12 deficiency. Inflammation was the predominant histopathologic finding in older adults with and without B12 deficiency. Our findings suggested that histopathologic findings were variable and not correlated with B12 status, however, a statistically significant difference in the degree of gastritis was noted between the B12-deficient patients and controls ($P=0.039$). Atrophic gastritis was more common in the B12-deficient patients, while superficial gastritis was more common in the controls. It should also be noted that atrophy was not seen when B12 levels were normal. In these samples of older adults, neither increasing age nor gender influenced gastric histopathology result, while endoscopic findings and gastric histopathology bore no relationship to Hct. There is a strong evidence that *H pylori* infection is associated with cobalamin deficiency; this is true even with non-ulcer dyspepsia or the presence of minimal to no GA[26]. Whether this is merely an association or a cause and effect relationship is unclear. In an earlier study, two-thirds of the patients with atrophic gastritis of the body had *H pylori* infection[27]. Eradication of *H pylori* infection alone has been reported to correct B12 status and improve anemia in B12-deficient individuals[28]. Thus, the possible role of *H pylori* infection in cases of severe food cobalamin malabsorption suggests specific options to prevent and treat B12 deficiency when associated with *H pylori* infection[29].

We observed a 40% rate of *H pylori* infection in patients with cobalamin deficiency compared to a 31% rate in individuals with normal status. Though this finding was not statistically significant (Figure 1E), the comparative trend supported the belief that *H pylori* infection occurs more frequently in B12 deficiency and is consistent with recent studies that implicate *H pylori* as an etiological factor for B12 deficiency[28]. The data may not be uniform in all countries as concluded in a Japanese study that noted higher rates of both *H pylori* infection and atrophic gastritis in Japan where PA is uncommon[30]. The presence of intrinsic factor antibodies were not correlated with the presence or absence of *H pylori* in the present study. Moreover, intrinsic factor antibodies were present in 29% (5/17) of cobalamin-deficient patients, suggesting a higher prevalence of PA than the previously reported prevalence of less than 5% in B12 deficiency patients[31]. Also, age had no effect on the presence or absence of intrinsic factor.

We recognized that this study had some limitations. The total number of subjects was not large, and not

every individual had biopsies from the gastric fundus, a site recognized for histopathologic changes in B12 deficiency[18]. In control subjects, although a small number of biopsies were obtained from the fundus, the reality was that a number of biopsies contained inadequate tissue for meaningful interpretation. In addition, intrinsic factor antibodies and ferro-kinetics were not obtained in all the patients. Iron deficiency is known to be associated with gastric histopathological changes and 4 of 19 patients tested with ferro-kinetics were noted to have iron deficiency as demonstrated by the ferritin assay. We also did not have precise data on the specifics of treatment for *H pylori* infection prior to endoscopy. Our aim was to evaluate endoscopic and histopathologic findings in B12 deficiency and controls irrespective of etiology and to attempt to decipher the changes related to cobalamin deficiency. This study made no attempt to delineate the etiology of B12 deficiency in our subjects. As stated, the indication for endoscopy was other than for an evaluation of B12 status. Thus, it is also possible that the primary disease process necessitating the endoscopy may have affected the gastric mucosa and influenced the histopathologic findings. Finally, the extent of injury from medications, bile reflux or infection also could not be assessed.

In conclusion, abnormal gastric endoscopic findings appear to be correlated with B12 levels, with GA being the predominant finding in cobalamin deficiency and gastritis being the common finding when B12 levels are normal. Normal en doscopic findings are also observed in B12-deficient as well as in control subjects. Histopathology results are variable, with a predominance of inflammation that lacks any correlation with B12 deficiency. However, gastritis type does correlate with B12 status in that atrophic gastritis is more prevalent in B12 deficiency and superficial gastritis is more common when B12 status is normal. GA is absent on both endoscopy and histopathology, when B12 levels are normal. Our findings are consistent with literature in that *H pylori* infection is associated with cobalamin deficiency, implicating *H pylori* as an etiological factor for B12 deficiency.

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• *BASIC RESEARCH* •

Reperfusion injury after critical intestinal ischemia and its correction with perfluorochemical emulsion "perftoran"

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Abstract

AIM: To investigate the anti-ischemic properties of perfluorochemical emulsion "perftoran" in mesenteric region.

METHODS: Experiments were conducted on 146 nonlinear white male rats weighing 200-350 g. Partial critical intestinal ischemia was induced by thorough atraumatic strangulation of 5-6 cm jejunal loop with its mesentery for 90 min. Global critical intestinal ischemia was made by atraumatic occlusion of the cranial mesenteric artery (CMA) for 90 min also. Perftoran (PF, 0.8-1.0 mL per 100 g) in experimental groups or 0.9% sodium chloride in control groups was injected at 75 min of ischemic period. Mean systemic arterial blood pressure (BP$_M$) registration, intravital microscopy and morphological examination of ischemic intestine and its mesentery were performed in both groups.

RESULTS: During 90 min of reperfusion, BP$_M$ progressively decreased to 27.3±7.4% after PF administration *vs* 38.6±8.0% in the control group of rats with partial intestinal ischemia (NS) and to 50.3±6.9% *vs* 53.1±5.8% in rats after global ischemia (NS). During the reperfusion period, full restoration of microcirculation was never registered; parts with restored blood flow had leukocyte and erythrocyte stasis and intra-vascular clotting, a typical "non-reflow" phenomenon. The reduction of mesenteric 50-400 μm feeding artery diameter was significantly less in the PF group than in the control group (24±5.5% *vs* 45.2±3.6%, *P*<0.05) 5 min after partial intestinal ischemia. This decrease progressed but differences between groups minimized at the 90th min of reperfusion (41.5±4.2% and 50.3±2.8%, respectively). In reperfusion of rat's intestine, a significant mucosal alteration was registered. Villous height decreased 2.5-3 times and the quantity of crypts decreased more than twice. In the group of rats administered PF, intestinal mucosal layer was protected from irreversible post-ischemic derangement during reperfusion. Saved cryptal epithelial cells were the source of regeneration of the epithelium, which began to cover renewing intestinal villi after 24 h of blood flow restoration. View of morphological alterations was more heterogeneous in CMA groups.

CONCLUSION: Systemic administration of perftoran promotes earlier and more complete structural regeneration during reperfusion in rats after partial and global critical intestinal ischemia.

INTRODUCTION

Prophylaxis and treatment of ischemia-reperfusion injuries in common and especially in mesenteric circulation are the most actual problems of modern-day surgery, intensive care medicine and pathology, and still require improved understanding of the pathophysiology and mechanisms of the development of these disorders[1,2]. Pathophysiological derangement, which occurs after restoration of a blood flow known as the reperfusion syndrome, continues to be responsible for the very high mortality rate in the fields of surgery associated with acute mesenteric occlusion or

intestinal strangulation[3,4]. There is no generally accepted opinion about the critical terms of intestinal ischemia. During this time, the answer to this question has not only theoretical but also important practical significance. For example, this problem is one of the main aspects in the field of intestinal transplantation[5,6].

There has been extraordinary progress in the treatment of ischemia-reperfusion injuries over the past two decades. Though advances have been made in the management of acute vascular occlusion, the optimal treatment of a reperfusion injury is still not defined. Perfluorocarbon emulsion (PFCE) is among the medicines administered in different cases of ischemia-reperfusion injuries. Investigations in this field are one of the most priority branches of scientific researches in the world. Designation and intensive investigation of the effects of PFCE in different fields of biology and medicine have been conducted by a number of Russian scientists since 1979 under the general direction of the Institute of Biological Physics of the Acad. Sci. USSR. Perftoran (OAO NPF "Perftoran", Puschino Russia) is a well-known PFCE containing 20% of perfluorodecalin and perfluoromethylcyclohexylpiperidin stabilized by proxanol-168. The diameter of an average particle is about 0.07 μm. Local (intraluminal or intraperitoneal) administration of PFCE on intestinal ischemia-reperfusion injury has been extensively elucidated[7-13].

MATERIALS AND METHODS

Procedures
All animal procedures were performed in accordance with the recommendations for the proper use and care of laboratory animals. Experiments were performed on 146 white nonlinear adult male pathogen-free rats weighing 200-350 g. Before the surgery, they were anesthetized with pentobarbital sodium (CPF, Tallinn, Estonia) (5-7 mg per 100 g, i m). Except for chronic experiments, tracheostomy cannula was inserted and animals were supported by time-cycled, volume-limited ventilation with room air by mechanical respirator 4601-1 Advanced (Technical and Scientific Equipment, GmbH, Germany) at the frequency of 70 breaths/min and the tidal volume of 5 mL/kg.

Animal models
The rats were then randomized into two groups depending on the type of chosen critical intestinal ischemia. Each group was divided into two subgroups. In the subgroup A, perftoran was used as a protective remedy before reperfusion. In the subgroup B, the same volume of 0.9% sodium chloride was injected as a control.

In group 1 (*n* = 110), animals were exposed to a critical segmentary (local) tourniquet ischemia of the intestine. This model corresponded to incarcerated hernia or different types of bowel strangulation such as commissural ileus, volvulus, formation of knots and others. After a midline abdominal incision was made, intestinal ischemia was produced by atraumatic strangulation of the jejunal loop (5-6 cm in length) and its mesentery (15-30 cm distal

after duodeno-jejunal flexion) with thick tourniquet until visual absence of regional artery pulsation for 90 min. In subgroup 1A (*n* = 48), perftoran (1 mL per 100 g) was injected slowly intra-arterially (i a) at 75 min of ischemic period. In subgroup IB (control group, *n* = 62), 0.9% sodium chloride solution was injected i.a. at the same time and volume.

In group 2 (*n* = 36), rats were exposed to a critical global (total) ischemia of the intestine. This model corresponded to acu 2A (*n* = 22), PF (1 mL per 100 g, 5-6% of circulating blood volume) was injected i a at 75 min of ischemic period. In subgroup 2B (control group, *n* = 14), 0.9% sodium chloride solution was injected i.a. at the same time and volume.

External hemoexfusion volume was thoroughly counted during the experiments and 0.95% sodium chloride (2:1) was injected i.a. in order to fill up the blood loss and maintain the volume of circulating plasma. In chronic experiments (*n* = 17), gut contents were replaced in the abdominal cavity, the incision was closed with suture technique and animals were returned to the prone position.

Blood pressure registration
Caudal or femoral arteries were prepared and cannulated by polyethylene (PE)-10 or PE-50 catheters (Becton Dickinson, USA) after i.a. heparin administration (30 ME per 100 g). During the experiments, mean systemic arterial blood pressure (BPM) was monitored by pressure transducers of Bentley Trantec and Statham P231D (Cobe Laboratories, Inc., USA) or MPU-05-290-0-III (San-EI, Japan) connected to the polygraph Schwarzer (Picker International GmBH, Munchen, Germany) or the automatic recorder 142-8 (San-EI, Japan).

Intravital microscopical examination
After a midline abdominal incision was made, the chosen segment of the proximal jejunum with mesentery was extracted on a transparent table of the microscope and covered by a transparent film to prevent tissue withering. Rectal temperature of the animal and intestinal loop was kept at 36.5 ℃. Rat's mesenteric microcirculatory bed was observed under a through-passage light bio-microscope MBI-15 (LOMO, Russia) supplied with photo-camera Zenit-ET (Zenit, Russia), an original device for registration of micro-vessel diameters by the splitting image method[14]. An analogous signal with amplitude proportional to the external vessel diameter was registered simultaneously with BPM line on the automatic recorder 142-8. Diameters of the mesenteric micro-vessels were registered before ischemia and during 90 min of reperfusion.

Morphological examination
Tissue samples were harvested from ischemic zone in experiments with tourniquet intestinal loop strangulation. In experiments with mesenteric occlusion, a very heterogeneous view of the injured intestine was registered. Morphological examination of the different segments of the intestinal wall was performed. Morphological samples were harvested just before the end of 1.5 ischemic period

and after 1.5 and 24 h of reperfusion.

Tissue samples of the intestinal wall were prepared as rings, fixed in 10% neutral buffered formalin and embedded in paraffin. Four-to six-micrometer-thick sections were stained with hematoxylin and eosin and examined under light microscope. PAS-reaction was also conducted. Morphological examination was performed on 243 sections of the rat's intestine by the "blind" method. The numerical six-step scale proposed in 1970 by Chiu et al[15]. was used for qualitative evaluation of the degree of the mucosal layer alterations.

Statistical analysis

Data were analyzed with "Microsoft Excel 97 SR-2" statistical software. All values were expressed as mean±SD. Paired and unpaired t-criteria were used to test the differences within and between groups, respectively. $P<0.05$ was considered statistically significant.

RESULTS

Microcirculatory disorders

Tourniquet application on the intestinal loop (group I, $n = 42$) led to complete blood flow interruption in the mesenteric bed and intestinal wall. Erythrocyte diapedesis around the mesenteric micro-vessels increased with terms of ischemia. Capillary network structure underwent significant alterations. One minute after tourniquet removal, blood flow was registered visually as a rule, only in the large mesenteric vessels and their branches directly before the intestinal wall, the so-called "feeding" arteries and veins with a diameter from 100 to 400 μm (Figure 1). In smaller vessels, full restoration of microcirculation was never registered; parts with restored blood flow had leukocyte and erythrocyte stasis and intra-vascular clotting, a typical "no-reflow" phenomenon. Hemorrhages occupied considerable areas of intestinal mesentery. During the reperfusion period, decreased blood flow velocity, increased number of leukocytes with activated aggregation and leukocyte-endothelial wall interactions were also registered in the micro-vessels.

During the initial phase of global intestinal ischemia (group 2, $n = 9$) in some large mesenteric arteries, no total stasis but slow blood flow was registered in contrast with total mesenteric occlusion. Probably, total mesenteric occlusion activated the collateral circulation of ischemic intestine in the first few minutes. After 15-20 min of ischemia, full stasis came in the mesenteric bed. After the removal of occluder from CMA, blood flow was restored in large arteries and veins which divides mesentery on transparent "windows" and in branches near the intestinal wall, the "feeding" vessels. In smaller blood vessels of mesentery, the "no-reflow" phenomenon progressed within 90 min of reperfusion.

In general, reperfusion injuries of mesenteric microcirculation did not change either after partial tourniquet ischemia or CMA occlusion, but impairments were more heterogeneous in rats of group 2.

Blood pressure dynamics

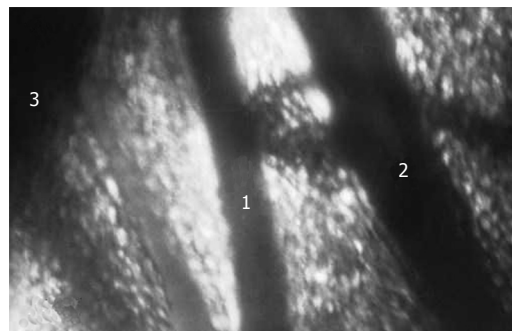Systemic BP$_M$ level during 90 min of partial and global

Figure 1 View of the "feeding" artery in rat's intestinal mesentery in light microscope. **1:** "feeding" vein; **2:** "feeding" arteries; **3:** mesenteric edge (wall) of intestine.

intestinal ischemia and reperfusion is shown in Figure 2A. BP$_M$ level did not change during the ischemic period but abrupt decrease was significant at 5 min of reperfusion (about 40% in cases with CMA occlusion). This systemic BP$_M$ level decrease continued till the end of the observation period (90 min of reperfusion).

Effects of perftoran

Administration of PF in subgroups 1A and 2A (partial and global intestinal ischemia) had no impact on the general picture of microcirculatory alterations in the intestinal mesentery during reperfusion. As in the control group, the systemic BP$_M$ level progressively decreased in the post-ischemic period (Figure 2B). As shown in Figures 2A and B, changes in the BP$_M$ level during reperfusion had no differences after the administration of PF before the end of the ischemic period and in control rats. There were no significant differences between subgroups 1A and B or between subgroups 2A and B.

Though there were no post-ischemic significant changes in microcirculation of rat's intestinal mesenteric bed, intestinal blood supply was restored. During this time, blood flow restoration was not complete because the diameters of the "feeding" arteries in reperfusion period were significantly smaller. In eight rats of the subgroup 1A injected with PF before the end of ischemia, diameter of 19 "feeding" arteries at 5 min of reperfusion became smaller. At the same time, in six rats of subgroup 1B (15 arteries) this value differed ($P<0.05$, Figure 3). During this time, the diameter of mesenteric "feeding" arteries continued to decrease, but differences between groups became less visible after 90 min of reperfusion. In nine rats of subgroup 2A injected with PF before CMA occluding, micro-clamp removing vascular reaction was heterogeneous. The diameter of 15 "feeding" arteries at 5 min of reperfusion was decreased by 10% in comparison with the initial one. In contrast, this value in 10 arteries was increased by 23%. The increase of some feeding artery diameters in post-ischemic period was typically exact for global model of rat's intestinal ischemia.

Morphological examination

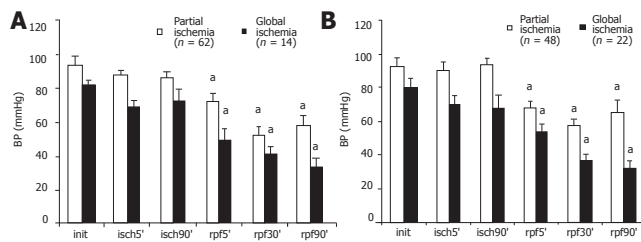Morphological examination of the intestinal wall sections

Figure 2 Systemic BPₘ at 90 min of partial and global intestinal ischemia and during reperfusion after 0.9% sodium chloride injection (**A**) and perftoran administration (**B**). BPₘ level decreased more significantly in groups after CMA occlusion. [a]*P*<0.05 *vs* initial values in each group.
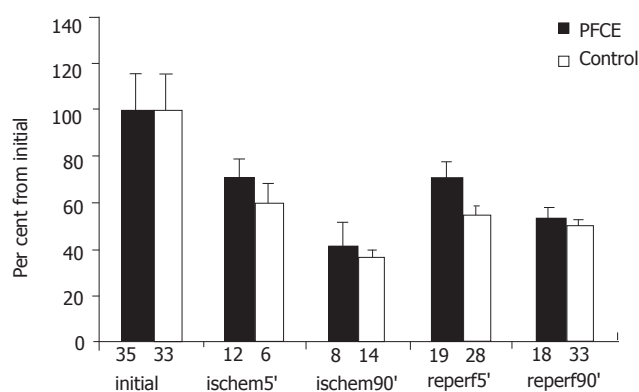

Figure 3 Diameter of "feeding" arteries at 90 min of partial intestinal ischemia and during reperfusion. The recovery of the diameter to initial value during reperfusion was more significant in the group administered PF in comparison with control group. [a]*P*<0.05 *vs* control group; initial – initial values, ischem-ischemic period, reperf-reperfusion.

harvested in group I (partial tourniquet ischemia) only revealed moderate alterations of mucosal layer such as slight apical swelling of intestinal villi with "lifting" of apical epithelium in the ischemic period. But within 90 min after tourniquet removal, degree of intestinal wall alteration progressively increased in comparison to the ischemic period. Therefore, impairment of crypts at 90 min of reperfusion was registered in rats of subgroup IB, spacious parts of intestinal luminal surface were completely denuded with marked villous architecture destruction (Figure 4A). At the same time, in rats of subgroup IA (administered PF) the intestinal wall structural safety was higher in the reperfusion period than in control rats (Figure 4B). Necrobiotic processes and structural changes in rats of subgroup IB progressed with the appearance of focal transmural necroses in the ischemic intestinal wall within 24 h after the end of ischemia (Figure 4C). In rats of subgroup IA (administered PF), alteration was not so significant and even reparative processes such as restoration of villous architecture and epithelial layer regeneration were also marked (Figures 4D and 5). Morphometric analysis data of intestinal wall sections in group I are presented in Table 1.

Morphological examination of intestinal tissue in group II (occlusion of CMA) revealed a number of characteristic

Table 1 Changes in the structure of rat's mucosal layer after 90 min of partial intestinal ischemia (mean±SD)

Groups	Subgroups		Morphometric indexes		
	(number of sections)		Height of villi, μm	number of crypts	safety of epithelium (%)	degree by Chiu scale
Intact	(*n* = 13)	497±25	143±16	100	0
Ischemia (90 min)	(*n* = 8)	524±16	135±44	100	II–III
Ischemia (90 min)	IA (*n* = 5)	200±11[a]	66±16	63±18	III
Reperfusion (90 min)	IB (*n* = 5)	152±20	73±30	56±27	IV
Ischemia (90 min)	IA (*n* = 8)	304±8[a]	77±27[a]	70±19[a]	III
Reperfusion (24 h)	IB (*n* = 9)	168±28	27±33	17±20	IV–V

[a]*P*<0.05 *vs* subgroups IA (with PF) and IB (control).

peculiarities. As in a partial intestinal ischemia, moderate changes in the mucosa appeared only at the end of the ischemic period. Then, damage of mucosal and other layers of the intestinal wall progressively increased during 90 min of reperfusion. Destruction of superficial mucosal layer, erythrocyte diapedes, massive hemorrhages, and leukocyte infiltration of the intestinal wall and mesentery occurred in subgroups 2A and B. In general, view was more heterogeneous than in the intestine of group I rats. Moreover, a lot of rats died after 90 min of CMA occlusion in early reperfusion period because of intoxication and progressing circulatory failure. Results of intestinal wall section morphometric analysis in group II are presented in Table 2.

DISCUSSION

The problem of post-ischemic or reperfusion impairments of organ blood flow restoration arises acutely after pharmacological and surgical intervention. Therapeutic measures have been concentrated on a critical threshold, minimization of cell metabolism and degree of tissue microcirculation reperfusion injuries[16-20]. If significant success is achieved in ischemic disorders, the problem of

Table 2 Changes in the structure of rat's mucosal layer after 90 min of global intestinal ischemia (mean±SD)

Groups	Subgroups		Morphometric indexes		
	(number of sections)		Height of villi, μm	number of crypts	safety of epithelium (%)	degree by Chiu scale
Intact	(*n* = 2)	525±19	125±24	100	0
Ischemia (90 min)	IIA (*n* = 3)	326±28	129±12	100	III
Reperfusion (90 min)	IIB (*n* = 7)	312±18	104±13	90±10	III
Ischemia (90 min)	IIA (*n* = 20)	296±9	118±9	97±3	III
Reperfusion (24 h)	IIB (*n* = 24)	285±9	107±6	91±4	III

There were no significant differences between subgroups 2A (with PF) and B (control).
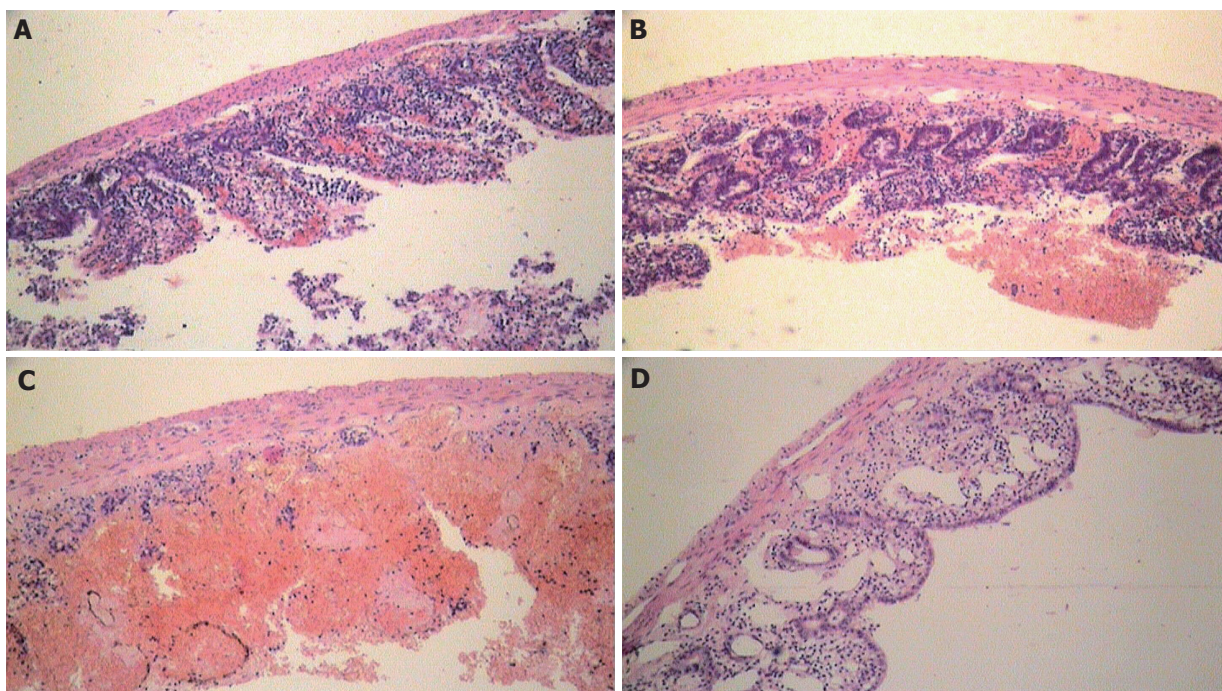
Figure 4 Changes during reperfusion following critical occlusive intestinal ischemia in rats. **A** almost complete denudation and insignificant destruction of villi; **B**: moderate changes after 90 min of reperfusion with perftoran; **C**: insignificant destruction, massive hemorrhages, and focal transmural necrosis formation after 24 h of reperfusion with 0.9% sodium chloride in the intestinal wall of controls; **D**: initial signs of the ischemic intestinal wall regeneration in rats after 24 h of reperfusion after perftoran injection.
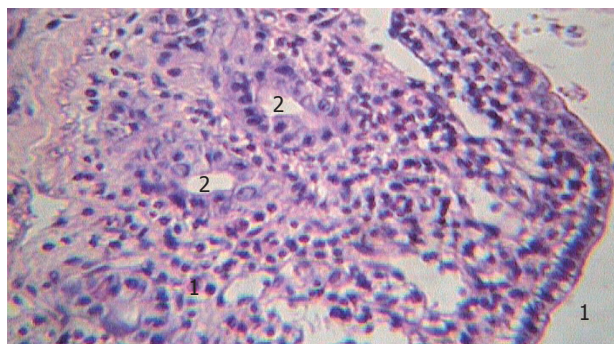

Figure 5 Villi of mucosal layer of the ischemic intestine covered by non-mature cubic epithelium after 24 h of reperfusion (**1**) and saved crypts in the post-ischemic period in rats administered perftoran (**2**).

reperfusion injury prophylaxis is far from its final solution and requires not only ways of correction but thorough investigation of its mechanisms. Investigation of the natural phenomena and main mechanisms of reperfusion injuries at "critical" terms of partial and global intestinal ischemia in rats was conducted in the current work.

The tourniquet partial intestinal ischemia model was chosen in the present work for more homogenous pathomorphological receipts. This model reproduces such disorders as incarcerated hernia and other types of bowel strangulation in clinical practice. The second model occluding CMA corresponds to global intestinal ischemia in consequence of arteriomesenteric thrombosis or embolism.

After significant and irreversible structural and functional changes occurred, the "critical term" (point-of-no-return) was about 90 min for tourniquet segmentary intestinal ischemia in rats. The distinct tendency to stable systemic BP_M level during ischemic period and its abrupt decrease in early reperfusion period were observed. This BP_M level falling was naturally more significant in experiments with CMA occlusion.

Anti-ischemic effect of PF was evaluated in intestinal wall sections from rats that received 0.9% sodium chloride solution. Morphometric analysis was used for quantitative evaluation of rats' intestinal wall alteration degree. A number of morphometric indexes such as the average villous height, number of crypts, epithelial layer safety, and degree of mucosal alteration were analyzed as previously described[15].

The destructive activity increased significantly during reperfusion of ischemic intestine. Mucosa was the most sensitive layer of the intestinal wall. Maximal degree in rat's ischemic intestine was noted at 1.5 h of reperfusion. At the same time, the reperfusion injury strength was significantly smaller in rats that received PF than in controls. In subgroup IA (PF), an explicit regenerative tendency was noted, while destructive process progressed in control rats. In general, luminal surface was covered by low non-mature cubic epithelium, suggesting that crypt's safety is a definitive factor in the viability of ischemic intestine.

It was reported that PFCE has multifunctional properties and is effective in preventing ischemic and reperfusion disorders[21-23]. At the same time, PFCE

administration is extremely limited and its mechanisms are poorly understood.

Differences in the intestinal morphologic structure of rats administered PF and control showed the protective role of PF in the reperfusion period. Though no significant destructive changes were found in mesenteric microvascular bed, intestinal circulation was restored during reperfusion period. Nevertheless, blood flow restoration during reperfusion was not complete because of "feeding" artery diameter and systemic BP$_M$ were significantly smaller than before. At 5 min of reperfusion, feeding artery diameter was decreased by 45% in average compared to the control rats. At the same time, feeding artery diameter was decreased by 24% in rats after PF administration, while BP$_M$ level was decreased in both groups (42%), suggesting that there are more feeding arteries after the "critical" ischemia in rats administered PF.

In the analysis of mechanisms of PF protective action on intestinal mucosa, polyfunctional properties of PFCE were taken into account. Therefore, some of them were protected from destruction and mucosal layer regeneration was maintained. Rheological and nitric oxide (NO) transporting properties of PF may take part in this phenomenon.

It is well known that powerful vasodilator NO can protect rat intestine against reperfusion injury[24]. Results of another investigation witness PFCE penetration from blood into endothelial cells of micro-vessels[25], suggesting that PFCE interacts directly with NO in tissues. PF in intestinal microvascular bed may play a role as a NO depot-regulator and protect intestinal wall tissues reperfusion against injury.

PF and NO interactions can be applied to vascular bed of the ischemic intestine. PF may support vasodilatatory potential of mesenteric feeding arteries as a NO depot-regulator during intestinal reperfusion.

In the present study, ischemia-reperfusion systemic and local pathological functional and structural changes were reproduced on experimental models of tourniquet segmentary intestinal loop strangulation and CMA occlusion in rats. "Critical" term (point-of-no-return) for normal thermic tourniquet segmentary intestinal ischemia in rats was about 90 min. Reperfusion could disregulate the systemic blood pressure support system and lead to its abrupt progressive decrease in the early reperfusion period.

In conclusion, the main pathophysiological reperfusion injury of rat's intestine is the significant mucosal alteration. The degree of intestinal changes is proportional to the duration of ischemia. Systemic administration of perftoran before the reperfusion promotes earlier and more complete structural regeneration in rats with critical partial intestinal ischemia.

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• *BASIC RESEARCH* •

Effects of arginine supplementation on splenocyte cytokine mRNA expression in rats with gut-derived sepsis

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Abstract

AIM: To investigate the effects of arginine (Arg)-enriched diets before sepsis and/or Arg-containing total parenteral nutrition (TPN) after sepsis or both on cytokine mRNA expression levels in splenocytes of rats with gut-derived sepsis.

METHODS: Rats were assigned to four experimental groups. Groups 1 and 2 were fed with a semipurified diet, while groups 3 and 4 had part of the casein replaced by Arg which provided 2% of the total calories. After the rats were fed with these diets for 10 d, sepsis was induced by cecal ligation and puncture (CLP), at the same time an internal jugular vein was cannulated. All rats were maintained on TPN for 3 d. Groups 1 and 3 were infused with conventional TPN, while groups 2 and 4 were supplemented with Arg which provided 2% of the total calories in the TPN solution. All rats were killed 3 d after CLP to examine their splenocyte subpopulation distribution and cytokine expression levels.

RESULTS: Plasma interleukin (IL)-2, IL-4, tumor necrosis factor-α (TNF-α) and interferon (IFN-γ) were not detectable 3 d after CLP. There were no differences in the distributions of CD45Ra+, CD3+, CD4+, and CD8+ cells in whole blood and splenocytes among the four groups. The splenocyte IL-2 mRNA expression in the Arg-supplemented groups was significantly higher than that in group 1. IL-4 mRNA expression in groups 3 and 4 was significantly higher than that in groups 1 and 2. The mRNA expression of IL-10 and IFN-γ was significantly higher in group 4 than in the other three groups. There was no difference in TNF-α mRNA expression among the

four groups.

CONCLUSION: The influence of Arg on the whole blood and splenic lymphocyte subpopulation distribution is not obvious. However, Arg administration, especially before and after CLP, significantly enhances the mRNA expression levels of Th1 and Th2 cytokines in the spleen of rats with gut-derived sepsis.

Shang HF, Hsu CS, Yeh CL, Pai MH, Yeh SL. Effects of arginine supplementation on splenocyte cytokine mRNA expression in rats with gut-derived sepsis. *World J Gastroenterol* 2005;11(45): 7091-7096
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INTRODUCTION

Sepsis is a major cause of death in critically ill patients. Under a condition of sepsis, different endogenous mediators are oversecreted, which may result in an imbalance of the metabolic pathway[1,2]. These metabolic abnormalities that occur during sepsis mainly result from the secretion of cytokines[3]. Cytokines belong to a superfamily of low molecular weight glycoproteins that act as important regulatory proteins, and play a key role in inflammatory responses either directly or by their ability to induce the synthesis of cellular adhesion molecules or other cytokines in numerous cell types[4-6]. Cytokines have beneficial properties in initiating immune responses and maintaining homeostasis in critically ill patients. However, exaggerated or prolonged secretion of cytokines may be detrimental for the host[4-6]. Modulation of cytokines is very important for patients under metabolic stress.

Arginine (Arg) is a semi-essential amino acid that possesses numerous useful physiologic properties[7]. Accumulating experimental and clinical evidence has suggested that Arg reduces protein catabolism and enhances immune function in severely injured animal models and critically ill patients[8–12]. Although a meta-analysis by Heyland *et al*[13]. suggested that immune-enhancing diets rich in Arg may be harmful in unstable critically ill patients, immunonutrition with Arg positively modulates postsurgical immunosuppressive and inflammatory responses[14]. Arg is considered to be an

essential amino acid for patients with catabolic diseases[7,15]. Previous reports by our laboratory showed that preventive use of a Arg-supplemented enteral diet or Arg administered both before and after cecal ligation and puncture (CLP) enhances peritoneal macrophage phagocytic activity and bacterial clearance[16]. Also, total lymphocyte yields in Peyer's patches and intestinal immunoglobin (Ig) A secretion are improved after Arg administration both before and after CLP[17]. In order to understand the protective mechanisms of Arg, the cytokine mRNA expression and protein secretion in septic hosts need to be investigated. However, cytokine protein detection is usually limited, due to lack of sensitive commercial ELISA kits and the short half-life of most cytokines in plasma. Wu et al[18]. have investigated the relation between cytokine mRNA expression and organ damage after sepsis. Cui et al[19]. reported that an Arg-supplemented diet decreases the expression of inflammatory cytokines in burn rats. As we know, there is no study investigating the effects of Arg on Th1 and Th2 cytokine mRNA expressions of splenocytes in septic rats. Therefore, the aim of this investigation was to study the effect of Arg-supplemented diets before sepsis and Arg-enriched total parenteral nutrition (TPN) after sepsis or both on plasma cytokine levels and splenic cytokine mRNA expression in gut-derived sepsis. Also, the lymphocyte subpopulations of whole blood and splenocytes were analyzed to understand the effects of Arg on the phenotype of lymphocytes in a septic condition.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 200-230 g were housed in stainless steel cages in a temperature- and humidity-controlled room with a 12:12 h light-dark cycle. Animals were allowed free access to standard rat chow for 3 d prior to the experiment. All procedures conducted in this study were approved by the Taipei Medical University Animal Care Committee.

Surgical procedure and grouping

All rats were divided into four groups. Groups 1 and 2 were fed a semipurified diet. Rats in groups 3 and 4 were fed an identical diet except that part of the casein was replaced by Arg, which provided 2% of the total energy intake (Table 1). After the rats were fed the experimental diets for 10 d, sepsis was induced by CLP according to the method of Wichterman et al[20]. Briefly, the rats were anesthetized with intraperitoneal pentobarbital (50 mg/kg), and the abdomen was opened through a midline incision. The cecum was isolated, and a 3-0 silk ligature was placed around it, ligating the cecum just below the ileocecal valve. The cecum was then punctured twice with an 18-gauge needle and placed back into the abdomen. The abdominal wound was closed in two layers.

Immediately after CLP, all rats underwent placement of a catheter for TPN infusion. A silicon catheter (Dow Corning, Midland, MI, USA) was inserted into the right internal jugular vein. The distal end of the catheter was

Table 1 Composition of the semipurified diet (g/kg)

Ingredients	Arg-supplemented	Non-supplemented
Casein	180	220
Arg	20	–
Total nitrogen	35.2	35.2
Corn starch	677	657
Soybean oi[1]	44	44
Vitamin[1]	10	10
Salt mixture[2]	35	35
Methyl-cellulose	30	30
Choline chloride	1	1
DL-methionine	3	3

[1]The vitamin mix contained 0.6 mg/g thiamine hydrochloride, 06 mg/g riboflavin, 0.7 mg/g pyridoxine hydrochloride, 3 mg/g nicotinic acid, 1.6 mg/g calcium pantothenate, 0.02 mg/g D-biotin, 0.001 mg/g cyanocobalamin, 1.6 mg/g retinyl palmitate, 20 mg/g DL-a-tocopherol acetate, 0.25 mg/g cholecalciferol, and 0.005 mg/g menaquinone. [2]The salt mixture contained 500 mg/g calcium phosphate diabasic, 74 mg/g sodium chloride, 52 mg/g potassium sulfate, 220 mg/g potassium citrate monohydrate, 24 mg/g magnesium oxide, 3.5 mg/g manganese carbonate, 6 mg/g ferric citrate, 1.6 mg/g zinc carbonate, 0.3 mg/g cupric carbonate, 0.01 mg/g potassium iodate, 0.01 mg/g sodium selenite, and 0.55 mg/g chromium potassium sulfate.

tunneled subcutaneously to the back of the neck, and exited through a coiled spring which was attached to a swivel, allowing free mobility of animals inside individual metabolic cages. TPN at 2 mL/h was administered on the first day. Full-strength TPN (56-64 mL/d, according to body weight) was given thereafter for 3 d. The infusion speed was controlled by a Terufusion pump (Model STC-503, Terumo, Tokyo, Japan). The TPN solution without fat was prepared in a laminar flow hood. Sterilized fat emulsions were added to the TPN solution daily just before use. The TPN solution was infused for the entire day at room temperature. No enteral nutrition was administered during the period of TPN. Groups 1 and 3 were infused with conventional TPN. Groups 2 and 4 were supplemented with Arg, which replaced 10% of the total amino acids, and provided 2% of the total energy of the TPN solution. TPN provided 280 kcal/kg body weight, and the energy (kcal):nitrogen (g) ratio was 119:1. The calorie density was almost 1 kcal/mL. The TPN solutions were isonitrogenous and identical in nutrient composition except for the difference in the amino acid content (Table 2). There were four groups in this study: group 1, no Arg supplementation before or after CLP (–/–); group 2, a semipurified diet given before and Arg-enriched TPN after CLP (–/+); group 3, an Arg-supplemented diet before and conventional TPN after CLP (+/–); and group 4, Arg supplementation both before and after CLP (+/+). There were 10 rats in each group.

Lymphocyte subpopulation distribution in whole blood and spleen

On d 3 after CLP, all surviving rats were weighed and anesthetized with pentobarbital. The survival rates in groups 1-4 were 10/17, 10/19, 10/18, 10/15,

Table 2 Composition of the TPN solution (mL/L)

	Arg-supplemented	Non-supplemented
50% Glucose	412	400
20% Fat emulsion	50	50
10% Moriamin-SN[1]	450	556
Arg (g)	5	–
Infuvita[2]	8	8
3% NaCl	35	35
7% KCl	10	10
8.7%K$_3$PO$_4$	10	10
Ca-gluconate	10	10
MgSO$_4$	4	4
ZnSO$_4$	2	2
Choline chloride (g)	1	1

[1]From Chinese Pharmaceuticals, Taipei, Taiwan. Contents per deciliter: 1 250 mg Leu, 560 mg Ile, 1 240 mg Lys acetate, 350 mg Met, 935 mg Phe, 650mg Thr, 130 mg Trp, 450 mg Val, 620 mg Ala, 790 mg Arg, 380 mg Asp, 100 mg Cys, 650 mg Glu, 600 mg His, 330 mg Pro, 220 mg Ser, 35 mg Tyr, and 1 570 mg aminoacetic acid (Gly). [2]From Yu-Liang Pharmaceuticals, Taoyuan, Taiwan. Contents per milliliter: 20 mg ascorbic acid, 660 IU vitamin A, 40 IU ergocalciferol, 0.6 mg thiamine HCl, 0.72 mg riboflavin, 8 mg niacinamide, 0.8 mg pyridoxine HCl, 3 mg D-panthenol, 2 mg dl-a-tocopheryl acetate, 12 μg biotin, 80 μg folic acid, and 1 μg cyanocobalamin.

respectively. There were no differences in the survival rates among the groups as described previously[16]. A middle abdominal incision was made, and the spleen was aseptically removed and teased on a stainless mesh immersed in chilled RPMI-1640 (Gibco, BRL, Grand Island, NY, USA). After filtration through a sterile nylon mesh, cell suspensions were washed thrice in HBSS and resuspended in RPMI-1640. Flow cytometry was used to determine the proportions of CD45Ra, CD3, CD4, and CD8 from the whole blood or splenocytes. One hundred microliters of heparinized whole blood or 10^5 splenocytes suspended in 100 μL HBSS were stained with fluorescein-conjugated mouse anti-rat CD3 (Serotec, Oxford, UK) and phycoerythrin-conjugated mouse anti-rat CD45Ra (Serotec) to distinguish T cells from B cells, respectively. Fluorescein-conjugated mouse anti-rat CD8 and phycoerythrin-conjugated mouse anti-rat CD4 (Serotec) were used to identify T helper cells and cytotoxic T lymphocyte cells, respectively. After staining for 15 min, 1 mL red blood cell (RBC) lysing buffer (Serotec) was added to lyse the RBCs and to fix the stained lymphocytes. Fluorescence data were collected on $5×10^4$ viable cells and analyzed by flow cytometry (Coulter, Miami, FL, USA).

Plasma cytokine immunoassay
Plasma interleukin (IL)-2, IL-4, IL-10, interferon (IFN-γ), and tumor necrosis factor (TNF-α) concentrations were determined by commercially available enzyme-linked immunosorbent assay (ELISA) kits (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Primers of cytokines
The primers of IL-2, IL-4, IL-10, TNF-α, and IFN-γ, and

the housekeeping gene (18S rRNA) of rats were purchased from PE Applied Biosystems (Foster City, CA, USA).

Real-time reverse-transcription polymerase chain reaction (RT-PCR) method
Total RNA from rat spleen was isolated using the TRIzol reagent according to the manufacturer's protocol. RNA was reverse-transcribed using the Reverse Transcript system (Frementas, Vilnins, Lithuania). Briefly, 20 μL water containing 2 μg RNA was mixed with 1 μL oligo (dT) primer (0.5 μg/μL) and incubated for 5 min at 70 ℃. To the mixture, 22 μL MgCl$_2$ (25 mmol/L), 10 μL 10× RT-buffer, 20 μL dNTP (10 mmol/L), 2 μL RNase inhibitor, and 2.5 μL MultiScribe-RT (50 U/μL) were added and incubated at 25 ℃ for 10 min, then at 42 ℃ for 30 min. The reaction was stopped by heating the samples for 5 min to 95 ℃. cDNA was used for the real-time PCR assay performed with an ABI 7700 Sequence Detection System (PE Applied Biosystems) according to the supplied guidelines. The PCR reaction for IL-2, IL-4, IL-10, IFN-γ and TNF-α was carried out using a TaqMan PCR kit (PE Applied Biosystems).

Statistical analysis
Data were expressed as mean±SD. Differences among the groups were analyzed by ANOVA using Duncan's test. $P<0.05$ was considered statistically significant.

RESULTS
There were no differences in initial body weights and body weights after feeding the experimental diets for 10 d and after TPN administration for 3 d (data not shown). In this study, we were unable to detect plasma IL-2, IL-4, TNF-α and IFN-γ 3 d after CLP. Plasma IL-10 levels could be detected, but there was no difference in IL-10 levels among the groups (data not shown).

No differences in the distribution of CD45Ra+, CD3+, CD4+, and CD8+ cells in blood and splenocytes were observed among the four groups (Figures 1A and 1B). The expression of IL-2 mRNA in splenocytes was significantly higher in the Arg-supplemented groups (groups 2, 3, and 4) than in group 1 (–/–) (Figure 2A). The mRNA expressions of IFN-γ and IL-10 in group 4 (+/+) were significantly higher than in the other three groups (Figures 2B and 2D). IL-4 mRNA expression in groups 3 and 4 was higher than that in groups 1 and 2 (Figure 2C). There were no differences in splenocyte TNF-α mRNA expression among the four groups (Figure 2E).

DISCUSSION
In this study, 2% of total energy was supplied in the diet by Arg. This amount of Arg has been proved to enhance immune functions in rodents with gut-derived sepsis[21]. We administered TPN for 3 d after CLP, because the severity of infection and mortality are the highest at this time point in an established septic animal model[20]. In this
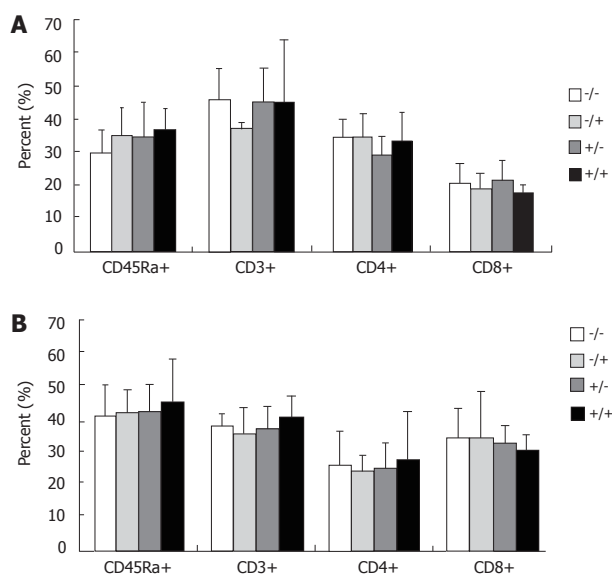
Figure 1 Distribution of CD45Ra+, CD3+, CD4+, and CD8+ lymphocytes in whole blood (**A**) and splenocytes (**B**) among the four groups 3 d after CLP (*n* = 10 in each group).

study, we did not include a sham-operation control (TPN without CLP) group; therefore, this study was not able to assess whether Arg supplementation could restore immune functions comparable to the control rats.

Circulating cytokine levels are usually used as a marker of injury or infection[3]. In order to understand the possible effects of Arg on the systemic cytokine expression in sepsis, IL-2, IL-4, IL-10, IFN-γ, and a pro-inflammatory cytokine TNF-α were measured. IL-2 and IFN-γ were produced by Th1 lymphocytes. Th1 cytokines enhance cell-mediated immunity. A predominant Th1 effect results in activation, growth, and differentiation of T and B lymphocytes as well as macrophages. Th2 cytokines, including IL-4 and IL-10, enhance humoral immunity. A predominant Th2 effect results in the activation of B lymphocytes, upregulation of antibody production, and mucosal immunity[22]. The effects of Th1 or Th2 lymphocytes were counter-regulatory.

In this study, we were unable to detect plasma IL-2, IL-4, TNF-α, and IFN-γ 3 d after CLP. Previous reports by our laboratory showed that IL-1β, IL-2, and IFN-γ were undetectable 24 h after CLP[23,24]. Cruickshank *et al*[25]. also reported that plasma IL-1, TNF, and IFN-γ are rarely detected in the plasma of injured patients. The inability to measure circulating cytokines may be due to a lack of highly sensitive assay kits and late assay times, or due to the fact that the quantities of cytokines do not enter the

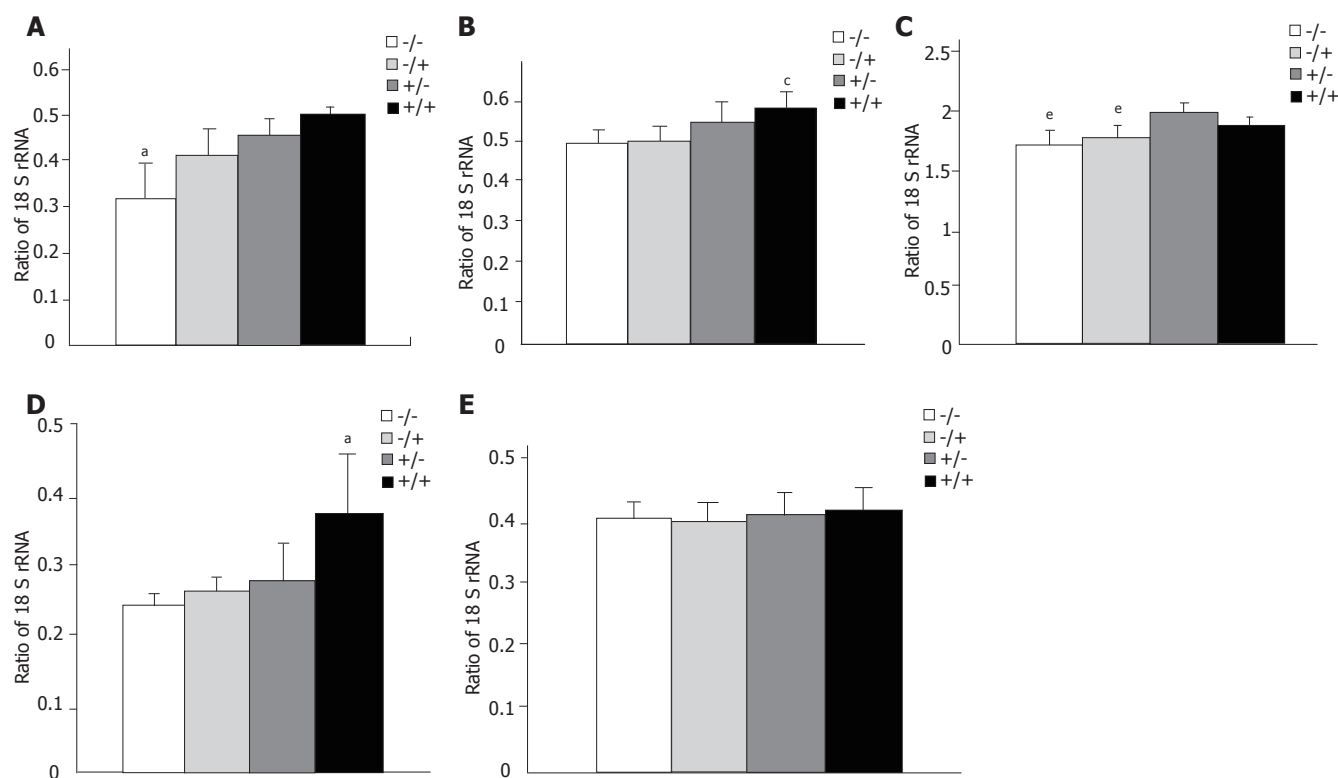**Figure 2** Expression of interleukin (IL)-2 (**A**), interferon (IFN-γ) (**B**), IL-4 (**C**), IL-10 (**D**) and tumor necrosis factor (TNF-α) (**E**) messenger RNA in the spleen as determined by real-time reverse-transcription polymerase chain reaction (*n* = 10 in each group). [a]*P*<0.05 *vs* other groups; [c]*P*<0.05 *vs* group 1 (–/–) and group 2 (–/+); [e]*P*<0.05 *vs* group 3 (+/–) and group 4 (+/+).

systemic circulation, even though the levels of cytokines in the tissues have increased[4]. Therefore, we used real-time RT-PCR to quantify splenic cytokine mRNA expression, this is a highly sensitive and reproducible tool to investigate the cytokine profiles at the mRNA level[26,27]. Our findings showed that Arg administration enhanced both Th1 (IL-2, IFN-γ) and Th2 (IL-4, IL-10) cytokine mRNA expression, and Arg given both before and after had a synergistic effect on enhancing cytokine mRNA expression in a septic condition. These results are consistent with our previous report that Arg supplementation promotes intestinal IgA secretion and enhances peritoneal macrophage phagocytic activity in septic rats[16,17]. Although Th1 type cytokines promote the differentiation of cytotoxic T cells, these cytokines usually respond to viral infection and intracellular pathogens. The secretion of IFN-γ may activate macrophages, promote microbicidal activity and stimulate expression of the secretory component for intestinal sIgA by epithelial cells[28]. IL-4 and IL-10 are anti-inflammatory cytokines released by Th2 cells. Furthermore, IL-10 induces the synthesis and secretion of sIgA[22,29,30].

In this study, Arg seemed to have no effect on splenocyte TNF-α mRNA expression. This result is inconsistent with a report by Cui *et al*[19]. who showed that dietary Arg supplementation decreases the mRNA expression of inflammatory cytokines in the spleen after thermal injury. Since the insults of scald burns and gut-derived sepsis differ, the inflammatory cytokine response may vary. Burn injury results in generalized inflammation in organs remote from the region of thermal injury due to activated neutrophil and reactive oxygen metabolites[31,32], whereas CLP causes peritoneal infection with mixed intestinal bacterial flora, which may result in a direct damage to the tissue and organs by circulating bacterial toxins.

In order to understand the effect of Arg on the distribution of lymphocyte subpopulations, the total B lymphocytes (CD45Ra+), total T cells (CD3+), helper T cells (CD4+), and cytotoxic T cells (CD8+) within the blood and splenocytes were evaluated. The results showed that there were no differences in the ratio of B cell and T cell subpopulations among the groups. This finding may indicate that the differences in cytokine mRNA expression after Arg supplementation do not influence the distribution of systemic and splenic lymphocyte subpopulations. Although the effects of Th1 and Th2 lymphocytes are counter-regulatory, cytokine mRNA expression and protein secretion may be regulated by different mechanisms in various tissues and organs, and the final performance of the immune response to specific tissues or organs may vary. Whether there are intracellular factors which regulate the post-transcriptional expression of these cytokines requires further investigation.

In conclusion, the influence of Arg supplementation on the distribution of whole blood and splenic lymphocyte subpopulations is not obvious. However, Arg supplementation, especially before and after CLP, significantly enhances the splenic mRNA expression of Th1 and Th2 cytokines in rats with gut-derived sepsis.

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ELSEVIER

• *BASIC RESEARCH* •

Secretory expression and characterization of a recombinant-deleted variant of human hepatocyte growth factor in *Pichia pastoris*

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Abstract

AIM: To study the secretory expression of human hepatocyte growth factor (hdHGF) gene in *Pichia pastoris*.

METHODS: The full-length gene of human cDNA encoding the deleted variant of hdHGF was cloned by RT-PCR and overlapping-fragment PCR technique using mRNA of human placenta as a template. The cloned hdHGF cDNA was inserted into the *Escherichia coli*-yeast shuttle vector of pPIC9. The constructed plasmid, pPIC9-hdHGF, was transformed into the GS115 cells of the methylotrophic yeast, *P pastoris,* using a chemical method. The Mut$^+$ transformants were screened to obtain high-expression strains by the test and analysis of expressed products of shake-flask culture. A secretory form of rhdHGF was made with the aid of the leader peptide sequence of *Saccharomyces cerevisiae* α-factor.

RESULTS: The expressed products, which showed a band of molecular mass of about 80 ku, were observed on 15% SDS-PAGE and identified by Western blotting and N-terminal amino acid sequencing. In the high cell density culture of 5 L fermentor by fed-batch culture protocol, the cell biomass was reached at approximately 135 g (DCW)/L. The productivity of secreted total supernant protein concentration attained a high-level expression of more than 8.0 g/L and the ratio of rhdHGF band area was about 12.3% of the total band area scanned by SDS-PAGE analysis, which estimated that the product of rhdHGF was 500-900 mg/L.

CONCLUSION: The *P pastoris* system represents an
attractive tool of generating large quantities of hdHGF for both research and industrial purposes.

INTRODUCTION

Hepatocyte growth factor (HGF) was identified initially as a mitogen for hepatocytes, called as scatter factor (SF) and fibroblast-derived tumor cytotoxic factor (F-TCF) as well as fibroblast-derived growth factor called plasminogen-like growth factor (PLGF)[1-3]. Nakamura *et al*[16]. reported that HGF could be purified from the serum of partially hepatectomized rats. Subsequently, HGF has been purified from rat platelets and its subunit structure is determined. The purification of human HGF from human plasma is described by Godowski *et al*[5].

The gene locus of human HGF is assigned to chromosome 7q21.1. The genomic gene consists of 18 exons and 17 introns, and spans about 70 kb. The whole length form of human hepatocyte growth factor (preproHGF) consists of 727 amino acids and the mature form of hHGF is composed of 674 amino acids, corresponding to the major form purified from human serum[5]. HGF is a disulfide-linked heterodimer derived by proteolytic cleavage of the human pro-hormone between amino acids R494 and V495. This cleavage process generates a molecule composed of an alpha-subunit of 440 amino acids (MW 69 ku) and a beta-subunit of 234 amino acids (MW 34 ku). The nucleotide sequence of hHGF cDNA reveals that both the alpha-and the beta-chains are contained in a single open reading frame coding for a pre-pro precursor protein. In the predicted primary structure of mature hHGF, an interchain S-S bridge is formed between Cys 487 of the alpha-chain and Cys 604 in the beta-chain. The N-terminus of the alpha-chain is preceded by 54 amino acids, starting with a methionine

group. This segment includes a characteristic hydrophobic leader (signal) sequence of 31 residues and the prosequence. The alpha-chain starts at amino acid (aa) 55 and contains four Kringle domains. The Kringle 1 domain extends from about aa 128 to about aa 206, the Kringle 2 domain is between about aa 211 and about aa 288, the Kringle 3 domain extends from about aa 303 to about aa 383, the Kringle 4 domain extends from about aa 391 to about aa 464 of the alpha-chain. It will be understood that the definition of the various Kringle domains is based on their homology with Kringle-like domains of other proteins (prothrombin, plasminogen). Therefore, the above limits are only approximate. Until now, the function of these Kringles has not been determined. The beta-chain of hHGF shows high homology to the catalytic domain of serine proteases (38% homology to the plasminogen serine protease domain). However, two of the three residues, which form the catalytic triad of serine proteases, are not conserved in hHGF. Therefore, despite its serine protease-like domain, hHGF appears to have no proteolytic activity and the precise role of the beta-chain remains unknown. HGF contains four putative glycosylation sites, which are located at positions 294 and 402 of the alpha-chain and at positions 566 and 653 of the beta-chain. Wild-type human HGF gene *in vivo* exists in the polymorphism. It has been observed that human HGF has a few natural variants. For example, hdHGF-encoded HGF molecule lacking five amino acids in the Kringle 1 domain (FLPSS) is fully functional[6-8].

HGF biological activity refers to any mitogenic, motogenic or morphogenic activities exhibited by wild-type human HGF or hdHGF, which have a broad spectrum of mitogenic cell specificity that can promote the proliferation of hepatocytes, endothelial cells, fibroblasts, melanocytes, and epithelial cells etc.[1,4], inhibit the growth of some tumor cell lines such as HepG$_2$, B6/F1, and KB from tumorigenic target cell lines[9]. Recent studies displayed that hdHGF can exert many important biological effects mediated via their specific tyrosine kinase receptor, C-met. Even to the extent, hdHGF has more significant biological effects on promoting the regeneration of hepatocytes and kidney epithelial cells compared to wild-type human HGF, suggesting that hdHGF has the therapeutic effect *in vivo* on liver injury[2].

Recently, methylotrophic yeast *Pichia pastoris* has become a dominant tool in molecular biology for the production of recombinant proteins. *P pastoris* is known for its high-level expression of heterologous proteins and its tightly regulated alcohol oxidase 1 (AOX1) gene promoter[10]. *P pastoris* can be easily grown to high cell densities using defined minimal media and is able to introduce eukaryotic post-translational modifications[11,12]. The techniques for molecular genetic manipulation are similar to those well established for *Saccharomyces cerevisiae*. At present, *P pastoris* as an efficient protein expression system can be fermented routinely in large scale to meet the industrial demands of interest proteins[13-15]. In the present report, we have described the recombinant production of hdHGF in *P pastoris* and its characterization.

MATERIALS AND METHODS
Strain, vector, reagents, and enzymes
P pastoris host strain GS115 (His$^+$Mut$^+$) and secretion expression vector pPIC9 were purchased from Invitrogen (San Diego, CA, USA). *E coli* DH5α was used for routine plasmid amplification and the cloned vector of pUC19 was maintained in our laboratory. Superscript™ II RNase H⁻Reverse transcriptase was purchased from GibcoBRL. Human placenta mRNA was obtained from Clontech Co. Expand™ High Fidelity PCR System was purchased from Boehringer Mannheim Co. Yeast nitrogen base, D-biotin, yeast extract and tryptone were obtained from Sangon (Shanghai, China). *EcoRI*, *NotI, SalI, XbaI, SphI,* T4 DNA ligase, and *Taq* DNA polymerase were obtained from TaKaRa Biotechnology (Dalian, China). Anti-hHGF antibody was purchased from Santa Cruz Biotechnology Co.

Molecular cloning of hdHGF
The whole length gene of human cDNA encoding the deleted variant of hdHGF was amplified by RT-PCR and overlapping fragments were amplified by PCR technique using mRNA of human placenta as the template. Three pairs of PCR primers for amplified hdHGF fragments were designed as follows.

In primer M1, single bottom line stands for SphI and two lines for SalI. Oblique boldface capital letters (included 8 codons) represent the frequently used codons in the highly expressed *P pastoris* genes. In primer M2, single bottom line stands for *XbaI* and two lines for *NotI*. Its complementary chain encodes the sequence for *TACAAGGTTCCACAGTCTTAA* (included 6 codons) and the oblique boldface capital letters represent the bias of codons in the highly expressed *P pastoris* genes. Therefore, the codons encoding N-and C-terminal amino acids of the deleted variant of hdHGF were amplified by RT-PCR and overlapping-fragment PCR technique, using mRNA of human placenta as the template, which has the advantage to acquire high-level expression of foreign genes in *P pastoris*.

A forward primer(P1: 5'TTCTTTCACCCAGGCATCTC3') and a reverse primer (P2: 5'CTATGTTTGTTCGTGTTGG AATCC3') as well as another forward primer (P3: 5'GTGG GACAAAGAACATGGAAGACTTAC3') and its reverse primer (P4: 5'GCTTCAGACACACTTACTT CAGCTA3') were designed to synthesize the two cDNA fragments based on the hdHGF sequence reported by Nakamura *et al*[16], namely one fragment (F1, about 1.6 kb) was amplified using a pair of primers P1 and P2 and the other fragment (F2, approximately 1.0 kb) was amplified using a pair of primers P3 and P4. Overlapping-fragment amplification using F1 and F2 fragments as templates was performed by routine PCR procedure using a pair of primers M1 and M2 (Figure 3). The cDNA product obtained from RT-PCR was modified by introducing *SphI* and *SalI* sites at the 5'end and *XbaI* and *NotI* sites as well as a TAA stop codon at the 3'end. Thirty-five cycles of PCR were performed: denaturation at 94 ℃ for 60 s, annealing at 55 ℃ for 60 s,

extension at 72 ℃ for 90 s, and then a further extension at 72 ℃ for 10 min. The PCR procedures were carried out according to the standard procedures published earlier[17].

Construction of expression plasmid

The PCR products were digested with *SphI* and *XbaI*, and cloned into the same enzyme digested vector pUC19. The recombinant vectors were transformed into *E coli* DH5 α . The recombinant transformants were acquired via the blue-white colony screening in the agar medium containing X-gal and characterized using restriction endonucleases *SphI* and *XbaI*. The gene sequence analysis of the recombinant pUC-hdHGF was carried out by Sanger's dideoxynucleotide DNA sequencing. The verified hdHGF cDNA fragment with *SalI* and *NotI* was cloned into the site of expression vector pPIC9 digested with *XhoI* and *NotI* enzymes. The recombinant plasmids were transformed into *E coli* strains of JM109. Screening and selection of expression plasmid clones containing hdHGF cDNA fragments through the identification with restriction endonucleases *Bam*HI and *NotI*, resulted in the plasmid pPIC9-hdHGF containing hdHGF gene under the control of AOX1 promoter and in-frame with α -factor signal sequence (Figure 1).

Yeast transformation

Plasmids used for transformation were linearized with *SalI*. The *SalI*-linearized pPIC9-hdHGF or parent pPIC9 was transformed into *his4* competent *P pastoris* GS115 cells by a chemical method. After the growth on minimal dextrose medium (MD) plates at 30 ℃ for 3 d, several colonies containing the linearized pPIC9-hdHGF fragment were selected for PCR confirmation by colony PCR, which was designed to amplify the 200-bp special sequence of pPIC9-hdHGF using a pair of primers, namely P5 (sense, 5'GTGGGACAAGAACATGGAAGA CTTA3') and P6 (antisense, 5'CTATGTTTGTTCGTGTTGGAATCC3').

Expression of hdHGF by recombinant Pichia in shake flask

Ten colonies were used to inoculate 10 mL buffered minimal glycerol-complex medium (BMGY) in a 50 mL shake flask, respectively. After being shook at 250 r/min for 2 d at 30 ℃, the cells were pelleted and resuspended in a 2 mL buffered minimal methanol-complex medium (BMMY). Following the additional 2 d of induction at 30 ℃, the samples of expressed hdHGF in culture supernatants were determined and hdHGF in culture supernatants was also analyzed by SDS-PAGE.

Fed-batch cultivation of P pastoris in a 5-L bioreactor

The clones exhibiting the highest level expression of hdHGF were selected for fed-batch fermentations which were carried out in a 5-L working volume bioreactor using a BIOFLO 3000 (New Brunswick Scientific) interfaced with AFS-Biocommand Bioprocessing software version 2.6 (New Brunswick Scientific) for data acquisition and supervisory control.

Seed culture for the bioreactor was started from the fresh glycerol stock and inoculated directly into 500-mL shake flasks (50-mL working volume) containing a minimum glycerol medium (1.34% YNB, 1% glycerol, and 1.61 μmol/L biotin). After 24 h of growth, seed culture was inoculated with 1% inoculum. After 16-20 h of growth, seed culture was used to inoculate the bioreactor. Ten percentage of the inoculum was used for the inoculation of a 5-L bioreactor containing 2.-L medium of high-cell density fermentation, comprising of 10× basal salts (42 mL/L 85% H_3PO_4, 1.8 g/L $CaSO_4·2H_2O$, 28.6 g/L K_2SO_4, 50 g/L glycerol, 23.4 g/L $MgSO_4·7H_2O$, 6.5 g/L KOH, and 4.35 mL/L 10·PTM_1 salts, 6.0 g/L $CuSO_4 · 5H_2O$, 0.08 g/L KI, 3.0 g/L $MnSO_4·H_2O$, 20.0 g/L $ZnCl_2$, 0.02 g/L HBO_4, 65 g/L $FeSO_4·7H_2O$, 0.2 g/L $Na_2MoO_4·2H_2O$, 0.5 g/L $CoCl_2$, 0.2 g/L biotin, and 5 mL/L H_2SO_4, buffered to pH 5.5 using 2 mol/L NH_4OH). Dissolved oxygen was maintained at over 20% air saturation at 30 ℃ and aeration was maintained at 2 vvm. pH was maintained at 5.5 and fermentation was carried out in two phases. Growth phase consisted of a glycerol batch phase and cells were grown batch-wise until glycerol in the medium was utilized. To achieve a high cell density, the glycerol (50% glycerol, 4.3 mL/L PTM_1, feeding rate: 18 mL/h·L) fed-batch phase was initiated and lasted for 6-10 h. Production phase consisted of a methanol fed-batch phase when cells were induced by methanol (100% methanol plus 12 mL/L PTM_1 salts). The methanol feed rate was gradually increased over a period of 6 h-6mL/h and the fermentation continued for an additional 46-92 h. Expressed hdHGF in culture supernatants was analyzed by SDS-PAGE and the concentration of secreted total supernant proteins was also determined at different intervals of induction phase using the standard curve analysis of human serum albumin (HSA).

Western blotting of rhdHGF

The purified recombinant hdHGF was run in 15% Tris-tricine electrophoresis[20] and then transferred onto a polyvinylidene difluoride (PVDF) membrane and probed with rabbit anti-hdHGF antibody as described previously[19].

RESULTS

Molecular cloning of hdHGF fragments

Based on the hdHGF sequence reported by Nakamura *et al*[16], we designed three pairs of PCR primers for amplified hdHGF fragments. A forward primer P1 and a reverse primer P2 and another forward primer P3 and

its reverse primer P4 were designed to synthesize the two fragments. The two fragments of hdHGF PCR products about 1 570 (F1) and 970 bp (F2), respectively, were also clearly seen in 1% agarose gel electrophoresis stained with 5 mg/mL ethidium bromide amplified with P1-P2 and P3-P4 primer pairs (Figure 2A). Overlapping-fragment PCR amplification using F1 and F2 fragments as templates was performed by routine PCR procedure using a pair of primers M1 and M2 and the PCR product of full-length gene of hdHGF was revealed (Figure 2B).

Construction of the expression plasmid

A cDNA encoding the mature form of hdHGF was used in these experiments. This cDNA consisted of 2.1 kb (Figure 2B) with an open-reading frame encoding a 669 amino acid peptide. The DNA fragment encoding the mature hdHGF was digested with *XhoI* and *NotI* from pUC19 vector and cloned into the same enzyme digested vector pPIC9 downstream of the alcohol oxidase I (AOX I) promoter (Figures 1 and 3). The resultant
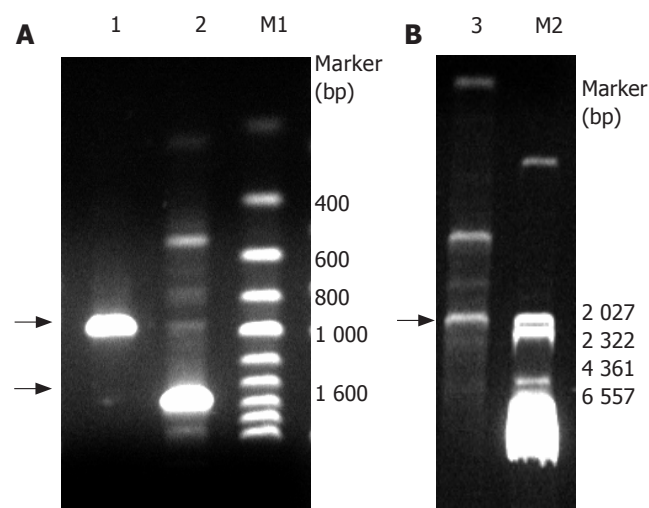

Figure 2 Analysis of PCR products by 1% agarose electrophoresis (**A** and **B**). Lane 1: fragment (F2) amplified with P3-P4 primer pair; lane 2: fragment (F1) amplified with P1-P2 primer pair; lane 3: fragment (whole length) amplified with M1-M2 primer pair; M1: 200-bp ladder marker; M2: λ+*Hind*III marker. The arrowheads show the bands of the interesting fragments

construct harbored a single open reading frame encoding a 85 amino acid translation product consisting of the α-factor secretion leader peptide (Figures 1 and 3). Prior to the secretion of the peptide into the culture medium, the signal peptide should be cleaved off by the *KEX2* gene products at the site (Glu-Lys-Arg-X) (Figure 1). The integrity of the recombinant plasmid was confirmed by direct DNA sequencing. Enzyme identification of the recombinant plasmids of pPIC9-hdHGF digested by *BamHI* and *NotI* is shown in Figure 4. This constructed vector was linearized with *SalI* and transformed into the competent cells of *P pastoris* GS115. The transformants were selected on MD plates and confirmed by colony PCR. Forty-one colonies presenting strong amplification

Figure 3 Cloning of hdHGF cDNA and construction of expression vectors pUC-hdHGF and pPIC9-hdHGF.

products were used for small-scale expression trials and the amount of the recombinant peptide was determined by SDS-PAGE.

Expression of recombinant hdHGF in shake flask

We investigated the expression of recombinant hdHGF by both Mut[s] and Mut[+] (GS115) strains in shake flasks. Since SDS-PAGE analysis revealed that the hdHGF level expressed by Mut[s] strain was much higher than that of Mut[+] (data not shown), Mut[s] was chosen for the expression of th e growth factor. In addition, Mut[s] phenotype was selected over the Mut[+] phenotype because

Figure 4 Agarose electrophoresis analysis of restriction enzyme mapping of recombinant plasmids of pPIC9-hdHGF. Lanes 1-5: recombinant plasmids of pPIC9-hdHGF digested by *Bam*HI and *Not*I; lane 6: λ-DNA/*Hind*III marker. The arrowhead reveals bands of the interesting fragment.

of the latter's higher oxygen requirement that could result in oxygen-deficient conditions within the bioreactor. Fifty transformants (Mut[s]) were used for the expression studies in shake flask experiments and secretion of the recombinant hdHGF into the culture medium was determined by SDS-PAGE analysis. The expression experiments were performed to screen out four high-level expression strains of hdHGF, which were named as HG209, HG211, HG305, and HG309 (Figure 5).

Expression of recombinant hdHGF in fermenter cultures

The selected clones with the highest expression level were chosen for fed-batch cultivation. A time-course study of
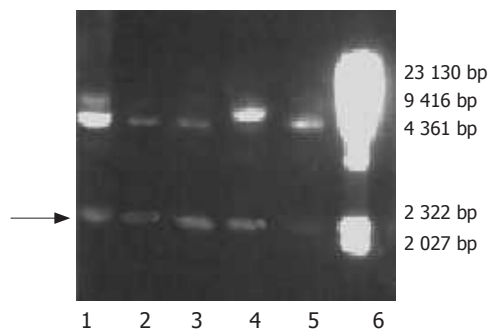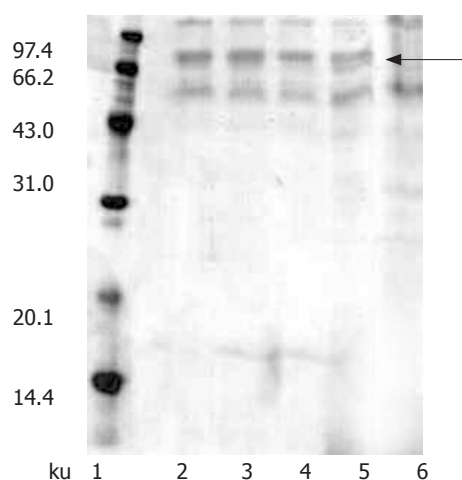

Figure 6 Fifteen percentage SDS-PAGE and Western blotting analysis of the 5-L fed-batch high cell density fermentation of GH209. Lane 1: LMW marker; lane 2: 10% SDS-PAGE result; lanes 3 and 4: Western blotting result. The arrowhead shows bands of the interesting protein.

Figure 5 Fifteen percentage SDS-PAGE analysis of high expression strains in shake-flask culture.Lane 1: LMW marker; lane 2: GH209; lane 3: HG211; lane 4: HG305; lane 5:HG309; lane 6: GS115. The arrowhead shows bands of the interesting protein.

secretion of hdHGF revealed a gradual accumulation of recombinant cytokines. The effect of induction pH on the production of recombinant cytokines was investigated. *P pastoris* is known to grow over a wide pH range from 3 to 7, with a minimal effect of pH on the growth rate. However, pH could significantly affect the productivity of secreted recombinant proteins in the fermentation broth. To find out the optimal pH for the expression of recombinant hdHGF, we conducted experiments with pH between 3.5 and 6.5 during the fed-batch production phase. The highest yield of recombinant hdHGF was observed at pH 5.5 (Figure 6).

Protein expression was initiated by changing carbon source from glycerol to methanol. At first, we attempted to express hdHGF in baffled shake flasks and obtained about 50 mg/L of hdHGF secreted into the medium after 72-h induction. For more systematic production of hdHGF, we expressed the protein using fermenter cultures. Figure 6 shows the secretion level of hdHGF using 5 L fermenter. Upon depletion of glycerol, the dry cell weight reached 45.6 g/L. A glycerol fed-batch phase was performed for an additional 6-12 h and the cell biomass reached at
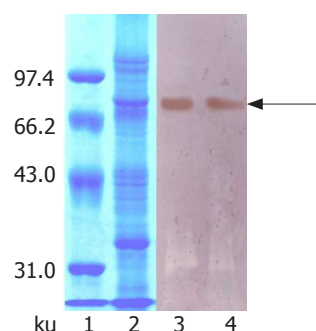
approximately 135 g (DCW)/L. Induction of hdHGF was initiated by the addition of 100% methanol containing 12 mL of PTM1 trace salts/L. Sample analysis at different intervals was also performed to show the increasing amounts of recombinant hdHGF presented in the culture medium with increasing induction time until 96 h. Dissolved oxygen (DO) was monitored by DO sensor and the oxygen transfer rate 1 min after turning off the carbon source feed. Dissolved oxygen was maintained between 20% and 30% air saturation in the two-phase fermentation. The secreted total supernant protein concentration in the induction phase was traced and observed to attain the high-level expression of more than 8.0 g/L after 84-96 h of induction cultivation, which was determined using the standard curve analysis of human serum albumin (HSA). The scanning result showed that the secreted rhdHGF protein band (in lane 3 of Figure 6) achieved about 12.3% of the total supernatant proteins. By comparison with the standard protein markers, the estimated product of rhdHGF was 500-900 mg/L. The maximum secretion yield was approximately 980 mg/L (Figure 6). Upon induction by methanol, four clones secreted a specific 80-ku protein with the same size as the standard HGF. The productivity varied among the four high-level expression strains of hdHGF.

These clones indicated that the highest product of rhdHGF was named HG209 (GS115/pPIC9-hdHGF) and selected for further analysis. Western blotting analysis showed that the 80-ku protein band from HG209 (GS115/pPIC9-hdHGF) reacted specifically with the rabbit anti-hHGF antiserum (Figure 6). The N-terminal sequence of the recombinant hdHGF was determined to be PALKI, which was identical to the N-terminal sequence of native hdHGF (Figure 1).

DISCUSSION

hdHGF is a large complex protein comprising of 669 amino acids. It is the most potent multifunctional cytokine on the regeneration of hepatocytes and kidney epithelial cells compared to wild-type human HGF[20]. It can promote cell division, migration, and differentiation.

Its receptor is the product of oncogene c-met. Besides being a nutritional factor of liver and kidney, hdHGF also promotes angiogenesis for peripheral artery disease and myocardial ischemia[20] and can affect synthesis of extracellular matrix and matrix metalloproteinases and tissue inhibitor of metalloproteinases in autosomal dominant polycystic kidney disease cyst-lining epithelial cells[21].

Since the concentration of native HGF in plasma is very low, purification of HGF from plasma is very difficult. It was reported that HGF is expressed in foreign gene expression systems such as mammalian cells, CHO cells[22,23], insect cells[24], and gene therapy[25]. Dang *et al*[26] and Li *et al*[27]. reported that the HGF gene is expressed in *E coli* and *P pastoris*. However, the expression of recombinant-deleted variants of human hepatocyte growth factor (hdHGF) has not been reported in yeast expression system. Therefore, in this investigation, we used the methylotrophic yeast *P pastoris* as the host for the high-level expression and secretion of recombinant hdHGF. Recombinant hdHGF was successfully secreted by *P pastoris* and the productivity of secreted total supernatant protein concentration attained high-level expression of more than 8.0 g/L and the ratio of rhdHGF band area was about 12.3% of the total band area scanned by SDS-PAGE analysis, which estimated the product of rhdHGF to be 500-900 mg/L. It had an approximately fivefold increase in productivity compared to that of HGF expressed in *P pastoris*[27]. Western blot analysis showed that the 80-ku protein band of GS115 (pPIC9-hdHGF) reacted specifically with the rabbit anti-hHGF antibody. N-terminal sequencing revealed that recombinant rhdHGF had the correct N-terminal amino acid sequence. These results suggest that the optimization of bias codons of *P pastoris* encoding N-and C-terminal amino acids of hdHGF via PCR-mediated codon replacement can acquire high-level expression of foreign genes in *P pastoris*. Nakamura *et al*[16]. reported that native hdHGF is composed of an alpha-subunit of 440 amino acids (MW 69 ku) and a beta-subunit of 234 amino acids (MW 34 ku). However, we found that the recombinant hdHGF produced by *P pastoris* was translated as a single-chain polypeptide comprising of 669aa, which was not cut by the proteolytic cleavage of the human pro-hormone between amino acids R494 and V495 in host cells of *P pastoris*.

In conclusion, though further characterization, bioassay, and optimization of the expression and cultivation of recombinant hdHGF by *P pastoris* are required, this expression system of hdHGF is expected to be a powerful tool in the industrial production of this foreign protein.

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ELSEVIER

• *BASIC RESEARCH* •

A novel gain of function mutant in C-kit gene and its tumorigenesis in nude mice

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Abstract

AIM: To transfect mutant C-kit cDNA at codon 579 into human embryonic kidney cell line to observe its role in the pathogenesis of gastrointestinal stromal tumor (GIST).

METHODS: Eukaryotic expression vectors of pcDNA3-Kit-NW and pcDNA3-Kit-W were constructed. Then pcDNA3-Kit-NW and pcDNA3-Kit-W plasmids were transfected into human embryonic kidney cell line by Lipofectamine. The resistant clone was screened by G418 filtration and identified by sequencing, Western blotting, and immunocytochemical staining. Human embryonic kidney cells were divided into three groups including pcDNA3-Kit-NW, pcDNA3-Kit-W, and vector control groups. Absorbency value with a wavelength of 574 nm was detected by MTT analysis. Mice were injected with three groups of cells. Volume, mass, and histological examinations of the tumors in different groups were measured and compared.

RESULTS: The C-kit gene and mutant C-kit gene were successfully cloned into the eukaryotic expression vector pcDNA3. pcDNA3-Kit-NW and pcDNA3-Kit-W were successfully transfected into human embryonic kidney cell line and showed stable expression in this cell line. Cell proliferating activity had significant differences between pcDNA3-Kit-NW and pcDNA3, pcDNA3-Kit-NW and pcDNA3-Kit-W ($P<0.05$), respectively. Tumors were only observed in nude mice implanted with cells transfected with pcDNA3-Kit-NW.

CONCLUSION: Mutation of C-kit gene increases the proliferation activity of human cells and plays an important role in the malignant transformation of GIST.

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INTRODUCTION

Gastrointestinal stromal tumor (GIST) is a designation for a major subset of mesenchymal tumors of the gastrointestinal tract. Their line of differentiation or cell of origin and clinical behavior are a persistent source of controversy[1-5]. Recently, specific mutations between transmembrane and tyrosine kinase domains in exon 11 at codon 550-560 of C-kit have been found in GISTs, which can lead to ligand-independent activation of the tyrosine kinase of C-kit and have a tumor promoting effect *in vitro*[4,6-10]. We studied the C-kit mutation type of exon 11 by PCR-SSCP and DNA sequencing in a series of Chinese GIST[11]. The results showed that C-kit mutations existed among GISTs. Compared to the reports in the published data, an insertion of 12 bp at codon 579 lies outside the hot spot area[12]. In order to fully characterize the activation of the insert mutation at codon 579 of C-kit gene, we constructed an expression vector containing mutant C-kit and evaluated its effect on implanted tumor in nude mice.

MATERIALS AND METHODS

Main reagents
Fetal bovine serum was produced by Hyclone and 96-well plates by Costa. Dimethyl sulfoxide (DMSO), ethylened iaminetetraacetic acid (EDTA), 3-(4,5-dimethyl-thiazol-2-yl), 2,5-diphenyl tetrazolium bromide (MTT), *N*-2-hydroxyethyl piperazine-*N*'-2-ethanesulfonic acid (HEPES) and trypsin were the products of Sigma. Rabbit polyclonal antibody against C-kit was obtained from DAKO. Envision two-step and DAB test kits were obtained from Beijing Zhongshan Biotechnology Inc.

Human embryonic kidney cell line
Human embryonic kidney cell (HEKC) line was established in our laboratory and prepared after long-term generation.

Construction of plasmids

Eukaryotic expression vector of pcDNA3 (Invitrogen, USA), plasmid pMD18-T-KIT-NW containing full-length of C-kit gene (C-kit cDNA was conducted in our laboratory) and plasmid pMD18-T-Kit-NW containing mutant-type C-kit cDNA with insertion of 12 bp at codon 579 (mutant C-kit cDNA was conducted in our laboratory) were digested by restriction enzymes *Xba*I *and Hind*III. The digested vector and plasmid cDNA fragment were ligated. Recombinant clones were identified by *Xba*I *and Hind*III double digestion. Positive clones named pcDNA3-Kit-W and pcDNA3-Kit-NW were further confirmed by sequencing.

Transfection of recombinant plasmid into HEKCs and grouping

Cultured HEKCs were divided into three groups, which were transfected with pcDNA3 vector, recombinant pcDNA3-Kit-W, and recombinant pcDNA3-Kit-NW, respectively. Transfection was performed according to the instructions of Lipofectamine TM2000 reagent kit (Gibco). Cells were cultured in RPMI 1640 medium containing 200mL/L fetal calf serum and 350 mg/L G418. Resistant clones could be detected 2 wk later.

Cell culture

HEKCs were inoculated in RPMI 1640 with 100 g/L fetal bovine serum and incubated at 37 ℃ in an incubator containing 50 mL/L CO_2 to measure the logarithmic growth phase. After the treatment with digestive fluid, HEKCs were suspended by adding D-Hank's fluid, deposited by centrifugation at 190 r/min for 5 min and then counted. Using RPMI 1640 containing 100 g/L fetal bovine serum, HEKCs were adjusted to a density of $5×10^4$/mL and added to a 24-well plate containing flying sheets, 0.2 mL/well. Flying sheets were taken out respectively at 24, 48, and 72 h, fixed with cold acetone for 10 min, dried and preserved at -60 ℃.

Cell fixation and immunocytochemical staining

Preserved cell flying sheets were immersed in PBS for 5 min, blocked with 10 mL/L H_2O_2 for 10 min, washed thrice with PBS (5 min each time) and then incubated with 10% goat serum for 30 min. C-kit antibody was diluted at a concentration of 1:50 and added as the first antibodies, staying 1 h at 37 ℃. After being washed thrice with PBS (5 min each time), horseradish peroxidase-conjugated goat anti-rabbit secondary antibody was added, staying 30 min at 37 ℃, washed thrice with PBS (5 min each time) and stained with DAB. Counterstaining with hematoxylin was performed before the final analysis. The first antibody was replaced with fetal bovine serum as a negative control.

Western blot analysis

Cell lysates were prepared in extraction buffer containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 10 g/L Triton-100, 1 g/L SDS, 1 mmol/L EDTA, 1 mmol/L AEBSF, 20 g/mL aprotinin, and 20 g/mL leupeptin. After centrifugation at 12 000 *g* for 10 min at 4 ℃, the supernatant was collected. Equal amounts of total protein (10 g) from each sample were loaded and separated by 120 g/L SDS-polyacrylamide gel electrophoresis and then transferred to Hybond-P polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). After being blocked with 50 g/L nonfat dry milk in PBS (pH 7.4) with 1 g/L Tween-20, membranes were probed with a rabbit anti-C-kit polyclonal antibody (1:500 dilution) followed by subsequent incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:1 000 dilution).

MTT assay

HEKCs with a density of approximately $2.5×10^5$/mL were seeded into each well of the 96-well plate (0.2 mL/well). Each group contained 12 wells. After 4 d of incubation at 37 ℃ in an incubator containing 50 mL/L CO_2, 50 mL of MTT (50 mg MTT) was added and the culture was continued for 4 h at 37 ℃. After the fluid at the top was removed, 150 mL DMSO was added to each well. After being concussed and dissolved, absorbency value with a wavelength of 574 nm was detected by enzyme-labeled instrument (BioRad 2250, Japan).

Animal experiment

Nine Balb/c male mice aged 4-6 wk (body mass 18-20 g) were purchased from Experimental Animal Center of Second Military Medical University, Shanghai and randomly divided into three groups, three mice in each group. The mice in vector-treated control group were injected with HEKCs transfected with pcDNA3 vector, the mice in Kit-W group received an injection of HEKCs transfected with recombinant pcDNA3-Kit-W, the mice in Kit-NW group received an injection of HEKCs transfected with recombinant pcDNA3-Kit-NW. After the cancer cells were cultured into the stage of logarithmic growth phase, they were digested with trypsin to make cancer cell suspension of $2.5×10^7$/L. Then, 0.2 mL of each suspension was subcutaneously injected into the right back of the nude mice.

Tumor volume measurement

The survival of nude mice was observed every day. After tumor cell injection, the nude mice were killed after 6 wk. The surrounding fatty tissues were dissected and the tumors were weighed. Tumors were fixed in 10% buffered formalin and processed in paraffin wax. Five-micrometer thick sections were stained with hematoxylin and eosin.

Statistical analysis

Data were treated with SPSS for LSD and one-way ANOVA test. $P<0.05$ was considered statistically significant.

RESULTS

Identification of recombinant plasmids pcDNA3-Kit-W and pcDNA3-Kit-NW

After digestion by *Xba*I *and Hind*III, bands at 3 kD could be detected for positive clones (Figure 1), suggesting

that Kit-W and Kit-NW fragments were inserted into the pcDNA3 vector, named as recombinant plasmids pcDNA3-Kit-W and pcDNA3-Kit-NW, respectively.

pcDNA3-Kit-W and pcDNA3-Kit-NW DNA were prepared for sequencing. The sequence obtained was the same as the reported sequence of C-kit cDNA and mutant
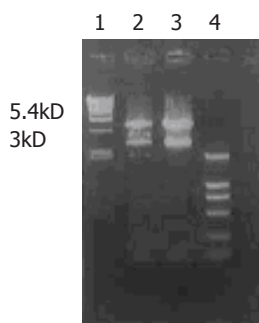

Figure 1 Restriction enzyme digestion of recombinant plasmids pcDNA3-Kit-W and pcDNA3-Kit-NW at *Xba*I and *Hind*III. Lane 1: DNA/*Eco*RI+*Hind*III marker; lane 2: pcDNA3-Kit-W clone; lane 3: pcDNA3-Kit-NW clone; lane 4: DL-2000 marker.

C-kit cDNA, indicating that the C-kit gene and mutant C-kit gene were successfully cloned into the eukaryotic expression vector pcDNA3.

Screening of C-kit gene-transfected cells

No significant differences were observed between the morphological characteristics of transfected cells and normal HEKCs.

Expression of C-kit protein

Supernatants of cultured cells in three groups were collected and analyzed by immunocytochemistry. The cells transfected with pcDNA3-Kit-W and pcDNA3-Kit-NW were positively stained for C-kit protein (Figure 2). However, the cells in vector control group were negatively stained for C-kit. In accordance with the findings on immunocytochemical staining, a band in a molecular mass of 145 ku was detected with rabbit anti-C-kit antibody from the cells transfected with pcDNA3-Kit-W and pcDNA3-Kit-NW, but no bands were detected in the vector control group. There was no significant difference in the protein level of C-kit between pcDNA3-Kit-W and pcDNA3-Kit-NW groups (Figure 3).

MTT assay

Absorbance values of the three groups were $0.1340\pm2.353\times10^{-3}$, $0.1830\pm3.888\times10^{-3}$, and $0.1274\pm3.537\times10^{-3}$, respectively. LSD analysis and one-way ANOVA analysis with SPSS10.0 software were used to compare the difference of absorbance in each group at 574 nm. Statistical analysis indicated that significant differences were detected between pcDNA3-Kit-NW and pcDNA3, pcDNA3-Kit-NW and pcDNA3-Kit-W ($P<0.05$), respectively. The results implied that the cell proliferating activity in pcDNA3-Kit-NW group was higher than that in

Figure 2 Positive immunocytochemical staining of C-kit transfected with pcDNA3-Kit-NW (×400).

Figure 3 Western blotting of pcDNA3-Kit-W, pcDNA3-Kit-NW, and pcDNA3 on HEKC line. Lanes 1-3: pcDNA3-Kit-NW; lanes 4-6: pcDNA3-Kit-W; lanes 7 and 8: pcDNA3.

control group.

Tumor growth in nude mice

Tumors were observed in the nude mice 3 wk after being implanted with cells transfected with pcDNA3-Kit-NW (Figure 4). Mice had no visible tumor after cell injection in pcDNA3-Kit-W transfection group and vector control group after 6 wk. Tumor volumes in pcDNA3-Kit-NW transfection group were approximately similar and no significant difference was observed between the three tumors ($t = 13.07$ $P>0.05$). Many areas of hemorrhage and necrosis were present, residual tumor cells were frequently found to have giant, bizarre-shaped pyknotic nucleoli, or prominent pathologic mitosis in the tumors (Figure 5).

DISCUSSION

GIST is the most common mesenchymal tumor in human gastrointestinal tract and is enigmatic in terms of its differentiation or cell origin and clinical behavior. In recent years, C-kit protein expression and activated gene mutation have been found in GIST[13-17]. C-kit encodes a growth factor receptor with ligand-dependent tyrosine kinase activity[18,19]. Kit ligand-stem cell factor (SCF) is the only known ligand of the Kit receptor[20]. SCF binding to the receptor mediates receptor dimerization, activation of kinase activity, and autophosphorylation. Subsequently, Kit activates several signaling cascades, leading to cell proliferation, cell survival, and other cellular responses. Kit and SCF are encoded at the white spotting (W) and steel (Sl) loci in mice, respectively[20-22]. Mutation at the murine W and Sl loci generates deficiencies in several major cell systems during embryogenesis and in postnatal animals. In hematopoiesis Kit receptor signaling is critical in the stem cell hierarchy, erythropoiesis, mast cell development and

Figure 4 Tumor growth in nude mice 6 wk after being implanted with cells transfected with pcDNA3-Kit-NW.

Figure 5 Giant, bizarre-shaped pyknotic nucleoli, or prominent pathologic mitosis in tumor (HE ×200).

function, megakaryopoiesis, and lymphopoiesis[23-26].

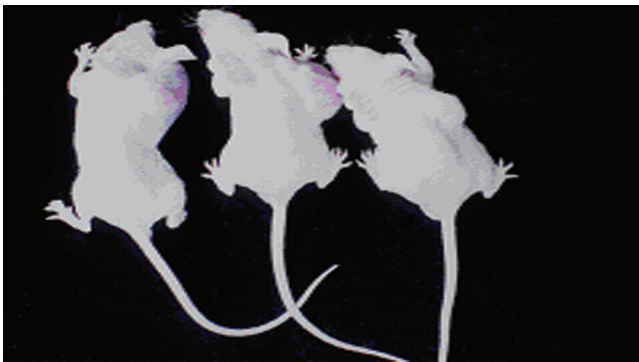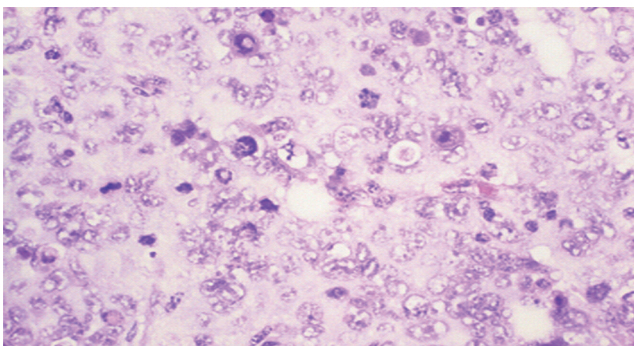GISTs most often harbor mutations at exon 11 codon 550-560 of C-kit cDNA, while the mutations in human mastocytosis predominantly involve the activation loop of the Kit kinase[27,28]. The C-kit mutations in this domain can lead to spontaneous ligand-independent activation of the tyrosine kinase of C-kit and was called "gain-of-function mutation". Development of GIST is highly associated with Kit-activating mutations, suggesting that the activated Kit receptor plays a critical role in tumor development[29]. In addition, gene mutation has shown a good correlation with biologic behavior in such tumors. For instance, it can provide valuable adjunctive prognostic information. In agreement with this notion, familial cases of GIST have been reported with Kit-activating mutations in the germ line[30]. We have found a mutation at codon 579 in malignant GIST, which lies outside the hot spot area[12]. In order to fully characterize the activation of the insert mutation at codon 579 of C-kit gene, we studied the expression of mutant C-kit gene in human embryonic kidney cell line and its effect on implanted carcinoma in the nude mice.

In the present study, we constructed a target fragment containing mutant-type C-kit cDNA with insertion of 12 bp at codon 579 and full-length of C-kit gene. The target gene fragment of 5.4 kD in length was cloned into *Xba*I *and Hind*III restriction sites of eukaryotic

expression vector pcDNA3. Restriction digestion and sequence analysis for positive clones indicated that the two recombinant eukaryotic expression vectors containing the gene were successfully constructed. These recombinant vectors were then transfected into HEKCs and screened by G418. The result of direct sequencing showed that the recombinant plasmids were stably integrated into the cells. Immunocytochemical staining revealed that HEKCs transfected with pcDNA3-Kit-W and pcDNA3-Kit-NW were stained positively for C-kit, indicating that C-kit protein is expressed in transfected HEKCs. Furthermore, a protein (145 kD) was detected by Western blotting and there was no significant difference in the expression level of C-kit protein between transfected pcDNA3-Kit-W and pcDNA3-Kit-NW HEKCs, indicating that the insert mutation at codon 579 of C-kit gene has no effect on protein expression of HEKCs.

In vitro characterization of the Kit V558 mutation in BaF/3 cells indicates that the Kit V558 mutation promotes cell proliferation and abolishes the growth factor dependence of BaF/3 cells[30]. Sommer *et al*[31]. showed that young bone marrow-derived mast cell cultures are refractory to growth factor deprivation-induced apoptosis. However, these cultures do not promote cell cycle progression. There is evidence that short kit protein product is more active than long Kit protein product. Therefore, it may be possible that the long-Kit variant is expressed predominantly in the young mutant bone marrow-derived mast cells, while the older cultures express the short-Kit variant, which may explain the progression from partial to complete growth factor independently of these cultures. Our results showed that the insert mutation at codon 579 of C-kit gene promoted HEKC proliferation. In addition, no significant differences between the morphological characteristics of transfected cells and normal HEKCs were observed, suggesting that mutant C-kit has no effect on the morphology of HEKCs.

In this experiment, HEKCs transfected with recombinant pcDNA3-Kit-NW and pcDNA3 vector were subcutaneously implanted into the nude mice. The implanted tumors appeared later in the pcDNA3-Kit-NW transfection group. Mice had no visible tumor after cell injection in pcDNA3-Kit-W transfection group and vector control group after 6 wk. These results suggest that the insert mutation at codon 579 of C-kit gene can significantly induce the growth of tumors and constitutive Kit signaling is critical and sufficient for the induction of neoplasia in the mice.

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• *CLINICAL RESEARCH•*

Associations between γ-glutamyl transferase, metabolic abnormalities and inflammation in healthy subjects from a population-based cohort: A possible implication for oxidative stress

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Abstract

AIM: To examine the relationships between γ-glutamyl-transferase (GGT), alanine-aminotransferase (ALT), aspartate-aminotransferase (AST) and various metabolic parameters, C-reactive protein (CRP) and an oxidative stress marker (nitrotyrosine, NT) in subjects without any metabolic abnormalities from a population-based sample.

METHODS: Two hundred and five subjects with normal body mass index (BMI), glucose tolerance, and without any metabolic abnormality were studied out of 1 339 subjects, without known liver diseases, alcohol abuse or use of hepatotoxic drugs, who are representative of the 45-64 aged population of Asti (north-western Italy).

RESULTS: In all patients metabolic parameters and hs-CRP levels linearly increase from the lowest to the highest ALT and GGT tertiles, while in subjects without metabolic abnormalities, there is a significant association between fasting glucose, uric acid, waist circumference, hs-CRP, triglyceride values, and GGT levels. In these subjects, male sex, higher hs-CRP and glucose levels are associated with GGT levels in a multiple regression model, after adjustments for multiple confounders. In the same model, median NT levels are significantly associated with the increasing GGT tertile (β = 1.06; 95%CI 0.67-1.45), but not with the AST and ALT tertiles. In a multiple regression model, after adjusting for age, sex, BMI, waist, smoking, and alcohol consumption, both NT (β = 0.05; 95%CI 0.02-0.08) and hs-CRP levels (β = 0.09; 95%CI 0.03-0.15) are significantly associated with

fasting glycemia.

CONCLUSION: GGT, an easy, universally standardized and available measurement, could represent an early marker of sub-clinical inflammation and oxidative stress in otherwise healthy individuals. Prospective studies are needed to establish if GGT could predict future diabetes in these subjects.

INTRODUCTION

Increased levels of the liver enzymes, γ-glutamyl transferase (GGT), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) have been found to be associated with diabetes, cardiovascular risk factors and components of the insulin resistance syndrome, even within normal reference intervals[1-12]. In many prospective studies, strong relationships between GGT or ALT concentrations and incident diabetes have also been observed in non-drinkers, in individuals with normal levels of liver enzymes, independently of classical cardiovascular risk factors[2-10]. However, a strong interaction between body mass index (BMI) and GGT has been described, suggesting that this enzyme acts as an intervening factor in the association between obesity and diabetes[1,4,5,7,9]. Some authors have speculated that visceral fat could play a role in the association of GGT with metabolic abnormalities, or that this enzyme could be considered as a reliable marker of visceral fat[1,2,13]. Furthermore, GGT and ALT might be interpreted as markers of hepatic steatosis,

a condition well known to be associated with insulin resistance, type 2 diabetes and the metabolic syndrome[14,15]. Thus, the associations recently found between the levels of liver enzymes and insulin resistance might be mediated by a fatty liver, which is accompanied by higher GGT and aminotransferase concentrations.

Increasing data are available about the associations between GGT levels and markers of oxidative stress (directly with F2-isoprostanes, an oxidative damage product of arachidonic acid; inversely with serum and dietary antioxidant vitamins), suggesting that the strong associations between cardiovascular risk factors might be explained by some oxidative mechanism[4,16-18]. Indeed, oxidative processes are key components of chronic inflammation, acting on multiple pathways and amplifying inflammatory reactions[19]. Recent reports have found associations between elevated liver enzymes and several inflammatory parameters[3,4,9,10,20,21].

However, previous studies have always included a group of overweight, or obese subjects, or did not take into account the deposition of body fat; thus adiposity might be a confounding factor. Two recent papers suggested that a non-alcoholic fatty liver could be considered as an early predictor of metabolic disorders also in non-obese non-diabetic Asiatic cohorts; however, in these studies, the control group included individuals with other components of the metabolic syndrome and Asians have a higher proportion of visceral fat and a lower lean body mass than white subjects with the same BMI[22,23]. It seems thus interesting to evaluate the associations between the liver enzyme levels and metabolic and inflammatory parameters in a cohort of Caucasian subjects without any metabolic abnormalities, which could potentially influence this association.

Therefore, the aims of the present study are to examine the relationships between liver enzyme concentrations and various metabolic parameters, a marker of inflammation (C-reactive protein, CRP) and a marker of oxidative stress (nitrotyrosine, NT) in subjects without any metabolic abnormalities from a population-based cohort of middle-aged individuals, recognizing that the cross-sectional design of the study does not definitively establish causal or temporal relationships, and should be considered hypothesis-generating only.

MATERIALS AND METHODS

All the patients aged between 45 and 64 years under the practice of six family physicians from the province of Asti (north-western Italy), whose patients are representative of the local health districts, were enrolled for a metabolic screening. Of these 1 877 subjects, 1 658 (88.3%) accepted to be interviewed on personal habits, and to be tested for several clinical and laboratory measurements, giving written consent, while 219 refused. The resident population of corresponding age, in the same area, and missing patients show the same percentage of males, level of education, known diabetes, and subjects living in a rural area than participating patients.

All procedures were in accordance with the Declaration of Helsinki.

Subjects with >30 g/d alcohol consumption, with known liver or gastrointestinal diseases, as recorded by the family physicians, with liver enzyme concentrations higher than three times the upper limit of the sex-related reference range, or on corticosteroids, methotrexate, amiodarone, tamoxifen or other hepatotoxic drugs were excluded. Estrogen users (both on contraceptive medications or estrogen replacement therapy) were included, all being on low-dose estrogen drugs.

One thousand and three hundred and thirty-nine subjects (80.8%) were evaluated.

In the morning, a fasting venous blood sample was drawn to measure glucose, total and HDL-cholesterol, uric acid, triglyceride, insulin, liver enzymes, and hs-CRP levels.

Weight and height were measured after an overnight fast. Overweight and obese subjects were those with BMI respectively, $\geqslant$25, <30, and $\geqslant$30 kg/m^2. Waist circumference was measured with a plastic tape meter at the level of the umbilicus. Systolic and diastolic blood pressures were measured twice with a standard mercury sphygmomanometer in a sitting position, after at least 10 min of rest. Values reported are the mean of the two determinations. A resting electrocardiogram (ECG) was performed in all the subjects, and interpreted according to the Minnesota Code criteria. If fasting serum glucose value was $\geqslant$6.1 mmol/L, a second fasting glucose determination was then performed. Diabetes and impaired fasting glucose (IFG) were diagnosed according to published recommendations[24].

The metabolic syndrome (MS) was defined as the presence of at least three of the following five criteria: fasting serum glucose $\geqslant$6.1 mmol/L; arterial blood pressure $\geqslant$130/85 mmHg; plasma triglycerides $\geqslant$1.69 mmol/L; HDL-cholesterol <1.29 mmol/L (females) and <1.04 mmol/L (males); waist >88 cm (females) and >102 cm (males), in line with the National Cholesterol Education Program (Adult Treatment Panel III) (NCEP-ATP III) criteria[25].

Insulin resistance was calculated from the homeostasis model assessment (HOMA-IR), according to published algorithm[26].

Vascular disease was assessed on the basis of the Rose questionnaire, ECG evidence of ischemic heart disease, and history of documented events, recorded by the family physician (angina, previous myocardial infarction, coronary artery by-pass graft or other invasive procedures to treat coronary artery disease, transient ischemic attacks, strokes, gangrene, amputation, vascular surgery, intermittent claudication, absence of foot pulses, and abnormal brachial and posterior tibial blood pressure using Doppler techniques).

Former or present smokers were defined as ever smokers. Alcohol intake was assessed by multiplying the mean daily consumption for each beverage by the ethanol content, to give grams of alcohol/d (one can/bottle/glass of beer = 13 g, one glass of wine = 12 g, one standard drink of spirit = 14 g).

Later, we identified 205 subjects with normal BMI (<25 kg/m^2) and without any component of the MS. To avoid considering as normoglycemic individuals who could be classified as hyperglycemic after the stimulatory test, these subjects were submitted to the 75 g oral glucose tolerance test (OGTT), performed and interpreted in accordance to the guidelines[24]. All of the subjects showed normal glucose tolerance at the OGTT. NT levels were measured in all these subjects.

Serum glucose was measured by the glucose oxidase method (HITACHI 911 Analyzer, Sentinel Ch., Milan, Italy), and serum insulin by immunoradiometric assay (Radim SpA, Pomezia, Italy; intra-assay CV: 1.6-2.2%, inter-assay CV: 6.1-6.5%). Plasma triglycerides and HDL-cholesterol were measured by enzymatic colorimetric assay (HITACHI 911 Analyzer), the latter after precipitation of LDL and VLDL fractions using heparin-MnCl$_2$ solution (Mn^{2+} concentration: 0.092 mol/L) and centrifugation at 4 °C.

AST was evaluated by a kinetic determination. Malate dehydrogenase catalyzes the reaction of oxalacetic acid with β-NADH2 by forming lactic acid and β-NAD (HITACHI 911 Analyzer). ALT was evaluated by a kinetic determination. Lactate dehydrogenase catalyzes the reaction of pyruvic acid with β-NADH2 by forming lactic acid and β-NAD (HITACHI 911 Analyzer). GGT was evaluated by an enzymatic colorimetric method (HITACHI 911 Analyzer).

Uric acid was evaluated by an enzymatic colorimetric method with uricase (HITACHI 911 Analyzer). Serum hs-CRP levels were determined via a high-sensitivity latex agglutination method on the HITACHI 911 Analyzer. The kit had a minimum detection of less than 0.05 mg/L and a measurable concentration range of up to 160 mg/L. The intra-assay and inter-assay CVs were respectively 0.8-1.3% and 1.0-1.5%. Plasma NT values were determined by an ELISA kit (HyCult Biotechnology b.v., sold in Italy by Pantec, Turin; inter-assay and intra-assay CV respectively: 7 ±4% and 5±2%). All samples were run in blind.

Statistical analysis

Since the distribution of GGT, hs-CRP, insulin, HOMA, triglyceride, and NT values were highly skewed, the levels of these variables were log-transformed, in order to obtain a normal distribution. In all the analyses, the log-transformed values of these variables were used. For an easy interpretation, median (and range) of not transformed values are reported.

Linear (unadjusted) and multiple regression analyses were performed to evaluate the associations between liver enzyme levels with metabolic, inflammatory, and oxidative parameters, after adjustments for multiple confounders.

RESULTS

BMI, waist, blood pressure, fasting glucose, and insulin, HOMA-IR, triglyceride, low HDL-cholesterol, uric acid, and hs-CRP levels, percentages of males and prevalence of the metabolic syndrome increase from the lowest to

the highest tertiles of ALT and GGT, and their values are significantly associated with these enzyme levels (Table 1). This trend is less evident within AST tertiles.

In subjects with normal BMI and without any component of the MS, there is a significant association of percentage of males, waist circumference, glucose, uric acid, hs-CRP, and triglyceride values with increasing GGT levels (Table 2). Waist, uric acid levels and percentages of males are significantly associated with ALT levels, while there is no significant association with AST values.

Table 3 describes the associations between the levels of each liver enzyme and age, gender, BMI, waist circumference, alcohol consumption, smoking habits, hs-CRP, HOMA-IR, and triglyceride uric acid levels (and hypertension in all the cohort) in a multiple regression model, after introducing all these as independent variables into the model. In all patients: waist levels are significantly associated with AST levels; male sex, waist, and higher levels of HOMA-IR, triglycerides, uric acid with ALT levels; male sex, alcohol and higher levels of hs-CRP, triglycerides, uric acid with GGT values.

In the subgroup of subjects with normal BMI, without any component of the MS, male sex and higher hs-CRP levels are associated with GGT levels, while no significant association is evident for the other liver enzymes. Fasting glucose is significantly associated with GGT levels in the same model, after adjusting for all previous variables, but insulin instead of HOMA-IR levels (β = 0.23; 95%CI 0.07-0.39); fasting glucose is not significantly associated with AST or ALT values.

In the subgroup of normal BMI subjects, the median NT levels are significantly associated with the increasing GGT tertile (β = 1.07; 95%CI 0.70-1.44), but not with the AST and ALT tertiles in a simple regression (Figure 1). After adjustments for age, sex, BMI, waist, smoking, and alcohol consumption, this association remains significant (β = 1.06; 95%CI 0.67-1.45).

Median NT levels are significantly correlated with: fasting glucose (β = 1.00; 95%CI 0.45-1.55) and waist circumference (β = 0.04; 95%CI 0.002-0.08). In a multiple regression model, after adjustments for age, sex, BMI, waist, smoking, and alcohol consumption, both NT (β = 0.05; 95%CI 0.02-0.08) and hs-CRP levels (β = 0.09; 95%CI 0.03-0.15) are significantly associated with fasting glycemia.

Correlations are similar also in non-drinkers, and after adjusting for AST or ALT levels (data not shown). Data do not change after excluding the subjects with CRP>10 (respectively $n = 48$ in the entire cohort and $n = 2$ in the subgroup of normal BMI), those with cardiovascular diseases (respectively $n = 50$ and $n = 3$) or those subjects on estrogen therapy (respectively $n = 73$ and $n = 12$).

DISCUSSION

Prospective studies have described that high levels of ALT and GGT[2-10] are associated with subsequent development of diabetes, while the association between AST levels and metabolic abnormalities is weaker and often attenuated or

Table 1 Clinical and laboratory characteristics of all the patients, according to liver enzyme tertiles (left); associations of the variables listed with liver enzyme levels, as a continuous variable, by unadjusted linear regression analyses (right)

AST	1st tertile (≤14 U/L)	2nd tertile (>14≤19 U/L)	3rd tertile (>19 U/L)	β; 95%CI	
Number	452	440	447		
Age (yr)	54.0±5.8	54.7±5.8	55.0±5.5	0.08; 0.002	0.15
Male (%)	31.9	34.1	47.2	1.88; 1.00	2.76
Alcoholics (%)	41.6	45.0	51.8	1.28; 0.42	2.14
Alcohol (g/day within drinkers)	14.4±8.7	15.8±8.9	16.3±8.5	0.03; -0.05	0.11
Ever smokers (%)	48.3	40.2	41.4	-0.41; -1.27	0.45
BMI (kg/m^2)	26.3±5.1	26.1±4.4	27.1±4.9	0.13; 0.04	0.22
Waist (cm)	88.6±13.4	89.3±12.4	92.5±13.0	0.09; 0.06	0.12
Systolic blood pressure (mmHg)	132.1±15.3	132.8±16.2	134.8±16.3	0.034; 0.007	0.06
Diastolic blood pressure (mmHg)	82.3±8.7	82.9±9.5	83.7±9.6	0.05;-0.02	0.078
Uric acid (mmol/L)	178.4±53.5	190.3±59.5	208.2±59.5	0.04;-0.007	0.087
Insulin (pmol/L)	10.2 (0.6-259.8)	10.8 (1.2-137.4)	11.4 (0.6-409.8)	0.74; 0.26	1.22
HOMA insulin resistance (mU/mL×mmol/L)	0.40 (0.02-31.8)	0.44 (0.05-16.6)	0.47 (0.02-25.9)	0.74; 0.30	1.18
Fasting glucose (mmol/L)	5.8±1.9	5.6±1.2	5.9±1.7	0.27; 0.0	0.54
HDL-cholesterol (mmol/L)	1.6±0.4	1.6±0.4	1.6±0.4	-0.46;-1.69	0.77
Triglycerides (mmol/L)	1.2 (0.5-5.2)	1.3 (0.6-8.7)	1.4 (0.4-5.4)	1.45; 0.5	2.5
Hs-CRP (mg/L)	1.40 (0.20-50.8)	1.30 (0.20-49.5)	1.60 (0.10-130.9)	0.33;-0.07	0.73
Metabolic syndrome (%)	20.1	19.3	26.4	1.54; 0.5	2.6
ALT	1st tertile (≤14 U/L)	2nd tertile(>14≤22 U/L)	3rd tertile(>22 U/L)	β; 95%CI	
Number	444	459	436		
Age (yr)	53.8±5.9	55.2±5.7	54.6±5.4	0.03; -0.09	0.16
Male (%)	23.4	35.1	55.0	5.1; 3.7	6.6
Alcoholics (%)	45.5	44.2	48.7	0.97; -0.46	2.4
Alcohol (g/day within drinkers)	15.2±8.4	16.0±9.0	15.5±8.8	0.03; -0.10	0.17
Ever smokers (%)	42.9	42.3	44.9	-0.07; -1.5	1.4
BMI (kg/m^2)	25.0±4.5	26.6±4.6	28.0±4.9	0.69; 0.55	0.83
Waist (cm)	85.4±12.9	89.7±12.1	95.3±12.3	0.29; 0.23	0.35
Systolic blood pressure (mmHg)	131.1±16.0	133.0±15.3	135.5±16.3	0.09; 0.05	0.13
Diastolic blood pressure (mmHg)	81.7±9.3	82.8±8.8	84.4±9.5	0.15; 0.07	0.23
Uric acid (mmol/L)	172.5±53.5	190.3±53.5	214.1±59.5	0.05; 0.04	0.06
Insulin (pmol/L)	10.2 (0.6-151.2)	10.8 (0.6-409.8)	15.0 (0.6-244.2)	3.4; 2.6	4.2
HOMA insulin resistance (mU/mL×mmol/L)	0.38 (0.02-14.3)	0.43 (0.02-31.8)	0.63 (0.02-19.9)	3.5; 2.8	4.2
Fasting glucose (mmol/L)	5.5±1.4	5.6±1.2	6.1±2.1	1.5; 1.1	1.9
HDL-cholesterol (mmol/L)	1.6±0.4	1.6±0.3	1.5±0.3	-6.8; -8.8	-4.8
Triglycerides (mmol/L)	1.2 (0.5-8.0)	1.3 (0.4-8.7)	1.5 (0.5-5.4)	5.8; 4.2	7.4
Hs-CRP (mg/L)	1.10 (0.10-50.8)	1.40 (0.20-49.5)	1.60 (0.10-130.9)	1.4; 0.8	2.1
Metabolic syndrome (%)	12.8	21.1	32.1	5.5; 3.8	7.2
GGT	1st tertile (≤12 U/L)	2nd tertile (>12≤20 U/L)	3rd tertile(>20 U/L)	β; 95%CI	
Number	412	460	467		
Age (yr)	53.6±5.5	54.8±5.8	55.1±5.7	0.009; 0.003	0.015
Male (%)	15.5	38.9	56.1	0.43; 0.37	0.50
Alcoholics (%)	36.6	47.9	52.7	0.15; 0.08	0.22
Alcohol (g/day within drinkers)	14.1±7.9	15.0±9.0	16.9±8.8	0.013; 0.007	0.019
Ever smokers (%)	36.9	43.6	48.8	0.13; 0.06	0.20
BMI (kg/m^2)	24.9±4.2	26.7±4.7	27.7±5.0	0.03; 0.02	0.04
Waist (cm)	84.4±12.5	90.4±12.4	94.8±12.2	0.016; 0.014	0.018
Systolic blood pressure (mmHg)	129.7±15.0	133.4±15.4	136.1±16.8	0.006; 0.004	0.008
Diastolic blood pressure (mmHg)	81.5±8.7	82.8±9.3	84.4±9.6	0.009; 0.005	0.013
Uric acid (mmol/L)	166.5±47.6	190.3±59.5	214.1±59.5	0.0037;0.003	0.004
Insulin (pmol/L)	10.2 (0.6-120.0)	10.8 (1.2-409.8)	13.8 (0.6-396.0)	0.13; 0.09	0.17
HOMA insulin resistance (mU/mL´mmol/L)	0.37 (0.02-5.13)	0.43 (0.04-25.9)	0.60 (0.02-31.8)	0.14; 0.10	0.18
Fasting glucose (mmol/L)	5.4±1.1	5.7±1.4	6.1±2.1	0.09; 0.07	0.11
HDL-cholesterol (mmol/L)	1.7±0.3	1.6±0.3	1.5±0.3	-0.32;-0.22	-0.42
Triglycerides (mmol/L)	1.1 (0.4-4.4)	1.3 (0.4-8.7)	1.5 (0.6-8.0)	0.44; 0.36	0.52
Hs-CRP (mg/L)	1.00 (0.10-20.0)	1.40 (0.20-50.8)	1.80 (0.20-130.9)	0.12; 0.09	0.15
Metabolic syndrome (%)	11.6	20.2	32.8	0.35; 0.27	0.43

Median (range) is reported for not-normally distributed variables; their log-transformed values and log-GGT values are used in the analyses.

abolished after adjustment for adiposity[2-6,8-10].

Accordingly, we have found in all our patients a worse metabolic pattern in subjects within the highest ALT and GGT tertiles than those within the highest AST tertile. Increased ALT and GGT levels are associated with hepatic insulin resistance and a subsequent decline in hepatic insulin sensitivity[1-5] and GGT seems implicated in oxidative stress[4,16-18].

Excess deposition of fat in the liver, the non-alcoholic fatty liver disease, shows strong cross-sectional associations with obesity, insulin resistance and type 2 diabetes[14,15] and is associated with liver enzyme elevation.

Table 2 Clinical and laboratory characteristics of the subjects with normal BMI and without any component of the metabolic syndrome, according to the tertile of liver enzyme concentrations (left); associations of the variables listed with liver enzyme levels, as a continuous variable, by unadjusted linear regression analyses (right)

AST	1st tertile (≤13 U/L)	2nd tertile (>13≤17 U/L)	3rd tertile (>17 U/L)	β; 95%CI	
Number	67	69	69		
Age (yr)	52.2±5.5	52.5±5.7	52.1±5.1	0.001;-0.16	0.16
Male (%)	22.4	34.8	42.0	0.8; -1.0	2.6
Alcoholics (%)	47.8	47.8	56.5	1.2; -0.5	2.9
Alcohol (g/day within drinkers)	15.5±9.1	13.8±9.0	18.5±9.1	0.05; -0.07	0.18
Ever smokers (%)	47.8	39.1	44.9	-0.63;-2.35	1.09
BMI (kg/m²)	21.8±2.0	22.1±1.9	22.1±2.1	0.07;-0.35	0.49
Waist (cm)	76.3±8.1	78.4±7.8	75.3±5.6	0.08;-0.02	0.18
Systolic blood pressure (mmHg)	117.4±7.7	116.7±7.4	116.6±9.1	-0.05;-0.15	0.05
Diastolic blood pressure (mmHg)	74.9±6.0	75.1±5.8	75.3±5.6	-0.01;-0.16	0.14
Uric acid (mmol/L)	154.6±47.6	160.6±41.6	172.5±53.5	0.014;-0.004	0.03
Insulin (pmol/L)	6.0 (0.6-49.2)	9.6 (1.2-108.6)	9.0 (3.0-72.0)	0.41;-0.90	1.72
HOMA insulin resistance (mU/mL´mmol/L)	0.25 (0.02-2.08)	0.34 (0.05-5.40)	0.30 (0.10-2.28)	0.48;-0.85	1.81
Fasting glucose (mmol/L)	5.1±0.4	5.1±0.5	5.2±0.7	0.84;-0.68	2.36
HDL-cholesterol (mmol/L)	1.8±0.3	1.7±0.4	1.8±0.3	1.73;-0.73	4.19
Triglycerides (mmol/L)	1.0 (0.5-1.7)	1.1 (0.6-1.7)	1.0 (0.4-1.7)	-0.48;-3.65	2.69
Hs-CRP (mg/L)	0.80 (0.10-20.0)	0.60 (0.20-12.6)	0.70 (0.20-8.90)	-0.16;-0.96	0.64
ALT	1st tertile (≤12 U/L)	2nd tertile (>12≤17 U/L)	3rd tertile (>17 U/L)	β; 95%CI	
Number	66	67	72		
Age (yr)	51.9±5.5	52.3±5.4	52.6±5.5	-0.04; -0.29	0.21
Male (%)	15.1	35.8	47.2	4.43; 1.57	7.29
Alcoholics (%)	45.4	50.7	55.6	2.02; -0.72	4.76
Alcohol (g/day within drinkers)	14.8±9.3	16.7±9.1	16.6±9.4	0.03; -0.2	0.26
Ever smokers (%)	47.0	46.3	38.9	-1.10; -3.86	1.66
BMI (kg/m²)	21.8±2.1	21.5±2.1	22.6±1.7	0.55; -0.13	1.23
Waist (cm)	76.8±7.2	75.4±8.8	81.2±7.3	0.23; 0.07	0.39
Systolic blood pressure (mmHg)	115.2±9.0	117.4±6.3	117.9±8.4	0.09; -0.08	0.26
Diastolic blood pressure (mmHg)	75.3±6.3	74.0±5.3	75.9±5.7	0.15; -0.08	0.38
Uric acid (mmol/L)	154.6±41.6	160.6±53.5	172.5±47.6	0.035; 0.008	0.06
Insulin (pmol/l)	7.5 (0.6-108.6)	9.6 (2.4-49.2)	8.4 (1.2-72.0)	1.24; -0.88	3.36
HOMA insulin resistance (mU/mL×mmol/L)	0.29 (0.02-5.40)	0.34 (0.09-2.08)	0.30 (0.05-2.56)	1.41; -0.73	3.54
Fasting glucose (mmol/L)	5.2±0.6	5.0±0.5	5.2±0.6	1.43; -1.02	3.88
HDL-cholesterol (mmol/L)	1.8±0.3	1.7±0.4	1.7±0.4	-2.07; -6.05	1.91
Triglycerides (mmol/L)	0.9 (0.5-1.7)	1.0 (0.4-1.5)	1.0 (0.4-1.7)	0.91; -4.22	6.04
Hs-CRP (mg/L)	0.80 (0.10-20.0)	0.50 (0.20-17.8)	0.95 (0.20-12.6)	1.15; -0.12	2.42
GGT	1st tertile (≤10 U/L)	2nd tertile (>10≤16 U/L)	3rd tertile (>16 U/L)	β; 95%CI	
Number	68	71	66		
Age (year)	51.7±5.2	52.3±5.6	52.8±5.5	0.009; -0.008	0.026
Male (%)	17.6	29.6	53.0	0.48; 0.29	0.66
Alcoholics (%)	36.8	54.9	60.6	0.24; 0.06	0.42
Alcohol (g/day within drinkers)	11.2±7.9	16.6±8.6	18.6±9.6	0.012; -0.002	0.026
Ever smokers (%)	35.3	45.1	51.5	0.14; -0.05	0.33
BMI (kg/m²)	21.7±1.9	22.0±2.0	22.3±2.1	0.034; -0.012	0.08
Waist (cm)	76.4±7.2	76.4±8.6	81.1±7.7	0.19; 0.18	0.20
Systolic blood pressure (mmHg)	116.0±9.0	117.3±6.3	117.4±8.7	0.008;-0.004	0.02
Diastolic blood pressure (mmHg)	74.6±6.3	74.9±5.7	75.8±5.4	-0.012;-0.004	0.03
Uric acid (mmmol/L)	148.7±35.7	166.5±41.6	178.4±59.5	0.004; 0.002	0.006
Insulin (pmol/L)	9.6 (3.0-72.0)	9.0 (0.6-63.6)	8.4 (1.2-108.6)	0.004;-0.14	0.14
HOMA insulin resistance (mU/mL×mmol/L)	0.34 (0.10-2.28)	0.30 (0.02-2.56)	0.30 (0.04-5.40)	0.047; -0.09	0.18
Fasting glucose (mmol/L)	4.8±0.4	5.1±0.5	5.5±0.5	0.35; 0.19	0.51
HDL-cholesterol (mmol/L)	1.8±0.3	1.7±0.3	1.7±0.4	-0.12; -0.39	0.15
Triglycerides (mmol/L)	0.9 (0.4-1.5)	1.0 (0.4-1.7)	1.1 (0.7-1.7)	0.52; 0.19	0.85
Hs-CRP (mg/L)	0.50 (0.20-8.30)	0.80 (0.10-8.90)	0.95 (0.20-20.0)	0.21; 0.13	0.29

Median (range) is reported for not-normally distributed variables; their log-transformed values and log-GGT values are used in the analyses.

Thus, the relationships previously found might reflect associations between ALT or GGT levels and obesity or insulin resistance. Accordingly, authors have found that the associations between GGT levels and diabetes or blood pressure have been attenuated on adjustment for known risk factors for diabetes or for plasma insulin levels and have speculated that visceral fat could play a role in the association of GGT with type 2 diabetes[2,4,27].
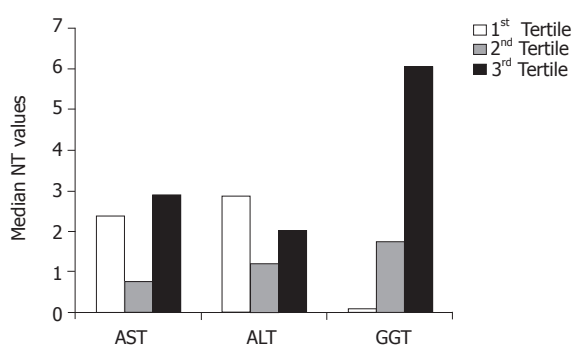
The subgroup of subjects free from abnormal glucose, lipid, pressure values and with normal BMI and waist circumference avoids these possible interference from confounding factors. Within this group, increased GGT levels are significantly associated with hs-CRP and fasting glucose values, after multiple adjustments.

Table 3 Associations of different clinical and laboratory variables with liver enzyme levels in all the patients (left) and in subjects with normal BMI and without any component of the metabolic syndrome (right) in a multiple regression model

	ALL			Normal	BMI and no component of the MS	
	β	95%CI		β	95%CI	
	AST					
Age	0.02	-0.06	0.10	-0.015	-0.17	0.14
Male	0.58	-0.54	1.70	-0.70	-3.05	1.65
Ever smoking	-1.06	-2.16	0.04	-0.54	-2.34	1.26
BMI	-0.13	-0.29	0.03	-0.25	-0.83	0.33
Waist	0.09	0.03	0.15	0.12	-0.04	0.28
G/day alcohol	0.04	0.0	0.08	0.07	-0.02	0.16
Hs-CRP	-0.015	-0.06	0.03	-0.29	-0.55	1.13
HOMA-IR	0.25	-0.28	0.77	0.26	-1.11	1.63
Triglycerides	0.17	-0.91	1.25	-1.39	-4.76	1.98
Uric acid	0.01	0.0	0.02	0.01	-0.009	0.03
	ALT					
Age	-0.09	-0.21	0.02	-0.12	-0.37	0.13
Male	3.32	1.58	5.06	3.36	-0.32	7.04
Ever smoking	1.13	-0.30	2.56	-2.27	-5.11	0.57
BMI	0.22	-0.01	0.45	0.07	-0.83	0.97
Waist	0.10	0.01	0.19	0.11	-0.14	0.36
G/day alcohol	0.015	-0.05	0.08	0.03	-0.11	0.17
Hs-CRP	-0.04	-0.74	0.66	1.16	-0.15	2.47
HOMA-IR	1.85	1.03	2.67	0.91	-1.25	3.07
Triglycerides	2.07	0.37	3.77	-0.88	-6.15	4.39
Uric acid	0.015	0.001	0.03	0.01	-0.02	0.04
	GGT					
Age	-0.0004	-0.006	0.006	-0.003	-0.019	0.013
Male	0.29	0.21	0.37	0.31	0.10	0.52
Ever smoking	0.02	-0.04	0.07	0.025	-0.16	0.20
BMI	-0.0003	-0.012	0.012	-0.02	-0.07	0.04
Waist	0.004	0.0	0.008	0.0035	-0.012	0.019
G/day alcohol	0.006	0.002	0.010	0.005	-0.003	0.014
Hs-CRP	0.09	0.05	0.12	0.19	0.11	0.27
HOMA-IR	0.03	-0.01	0.07	-0.05	-0.19	0.09
Triglycerides	0.21	0.13	0.29	0.26	-0.05	0.57
Uric acid	0.0012	0.0006	0.0018	0.002	0.0	0.004

Multiple regression analyses with adjustments for all the variables listed.

Figure 1 Median NT values for tertile of liver enzyme levels in subjects with normal BMI and without any component of the metabolic syndrome. At simple and multiple regression analyses NT values are associated with increasing GGT tertiles ($P<0.001$), but not with ALT or AST tertiles.

There is a progressive and significant increment of NT levels with increasing GGT tertile, not evident within the tertiles of the other liver enzymes that were tested (Figure 1). Furthermore, NT levels are significantly associated to fasting glucose values.

NT, generated from the oxidation of tyrosine, has been considered as a measure of oxidative injury from peroxynitrite (deriving from the reaction of nitric oxide with superoxide anion radicals), and reported to be elevated in diabetes, a condition associated with oxidative stress[28,29].

It could be speculated that the association between GGT levels and fasting glucose, not confounded by other metabolic abnormalities in the subgroup of "metabolically" healthy individuals, could be due to the adverse oxidative pattern of these subjects, suggested by the sharp increase of NT levels in the individuals within the highest GGT tertile.

In line with this, previous studies have reported a primary role for GGT in metabolizing extracellular reduced glutathione, a cellular antioxidant, allowing for precursor amino acids to be reutilized for the intracellular synthesis of glutathione[4,5,18]. Furthermore, dietary and serum antioxidants inversely predicted future GGT levels, while these latter are associated to F2-isoprostanes, a marker of oxidative damage[4,16-18]. It has been speculated that elevated GGT levels might be a defensive response to oxidative stress or, otherwise a marker of oxidative stress, being involved directly in the generation of reactive oxygen species, especially in the presence of iron or other transition metals, inducing lipid peroxidation in

human biological membranes[17,18,30,31]. Whether GGT is a causative factor for oxidative stress or a marker is currently unknown.

Aminotransferases were not associated with NT levels (Figure 1), in agreement with studies that showed lower associations between ALT levels and antioxidant vitamins and micronutrients, at least in individuals with normal BMI or fat distribution[18,32].

Thus, in subjects without other metabolic abnormalities, serum GGT might represent a marker of oxidative stress, and justify the associations found with higher fasting glucose values in these healthy individuals.

We cannot exclude the possibility that abnormal GGT values reflect fatty liver deposition, predating the development of subsequent diabetes; furthermore, hepatic ultrasonography or more invasive instrumental methods have not been performed in our subjects. Otherwise, they could be considered as "metabolically obese, normal weight" individuals, in consideration of their higher, though within the normal range, waist values[33]. Indeed our subjects with elevated GGT levels do not show higher insulin levels or insulin resistance (Table 2) and fatty liver has been associated with hyperinsulinemia and insulin resistance[14,15], and associations remain significant, after adjustments for waist circumference and BMI. Accordingly, abnormal liver enzymes have been found in a proportion of normal BMI individuals without fatty liver by ultrasound[34].

Furthermore, the correlations found are independent by adjustment for aminotransferase levels, which are better indicators of hepatocellular statuses and these latter are not associated with glycemia in the subgroup of normal BMI individuals.

Finally, increased GGT levels are conventionally considered as a marker of alcoholic abuse; however, only moderate drinkers have been included in the study, adjustments for alcohol intake have been performed, and correlations do not change after including only non-drinkers.

Another possible mechanism implicated is chronic inflammation: hs-CRP, an acute-phase reactant of hepatic origin and a sensitive marker for systemic inflammation, predicts the occurrence of diabetes, the metabolic syndrome and atherosclerotic diseases in healthy subjects[35]. Previous studies have found associations between GGT and CRP or other inflammatory parameters, suggesting that this enzyme represents the expression of sub-clinical inflammation, and has a role in cellular stress[3,4,9,10,20,21]. In our healthy cohort, hs-CRP levels are significantly associated either with highest GGT values or with fasting glucose. Oxidative processes might have an implication in chronic inflammation[19]; it has been hypothesized that elevation in GGT might occur before an elevation in CRP, and the related oxidative stress would give rise to a subsequent inflammatory response[21].

Again, aminotransferases are not associated with hs-CRP in normal BMI individuals, in line with previous reports, which did not find such an association[6,8,21].

Another explanation might be related to the liver response to pro-inflammatory cytokine tumor necrosis factor-α, giving fatty hepatic changes, or to the inflammatory processes accompanying non-alcoholic fatty liver and contributing to the systemic inflammation observed in these subjects, frequently affected by the MS[20,36]. However, the lack of a significant association between hs-CRP and ALT (a better marker for liver fat accumulation) levels in our normal BMI individuals is against this pathogenetic hypothesis at least in this subgroup. Accordingly, other authors demonstrated that CRP and aminotransferases predicted diabetes independent of each other, with similar magnitude associations[8].

Since the majority of healthy subjects within the highest GGT tertile show enzyme levels within targets of normality (89%), it could be suggested that variations within the normal ranges of GGT are associated with a worse oxidative or inflammatory pattern.

A single measurement of glucose, insulin, hs-CRP or liver enzyme levels represents a limitation of the present study, although common to most epidemiological studies. However, random errors due to the fluctuations of laboratory measurements usually lead to a reduced estimate of the associated strength. Serum oxidative markers could not directly reflect hepatocellular levels, and the existence of oxidative stress depends on the relative balance of reactive oxygen species and all the microenvironment antioxidant defenses, that could not be evaluated in such an observational study.

Whether elevated GGT levels should be added to the cluster of cardiovascular risk factors that form the metabolic syndrome and link insulin resistance to cardiovascular disease, as supported by literature[37,38] or if elevated GGT levels represent a marker of oxidative stress or inflammation could not be established by a cross-sectional study. Nevertheless, the hypothesis that elevated GGT levels may represent an early, easy, and inexpensive marker for a higher subsequent metabolic risk in apparently healthy subjects seems intriguing and worthy of future investigation.

In conclusion, in adult healthy subjects without any measurable metabolic abnormality, those with the highest GGT levels present with either higher fasting glucose values (even within the range of normality) and evidence of some oxidative stress or inflammation; aminotransferase levels do not show these correlations. The follow-up of these individuals would determine if GGT values might be considered as an early predictor of subsequent diabetes occurrence.

ACKNOWLEDGMENTS

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ELSEVIER

• *CLINICAL RESEARCH*•

Usefulness of ω-3 fatty acid supplementation in addition to mesalazine in maintaining remission in pediatric Crohn's disease: A double-blind, randomized, placebo-controlled study

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Abstract

AIM: To assess the value of long-chain ω-3 fatty acids (FAs) supplementation in addition to amino-salicylic-acid (5-ASA) in pediatric patients with Crohn's disease (CD).

METHODS: Thirty-eight patients (20 males and 18 females, mean age 10.13 years, range 5-16 years) with CD in remission were randomized into two groups and treated for 12 mo. Group I (18 patients) received 5-ASA (50 mg/kg/d)+ω-3 FAs as triglycerides in gastro-resistant capsules, 3 g/d (eicosapentanoic acid, EPA, 400 mg/g, docosahexaenoic acid, DHA, 200 mg/g). Group II (20 patients) received 5-ASA (50 mg/kg/d)+olive oil placebo capsules. Patients were evaluated for fatty acid incorporation in red blood cell membranes by gas chromatography at baseline 6 and 12 mo after the treatment.

RESULTS: The number of patients who relapsed at 1 year was significantly lower in group I than in group II ($P<0.001$). Patients in group I had a significant increase in the incorporation of EPA and DHA ($P<0.001$) and a decrease in the presence of arachidonic acids.

CONCLUSION: Enteric-coated ω-3 FAs in addition to treatment with 5-ASA are effective in maintaining remission of pediatric CD.

Key words: Crohn's disease; ω-3 fatty acids; Remission maintenance; Treatment

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INTRODUCTION

The etiology and pathogenesis of CD are still unknown[1]. First-line therapy is still based on the combinations of glucocorticoids and amino-salicylic acid (5-ASA) derivatives. Knowledge of inflammatory mechanisms and the high incidence of side effects with steroids have induced researchers to look for valid alternatives, especially in maintaining remission secondary to steroid treatment[2]. The rationale for the use of ω-3 FAs in uncomplicated CD is that metabolites of the arachidonic acid (AA), which are considered as primarily responsible for inflammatory process and intestinal inflammation in CD, are produced from fatty acids[3]. Administration of EPA and DHA, the predominant fatty acids in fish oil, causes competition with AA for the utilization of 5-lipoxygenase enzyme cascade, thus inhibiting the production of inflammatory metabolites such as LTB4[4].

Data in literature on the use of ω-3 fatty acids are controversial. Belluzzi *et al*[5]. claimed that ω-3 FAs seem prudent to promote a diet rich in fish oil in patients with CD, while Lorenz-Meyer *et al*[6] did not demonstrate any effect of ω-3 FA supplementation on the remission of CD in adults.

Since the use of fish oil has not yet been intensively studied in children with CD, we carried out a placebo-controlled clinical trial in children with CD by administering either ω-3 fatty acid or olive oil capsules used as a placebo in addition to 5-ASA.

MATERIALS AND METHODS

A double-blind, randomized study was carried out in 38 patients with CD (20 males and 18 females, mean age 10.13 years, range 5-16 years) recruited from eight Italian Pediatric Gastroenterology Centers. All of them had a definite diagnosis of CD based on accepted endoscopic and histological criteria if available (27 patients)[7].

Inclusion criteria were pediatric Crohn's disease activity index (PCDAI) score <20 for at least 2 mo obtained by an 8-wk course of corticosteroid treatment (1 mg/kg/d), no extraintestinal involvement of the disease, no previous intestinal resection (apart from appendectomy), no previous immunosuppressant treatment and a wash-out

period of at least 3 mo from corticosteroids.

All patients were non-smokers. After enrollment, patients were randomized into two groups according to the intestinal localization of the disease (Table 1) and treated for 12 mo as follows. Eighteen patients (9 males and 9 females) in group 1 were treated with time-dependant 5-ASA (50 mg/kg/d)+ω-3 FAs as triglycerides in gastro-resistant capsules, 3 g/d (TRIOLIP - SOFAR, Italy). Each capsule contained 400 mg/g EPA and 200 mg/g DHA. Eighteen patients (11 males and 9 females) in group 2 were treated with time-dependant 5-ASA (50 mg/kg/d)+olive oil placebo capsules. Patients who had a relapse were excluded from the study.

Clinical evaluations and several laboratory tests [assessment of blood cell count, erythrocyte sedimentation rate (ESR), C-reactive protein (PCR) and electrophoresis] were carried out at enrollment (T0) and after 6 (T1) and then 12 (T2) months of treatment. Fatty acids in the red blood cell membranes were also evaluated with gas chromatography on capillar column (SP 2340 Supelco). Analysis temperature was set at 160 ℃ and 210 ℃ with a gradient of 8 ℃/min, while the gas carrier (He) flow was 2 mL/min. The percentage (% mol) of each single fatty acid was compared to an external standard.

Statistical analysis

Data were analyzed by the statistical program 7.0 for Windows. The significance of the differences between

Table 1 Characteristics of patients at the beginning of the study

	Group 1 (5-ASA+ω-3)	Group 2 (5-ASA+placebo)
Number of patients	18	20
Mean age (yr)	9.33	10.85
Sex (M/F)	9/9	11/9
Disease localization		
Ileal	10	7
Ileo-colic	6	8
Colic	2	5

averages and proportions was calculated by bivariate analysis (χ^2 square). $P<0.005$ was considered statistically significant.

RESULTS

Relapse (PCDAI score >20) after 1 year occurred in 11 patients (61%) of group 1 and 19 patients (95%) of group 2 (Figure 1A) with a statistically significant difference ($P = 0.0016$). However, no relapse occurred after 6 mo of follow-up in patients of group 1 who received capsules of EPA and DHA. The disease relapse occurred 8 mo after the treatment in group 1 and 1 mo in group 2 (Figure1B) with a statistically significant difference ($P<0.001$). Patients in group 2 and those with ileo-colonic localization presented a greater number of relapses (Figure 1C)

Figure 1 Relapses of CD after 1 yr (**A**), throughout the follow-up (**B**) and in those with ileo-colonic CD (**C**) (%).Characteristics of patients at the beginning of the study

compared to those under treatment (group 1) who had the same initial localization of the disease ($P = 0.001$). Compliance to the assumption of the capsules was optimal in all the patients and no side effects were reported in patients of either group.

All subjects in group 1 showed a significant increase in the range of incorporation of EPA (%/mol): 1.1 ± 1, 2.48 ± 1.83, 2.07 ± 0.5 ($P<0.005$) and DHA (% mol): 1.81 ± 0.68, 2.55 ± 0.97, 3.54 ± 0.85 ($P<0.001$) and a decrease in the presence of AA (% mol): 6.17 ± 2.63, 5.5 ± 2.33, 5.56 ± 2.05 between T0 and T2.

DISCUSSION

Since no definite treatment for CD is available and the precise mechanism which triggers and maintains the intestinal inflammation is not yet well known, the aim of the treatment is to induce and maintain clinical remission[8]. There are several therapeutic options for the treatment of CD. Mild-to-moderate disease has been managed with nutritional support, 5-ASA and antibiotics[9], while the mainstay therapy for severe or complicated disease includes steroids, immunosuppressants and biological therapy[10].

Drugs most commonly used in the treatment of

inflammatory bowel disease (IBD) act by interfering with the AA metabolic pathway. Glucocorticoids prevent the formation of free AA through the inhibition of the activity of phospholipases A2 and C on the membrane phospholipids, while mesalazine acts with its N-acetyl-5-ASA metabolite through the inhibition of 5-lipoxygenase in the colonic mucosa. However, both drugs have not proved to be effective in maintaining the remission of CD[11] and many relapses of the disease can occur[12]. Biological agents introduced in recent years seem to be effective in inducing remission of active disease resistant to standard treatment but not in maintaining the remission[13].

The most prolonged remission has been obtained with 6-mercaptopurine and its pro-drug azathioprine[14]. Their efficacy in maintaining remission is widely accepted, but toxicity and possible side-effects have been reported[15]. Another therapeutic option to maintain CD remission is represented by w-FAs. In 1986, Lee et al[16]. reported that fatty acids contained in fish oil can limit the AA pathway. Kim[17] reported that patients with active CD have increased levels of LTB4 in the colic mucosa associated with the severity of the disease. The anti-inflammatory effect of fish oil has been tested on animals (rats with induced colitis) and ω-3 FAs are observed to determine other biochemical effects such as reduction of mucosal and circulating levels of the main powerful pro-inflammatory cytokines (IL-1, TNF-α) and reduction of synthesis of the platelet activating factor (PAF), which has a strong pro-inflammatory activity. In addition, it has even been demonstrated that it acts as a free radical scavenger[18]. Fish oil reduces the inflammatory process competing with the AA. In fact, EPA, a polinsaturi fatty acid of 20 carbon atoms produced by the metabolism of α-linoleic acids, is different from the AA only for the presence of various double bonds in position-17. It is a good substratum for 5-lipoxygenase and competes with the AA for the utilization of this enzyme together with its corresponding DHA. The incorporation of EPA and DHA into the erythrocyte membranes is necessary to stimulate a competitive action on the AA. This effect involves a reduced LTB4 production and synthesis of a new leukotriene, LTB5, which has no inflammatory effects[19]. In 1990 Mc Call et al[20]. and Salomon et al[21]. carried out the preliminary clinical studies, showing that administration of fish oil in patients with IBD causes a significant reduction of the disease activity score (CDAI score). Hawthorne et al[22]. have verified how fish oil supplementation in patients with IBD in the active phase significantly reduces steroid consumption and results in a longer clinical remission. Confirmation of the usefulness of EPA in IBD is demonstrated by the significant decrease in the production of LTB4[23]. Since there are no adequate formulations of fish oil which could be used as a drug without side-effects, its widespread use in the treatment of IBD is limited. Belluzzi et al[5]. showed that capsules of EPA and DHA, a free fatty acid mixture, are well tolerated and can be used to treat patients for long periods instead of administering pure fish oil. Other reports are controversial, showing contrasting results in maintaining

the remission of CD[24,25].

All these experiences reported in literature are referred to adult CD, but ω-3 FAs have not yet been evaluated in children with CD. Our study is the first placebo-controlled clinical trial in pediatric CD and has achieved several interesting results though the number of patients enrolled was less. In fact, the addition of enteric-coated capsules to a conventional therapy with 5-ASA in pediatric CD delayed the relapse of the disease even if it could not prevent it. This association seems that the addition of enteric-coated capsules is more effective than 5-ASA alone probably due to the synergistic effect of the drugs used. 5-ASA can inhibit some key factors of the inflammatory cascade (cyclooxygenase, thromboxane-synthetase and PAF-synthetase) as well as production of interleukin 1 and free radicals[26-28] and has an intrinsic anti-oxidant activity[29]. Furthermore, ω-3 FAs inhibit PAF-synthetase and thromboxane-synthetase acts as a free radical scavenger decreasing mucosal and circulating levels of some powerful pro-inflammatory cytokines (such as IL-1 and TNF-α)[18]. In light of these biochemical characteristics, it is clear that the association of these two anti-inflammatory compounds may strengthen their single anti-inflammatory role and explain the better results obtained with 5-ASA alone in maintaining the remission of pediatric CD. 5-ASA/ω-3 FAs seem to play the same role in pediatric CD as the balsalazide/VSL#3 in the treatment of adult ulcerative colitis. 5-ASA plus a probiotic mixture can obtain significantly better results than balsalazide alone or mesalazine alone in maintaining remission of mild-to-moderate ulcerative colitis due to the synergistic effect of the two powerful anti-inflammatory compounds[30].

In conclusion, supplementation of ω-3 fatty acids to a standard treatment with 5-ASA is a good choice for maintaining remission of pediatric CD.

ACKNOWLEDGMENTS

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Nutr Rev 1996; **54:** 248-252

• CLINICAL RESEARCH •

Outcome of non-variceal acute upper gastrointestinal bleeding in relation to the time of endoscopy and the experience of the endoscopist: A two-year survey

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Abstract

AIM: To prospectively assess the impact of time of endoscopy and endoscopist's experience on the outcome of non-variceal acute upper gastrointestinal (GI) bleeding patients in a large teaching hospital.

METHODS: All patients admitted for non-variceal acute upper GI bleeding for over a 2-year period were potentially eligible for this study. They were managed by a team of seven endoscopists on 24-h call whose experience was categorized into two levels (high and low) according to the number of endoscopic hemostatic procedures undertaken before the study. Endoscopic treatment was standardized according to Forrest classification of lesions as well as the subsequent medical therapy. Time of endoscopy was subdivided into two time periods: routine (8 a.m.-5 p.m.) and on-call (5 p.m.-8 a.m.). For each category of experience and time periods rebleeding rate, transfusion requirement, need for surgery, length of hospital stay and mortality we compared. Multivariate analysis was used to discriminate the impact of different variables on the outcomes that were considered.

RESULTS: Study population consisted of 272 patients (mean age 67.3 years) with endoscopic stigmata of hemorrhage. The patients were equally distributed among the endoscopists, whereas only 19% of procedures were done out of working hours. Rockall score and Forrest classification at admission did not differ between time periods and degree of experience. Univariate analysis showed that higher endoscopist's experience was associated with significant reduction in rebleeding rate (14% vs 37%), transfusion requirements (1.8±0.6 vs 3.0±1.7 units) as well as surgery (4% vs 10%), but not associated with the length of hospital stay nor mortality. By contrast, outcomes did not significantly differ between the two time periods of endoscopy. On multivariate analysis, endoscopist's experience was independently associated with rebleeding rate and transfusion requirements. Odds ratios for low experienced endoscopist were 4.47 for rebleeding and 6.90 for need of transfusion after the endoscopy.

CONCLUSION: Endoscopist's experience is an important independent prognostic factor for non-variceal acute upper GI bleeding. Urgent endoscopy should be undertaken preferentially by a skilled endoscopist as less expert staff tends to underestimate some risk lesions with a negative influence on hemostasis.

INTRODUCTION

Acute upper gastrointestinal bleeding (AUGB) is the most common emergency managed by gastroenterologists, with an incidence ranging from approximately 50 to 150 per 100 000 per year in the Western population[1,2]. The treatment of this condition has made important progress since the introduction of emergency endoscopy and endoscopic techniques for hemostasis along with

the application of specific post-endoscopic protocols, significantly decreases rebleeding and the need for surgery[3-7], whereas mortality rates associated with AUGB still range as 5-15%[8,9].

Several clinical and endoscopic score systems have been proposed to risk-stratify patients with AUGB in order to predict outcome and several factors such as age, shock and tachycardia at presentation, the presence of severe medical comorbidity and the lesion's appearance at endoscopy have been shown to be associated with adverse prognosis[10-13]. In particular, Forrest's classification of endoscopic findings closely associated with peptic ulcer disease but sometimes seen with other causes of AUGB is associated with specific recurrent bleeding rates and is commonly used to assess the need for endoscopic therapy[14].

As far as the effectiveness of various endoscopic therapies for AUGB is concerned, a recent review indicates that differences in terms of hemostatic results using the same treatment modality, exist between research studies and clinical practice as well as among various randomized clinical trials and are probably related to surgeon-dependent factors[15]. Surprisingly, the experience of surgeons in achieving endoscopic hemostasis has not yet been examined, whereas the time of endoscopy has received so far little attention as a possible variable influencing the outcome of AUGB[16-19].

We therefore undertook a 2-year survey in order to assess prospectively the impact of surgeon's experience and time of endoscopy on the outcome of acute non-variceal upper gastrointestinal (GI) bleeding patients in a large tertiary referral center of western Milan.

MATERIALS AND METHODS

Patients
Two hundred and seventy-two (mean age 67.3 years) patients who presented with AUGB to L. Sacco University Hospital, Milan, between June 2001 and July 2003 were included in this study. L. Sacco Hospital is a large teaching hospital located in western Milan with a catchment area of nearly 250.000 inhabitants and provides 24 h emergency endoscopy for two district neighboring hospitals serving an additional population of approximately 200 000 inhabitants.

The treatment protocol for patients with AUGB did not change during the study period and could be summarized in short as follows. All patients who arrived or were referred from a district hospital to the Accident and Emergency Department of our hospital with clinical manifestations of AUGB were managed according to a three-stage scheme: stage I: initial clinical and laboratory evaluation in the Emergency Department including placement of a double-bore nasogastric tube; stage II: hemodynamic stabilization including infusion of crystalloid fluids to maintain adequate blood pressure; stage III: urgent endoscopy within 12 h from presentation in patients who had at least one of the following presenting features: hematemesis with red blood or coffee

grounds, passage of melena and a hematocrit below the normal range with a nasogastric aspirate demonstrating red blood or coffee ground material. Recommendations regarding admission to the various hospital departments were made to the attending physicians by the endoscopist mainly based on the assessment of clinical and endoscopic criteria.

We used the Forrest's classification[14] for endoscopic grading of bleeding lesions as follows: class 1A: active ulcer bleeding presenting as arterial spurting or pulsatile bleeding from the ulcer base; class 1B: milder forms present as continuous oozing either from a visible vessel or from underneath an adherent clot; class 2A: in the absence of active bleeding, the stigmata of recent hemorrhage including a non-bleeding visible vessel seen as a red or whitish-gray elevated lesion at the base of the ulcer; class 2B: an adherent clot covering the base of an ulcer; class 2C: a flat pigmented spot or a black membrane covering the ulcer base; class 3: a clean ulcer bottom (i.e, without vessel nor clot).

Patients with endoscopic stigmata of recent hemorrhage, regardless of whether they received endoscopic hemostasis or not were usually admitted to the surgical or gastroenterological ward. The allocation choice was mainly based on the presence of additional medical comorbidity (such as diabetes mellitus, renal failure, etc.) and available space at the different services. Patients with hemodynamic instability after the endoscopic procedure were usually admitted to the intensive care unit (ICU).

Study population
All patients aged 16-95 years undergoing urgent endoscopy (within 12 h after admission) for non-variceal AUBG and those who presented endoscopic stigmata of recent hemorrhage were potentially eligible for this study. Patients were excluded if they had variceal bleeding (both from esophagus and stomach), those who bled from an evident digestive tumor and other non-ulcer lesions such as Dieulafoy's lesions and Mallory-Weiss tears. In particular, Mallory-Weiss tears could not enrolled be since they have a low risk of recurrent bleeding[20]. Dieulafoy's lesions were excluded since we preferred to treat these lesions by mechanical methods (such as banding or clipping) or APC rather than by epinephrine injection plus heat probe as in this study. Even patients with clearly malignant ulcers at endoscopy (i.e, patients with large flat, plaque-like, ulcerated tumors) were excluded due to the difficulty of standardizing the endoscopic hemostatic maneuvers under these circumstances. Finally, patients with ASA grade 5 were not enrolled in the study due to their severe clinical conditions as defined in this category.

Patients were evaluated at the time of admission using an extensive standardized-item list. The medical history (including concomitant disease, smoking habit, previous peptic disorders) and complaints (melena, hematemesis, hematochezia, dyspeptic complaints, syncope) were recorded. Concomitant diseases were categorized into six main classes (Table 1). The findings on physical examination were also recorded. Hemodynamic instability

Table 1 Distribution of 272 patients with acute non-variceal upper GI bleeding according to age, sex, and other selected covariates

Covariates		n (%)
Sex, male		186 (68.4)
Mean age (yr)		67.4±15.8
<40		19 (7.0)
40–49		24 (8.8)
50–59		26 (9.6)
60–69		51 (18.8)
70–79		88 (32.4)
≥80		64 (23.5)
Shock grade[1]: 0		213 (78.3)
1		35 (12.8)
2		24 (8.8)
Comorbidities: cardiovascular		73 (26.8)
Neoplastic		30 (11.0)
Hepatic		26 (9.5)
Nephropathic		17 (6.2)
Multiple		48 (17.6)
Others		37 (13.3)
Main symptom at presentation[2]: hematemesis		114 (42.0)
Melena		89 (33.0)
Anemia		69 (25.0)
Epigastric pain		19 (7.0)
Time of endoscopy: 8.00 a.m.–5.00 a.m.		221 (81.0)
5.00 p.m.–8.00 a.m.		51 (19.0)
Rockall score: 1–3		58 (21.3)
4–6		146 (53.7)
>6		68 (25.0)
Ulcer location: esophagus		21 (7.7)
Stomach		121 (44.5)
Duodenum		130 (47.8)
Ulcer size[3]: <20 mm		152 (68.5)
≥20 mm		70 (31.5)
Forrest classification: 1A		6 (2.2)
1B		59 (21.7)
2A		19 (7.0)
2B		63 (23.2)
2C		125 (45.9)
Transfusion requirements before		
endoscopy: (number of blood units)	0	229 (84.2)
	1–4	37 (13.6)
	≥5	6 (2.2)

[1]Shock grade: grade 0 = no shock signs (systolic BP >100, pulse <100), grade 1 = tachycardia (systolic BP >100, pulse >100), grade 2 = hypotension (systolic BP <100, pulse >100). [2]The sum of main symptoms is higher than the number of patients as some patients had more than one main symptom at presentation. [3]The sum does not add up to the total because of some missing values.

was defined as a systolic blood pressure <100×0.133 kpa or a heart rate >100 r/mm.

The Rockall score was calculated from age, hemodynamic characteristics, endoscopic findings, and comorbidity as previously described[21].

Medications before and during the hospital stay [apart from intravenous proton pump inhibitors (PPIs) used for the bleeding episode] as well as the number of units of blood transfused before and after endoscopy were specifically noted.

Endoscopists and endoscopic procedures

We limited the endoscopists to seven gastroenterologists who were on a 24-h call for emergency endoscopy and whose experience was arbitrarily categorized into two levels according to the number of hemostatic endoscopic procedures done at the time of study. Five endoscopists (three consultant gastroenterologists and two senior registrars) had high experience (each one had performed more than 3 000 upper GI endoscopies and more than 100 emergency procedures). The three consultants had been in practice for more than 8 years, whereas the two senior registrars had completed 6 years of training in general gastroenterology and gastrointestinal endoscopy. The other endoscopists (young senior registrars in gastroenterology) had less experience as they had just completed only 4 years of training in general gastroenterology and had performed less than 1 000 upper GI endoscopies and 40–70 emergency procedures, as a principal surgeon before participating in this study. All emergency endoscopies were undertaken by the gastroenterologists on call in a separate endoscopic suite. Endoscopies performed between 8 am and 5 pm from Monday to Friday were defined as done within working hours (routine), whereas those performed at other time points were classified as done out of working hours (on call).

The endoscope employed was a Pentax EG 3440 video endoscope with a large operative channel (3.5 mm, Pentax, GmbH, Hamburg, Germany).

To improve the visual field, gastric lavage with a broad double-bore nasogastric tube was used and continuous normal saline infusion was carried out before endoscopy. Ulcers with bleeding stigmata were cleaned by water irrigation through the biopsy channel. Adherent clots were washed with a jet of water delivered through a catheter passed through the endoscope.

Only ulcerative lesions with endoscopic stigmata of acute bleeding, visible vessels or adherent clot (Forrest Ia–IIc) were included in the present study. Since previous studies have shown medium to poor inter observer variability in assessing endoscopic stigmata of bleeding[21],we attempted at reducing interobserver bias onthe grading of endoscopic stigmata by reviewing the video records of 20 explicative cases at a pre-study meeting.

Endoscopic therapy was standardized as follows: initial injection of 1:10 000 adrenaline around the bleeding lesion (up to a maximum of 20 mL) to achieve a tamponade effect, followed by application of a 3.2-mm heater probe

(Olympus, CD-120 U, Tokyo, Japan) at settings of 30 J per goal until the achievement of a coaptive effect[22]. This method was used whenever endoscopic stigmata of hemorrhage such as acute spurting, oozing, visible vessel or adherent clots were present. Written guidelines containing the above mentioned recommendations concerning the hemostatic maneuvers to be undertaken were circulated among the endoscopists participating in this survey before the study.

The success of endoscopic hemostasis was defined as the cessation of bleeding together with the achievement of cavitation over the lesion after the application of the heater probe.

After endoscopy, the surgeon filled in a specific form with all the details concerning the procedure with specific reference to the appearance of bleeding lesions (according to the Forrest's classification) and the hemostatic maneuvers that were undertaken. Epinephrine solution injected and the number of pulses with the heater probe whenever employed were recorded. The time of endoscopy after admission was also recorded.

No patient was initially treated either with endoscopic band ligation or with hemoclipping, whereas these therapies were occasionally used by a senior endoscopist to obtain hemostasis in case of recurrent bleeding.

All patients received high dose intravenous PPIs: omeprazole or pantoprazole, 80 mg bolus within 12 h of endoscopy followed by 8 mg/h for 3 d [23] and then an oral PPI, 40 mg once daily for the remainder of their hospital stay. Patients were closely monitored and underwent clinical reviews with their blood pressure, pulse, respiratory rate, and urine output measured hourly for the first 24 h followed by close observation for symptoms and signs of recurrent bleeding throughout their stay in the hospital. Subsequent management decision was made by the attending physician. No attempt was made to persuade the attending physician to follow a specific course of action beyond the recommendations quoted above and to determine the length of hospital stay.

Outcome measures

The major outcome parameters were rebleeding, surgical intervention, and mortality. Rebleeding was defined as repeated melena, hematemesis or a drop in hemoglobin concentration (>2 g/dL in 24 h) after a period of stabilization and unexplained by fluid replacement within 28 d of the initial bleeding episode. We performed a second endoscopy to confirm clinical recurrent bleeding which was defined as persistent endoscopic stigmata of acute spurting or oozing, visible vessels or adherent clots with the appearance of blood clots or coffee ground material in the stomach or duodenum. Our study had a result greater than 95% to reject the null hypothesis that the proportion of rebleeding patients was the same in endoscopists with high and low experience.

Surgery was performed in those patients whose bleeding could not be stopped by primary endoscopic hemostasis or by a second or third endoscopic therapy. Interventional radiology was not available for patients who did not respond to endoscopic therapy when the study started. The choice of surgery was left to the individual surgeon though gastrectomy was the most preferred operation for the control of ulcer bleeding. The mortality was defined as death within 28 d of the bleeding episode.

Secondary outcome measures included the number of packed red cell units transfused after endoscopy and the length of hospital stay.

Appropriateness of endoscopic hemostatic maneuvers

The appropriateness of each endoscopic procedure was evaluated jointly by the two consultant gastroenterologists who did not participate in the procedures and were blinded to the outcome of patients and name of the endoscopist. Treatment in accordance with the implemented guidelines[6] was classified as appropriate, treatment in disagreement as inappropriate. In many instances video taping of the cases was also used with regard to the appropriateness.

Statistical analysis

Univariate analysis was performed by the χ^2 test for frequencies and by the Man-Whitney rank sum test for means.

Odds ratios (OR) and corresponding 95% confidence intervals (CI) were estimated by unconditional multiple logistic regression models[24] after adjustment for age (<40/40-49/50-59/60-69/70-79/≥80 years), sex, Rockall (<6/≥6) and Forrest's score (IA,IB/2A-2C), blood transfusion (no/yes), endoscopist's experience (high/low), and time of endoscopy (routine/on-call). Moreover, we estimated ORs after a further adjustment for appropriateness of treatment (appropriate/inappropriate).

The significance of OR estimates was represented by the corresponding 95%CI. If OR did not include unity, the estimate was statistically significant.

RESULTS

Overall results

Between June 2001 and July 2003, we recruited 272 patients (mean age 67.3 years, 186 males, 94% Caucasians) who presented to the A&E, Department of Luigi, Sacco University Hospital for non-variceal AUGB and underwent upper GI endoscopy within 12 h after admission with the presence of endoscopic stigmata of hemorrhage. They satisfied all the inclusion criteria of the study. Table 1 describes the distribution of these patients according to sex, age, and other selected covariates.

Overall, the rebleeding, surgical intervention, and mortality rates were 18.7%, 5.5%, and 13.2%, respectively. Initial endoscopic hemostasis was achieved in 268 of the 272 patients (98.5%) and four patients required immediate surgery for failure of primary endoscopic hemostasis. Fifty-one patients had at least a rebleeding episode after initial hemostasis (Figure 1). Thirty-six patients died within 28 d from the initial episode of bleeding. Their ultimate causes of death were cardiac failure (15), pulmonary failure (9), liver failure (4), multi organ failure (2), renal failure (4), and cerebral edema (2).
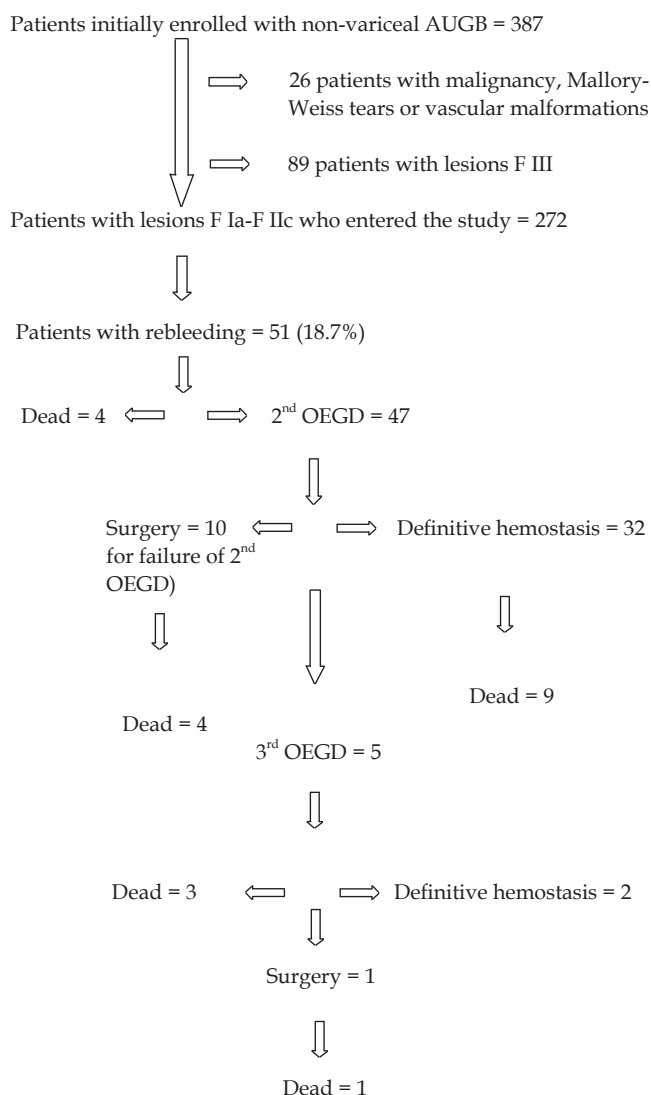
Patients initially enrolled with non-variceal AUGB = 387

⇒ 26 patients with malignancy, Mallory-Weiss tears or vascular malformations

⇒ 89 patients with lesions F III

Patients with lesions F Ia-F IIc who entered the study = 272

⇓

Patients with rebleeding = 51 (18.7%)

⇓

Dead = 4 ⇐   ⇒ 2nd OEGD = 47

⇓

Surgery = 10 ⇐   ⇒ Definitive hemostasis = 32
for failure of 2nd
OEGD)

⇓   ⇓   ⇓

Dead = 4   Dead = 9

3rd OEGD = 5

⇓

Dead = 3 ⇐   ⇒ Definitive hemostasis = 2

⇓

Surgery = 1

⇓

Dead = 1

Figure 1 Flow chart illustrates the study design and how the 51 patients who had at least one rebleeding episode were managed and their clinical outcomes.

Outcome of patients

Eighty-one percent of the emergency procedures were performed between 8 a.m. and 5 p.m. whereas only 19% were done out of working hours.

Forrest's classification of lesions at admission did not significantly differ between the two endoscopist categories, though no significant trend towards a higher mean Rockall's score was found in patients undergoing endoscopy out of working hours.

Univariate analysis showed that higher endoscopist's experience was associated with significant reduction in rebleeding rate (14% *vs* 37%), transfusion requirements (1.8 ± 0.6 *vs* 3.0 ± 1.7 units) as well as the need for surgery (4% *vs* 10%), but not associated with the length of hospital stay and mortality (Table 2). In contrast, outcomes did not significantly differ between the two time periods of endoscopy.

Table 3 shows the distribution of patients according to the experience of endoscopists and the time of

Table 2 Rebleeding rate according to age, sex, Rockall score, Forrest's classification, ulcer size, operator's experience, and time of endoscopy

	Total NO. of patients	Rebleeding rate		P	
Age (yr)					
<65	88	8	9.1% (A)	0.046	A *vs* B
65-74	75	15	20.0% (B)	0.370	B *vs* C
> 74	109	28	25.7% (C)	0.003	A *vs* C
Sex					
Males	186	36	19.4%	0.707	
Females	86	15	17.4%		
Rockall score					
1–4	92	6	6.5% (A)	0.016	A *vs* B
5–6	112	20	17.8% (B)	0.005	B *vs* C
>6	68	25	36.7% (C)	<0.001	A *vs* C
Forrest's classification					
I A– I B	65	9	13.8% (A)	0.001	A *vs* B
II A– II B	82	33	40.2% (B)	<0.001	B *vs* C
III C	125	9	7.2% (C)	0.138	A *vs* C
Ulcer size [a]					
< 20 mm	152	24	15.8%	0.079	
≥ 20 mm	70	18	25.7%		
Surgeon's experience					
High	214	29	13.6%	< 0.001	
Low	58	22	37.9%		
Time of endoscopy					
Working hours	221	41	18.5 %	0.862	
Out of working hours	51	10	19.6%		

[1] The sum does not add up to the total because of some missing values.

endoscopy. Univariate (Table 3) and multivariate (Table 4) analyses showed that low endoscopist's experience was associated with the higher rebleeding rate and transfusion requirements, but not significantly associated with the need for surgery, the length of hospital stay, and higher mortality rate. After adjustment for age, sex, and other selected covariates, the OR of low experienced endoscopist was 4.47 for rebleeding, 6.90 for need of transfusion after endoscopy, 2.36 for need of surgery, 0.47 for mortality, and 0.88 for length of hospital stay, respectively (Table 4). The estimates were statistically significant for rebleeding and need of blood transfusions. However, after further adjustment for appropriateness, the association with rebleeding disappeared (OR = 1.33), whereas it was still significant with the need of transfusion (OR = 5.07). The OR of making endoscopy in non-ordinary time period was 0.94 for rebleeding, 0.89 for need of transfusion after endoscopy, 1.85 for need of surgery, 1.96 for mortality, and 1.51 for length of hospital stay, respectively. None of the estimates were statistically significant even after further adjustment for appropriateness.

Table 5 shows the effect of an inappropriate endoscopic treatment on the occurrence of events of interest. A direct association was found between inappropriateness of treatment and risk of subsequent rebleeding (OR = 43.49),

Table 3 Distribution of patients by experience of endoscopists and time period of treatment, according to selected outcomes. Univariate analysis.

	Experience of endoscopist			Time period		
	High *n* (%)	Low *n* (%)	*P*	8AM-5PM *n* (%)	5PM-8AM *n* (%)	*P*
				n	*n*	
Rebleeding						
Yes	29 (58.9)	22 (43.1)	< 0.001	41 (80.4)	10 (19.6)	0.862
No	185 (83.7)	36 (16.3)		180 (81.5)	41 (18.6)	
N. of blood units transfused after endoscopy						
≥ 3	14 (48.3)	15 (51.7)	< 0.001	23 (79.3)	6 (20.7)	0.777
< 3	200 (82.3)	43 (17.7)		198 (81.5)	45 (18.5)	
Need for Surgery						
Yes	9 (60.0)	6 (40.0)	0.069	10 (66.7)	5 (33.3)	0.137
No	205 (79.8)	52 (20.2)		211 (82.1)	46 (17.9)	
Mortality						
Death	31 (86.1)	5 (13.9)	0.242	26 (72.2)	10 (27.8)	0.136
Alive	183 (77.5)	53 (22.5)		195 (82.6)	41 (17.4)	
Length of hospital staying						
≥ 13 d	107 (75.9)	34 (24.1)	0.244	120 (85.1)	21 (14.9)	0.091
< 13 d	107 (81.7)	24 (18.3)		101 (77.1)	30 (22.9)	
Rockall score						
< 6	111 (83.5)	22 (16.5)	0.06	108 (81.2)	25 (18.8)	0.985
≥ 6	103 (74.1)	36 (25.9)		113 (81.3)	26 (18.7)	
Forrest's classification						

Table 4 OR and corresponding 95%CI for patients treated by less *vs* well experienced endoscopists and for patients treated between 5 p.m. and 8 a.m. compared to those treated between 8 a.m. and 5 p.m. according to selected outcomes

	Low *vs* high experience of endoscopist		8 a.m.-5 p.m. *vs* 5 p.m.-8 a.m. time period	
	OR[1] (95%CI)	OR[2] (95%CI)	OR[3] (95%CI)	OR[4] (95%CI)
Rebleeding				
Yes	4.47	1.33	0.94	1.40
No	(2.11-9.47)	(0.46-3.83)	(0.38-2.30)	(0.51-3.83)
Number of blood units transfused after endoscopy				
≥3	6.90	5.07	0.89	0.92
<3	(2.80-16.98)	(1.89-13.61)	(0.31-2.56)	(0.32-2.70)
Need for surgery				
Yes	2.36	1.67	1.85	1.99
No	(0.74-7.50)	(0.43-6.41)	(0.54-6.37)	(0.56-7.04)
Mortality				
Dead	0.47	0.64	1.96	1.92
Alive	(0.17-1.34)	(0.20-2.11)	(0.80-4.78)	(0.79-4.69)
Length of hospital stay				
≥13 d	0.88	1.66	1.51	0.52
<13 d	(0.51-1.53)	(0.85-3.28)	(0.84-2.72)	(0.27-1.01)

[1]Estimates from multiple logistic regression after adjustment for age, sex, Rockall and Forrest score, blood transfusion, and time period. Reference category is highly experienced endoscopists. [2]As in 1 after further adjustment for appropriateness. Reference category is highly experienced endoscopists [3]Estimates from multiple logistic regression after adjustment for age, sex, Rockall and Forrest score, blood transfusion, and experience of endoscopists. Reference category is 5 p.m.-8 a.m. [4]As in 3 after further adjustment for appropriateness. Reference category is 5 p.m.-8 a.m.

whereas no significant association was found between inappropriateness and need of transfusion after endoscopy (OR = 2.98), need of surgery (OR = 2.62), mortality (OR = 0.47), and length of hospital stay (OR = 0.60). The latter findings showed that appropriateness of endoscopic therapy rather than the endoscopist experience was the main variable associated with rebleeding. Of course,

the lower the endoscopist's experience, the higher the inappropriateness in hemostatic maneuvers. Again, the two time periods of endoscopy were not associated with any of the variables evaluated.

When considering subjects with lesions 2A and 2B of the Forrest's classification, we found a stronger association between low endoscopist's experience and rebleeding, the

Table 5 OR and corresponding 95%CI for patients cured with inappropriate and appropriate treatment according to selected outcomes

	Treatment used		
	Appropriate	Inappropriate	
	n (%)	n (%)	OR[1] (95%CI)
Rebleeding			
Yes	27 (52.9)	24 (47.1)	43.49
No	216 (97.7)	5 (2.3)	(11.29-167.60)
Number of blood units transfused after endoscopy			
≥3	21 (72.4)	8 (27.6)	2.98
<3	222 (91.4)	21 (8.6)	(0.82-10.78)
Need for surgery			
Yes	11 (73.3)	4 (26.7)	2.62
No	232 (90.3)	25 (9.7)	(0.46-14.94)
Mortality			
Dead	33 (91.7)	3 (8.3)	0.47
Alive	210 (88.9)	26 (11.0)	(0.10-2.25)
Length of hospital stay			
≥13 d	125 (88.7)	16 (11.4)	0.60
<13 d	118 (90.1)	13 (9.9)	(0.24-1.54)

[1]Estimates from multiple logistic regression after adjustment for age, sex, Rockall and Forrest score, blood transfusion, time period, and experience of endoscopists. Reference category is appropriate endoscopic treatment.

multivariate OR being 9.82 (95%CI: 2.62-36.89).

Finally, there was a trend towards more adrenaline injected and a significant difference in the number of heater probe pulses used by endoscopists with high and low experience, respectively. The mean adrenaline injected was 9.4 and 8.6 mL, whereas the median number of heater probe pulses was 7 (range 0-15) vs 4 (range 0-10) for high- and low-experienced endoscopists, respectively.

DISCUSSION

Over the past two decades, advances have been made in the management of non-variceal AUGB[5]. Various endoscopic single-modality treatments including epinephrine injection, thermal therapy (heater probe, electrocoagulation, laser, etc.) and their combination are efficacious in achieving hemostasis in clinical trials[7]. These endoscopic procedures can control active bleeding in 85-90% of patients with a significant decrease in the rate of recurrent bleeding and need for surgery[6,7,25]. Therefore, the management decision for preventing recurrent bleeding is a rapid endoscopic diagnosis with adequate hemostasis. Recent data demonstrate that the best predictor of recurrent bleeding is the endoscopic finding of ulcer since the presence of a visible vessel or sentinel clot in the ulcer base indicates a high likelihood of rebleeding (43% and 22%, respectively), whereas ulcers with a clean base have a recurrent bleeding risk that is negligible[25]. The above rebleeding rates can be lowered by 60-80% by the application of aggressive endoscopic therapeutic modalities (such as a combined injection/thermal therapy[26]). However, one should keep in mind that these results are obtained in centers of

excellence, often known internationally for their expertise in the treatment of peptic ulcer bleeding. Whether the aforementioned results are reproducible in centers with less endoscopic expertise is currently a matter of debate[15].

The influence of endoscopy time on the outcome of patients with non-variceal AUGB remains a subject of debate as very few studies have addressed the question so far. Adler et al[17] showed that complications are significantly more frequent after emergency endoscopy between 7 p.m. and 11 p.m. as compared to regular working hours, which may be due to the fact that during non-working hours endoscopy is performed by more fatigued personnel. Choudari et al[16] and Ramage et al[18] found that there is no difference in the outcome of patients with non-variceal AUGB who had undergone endoscopy during working or non-working hours and weekdays vs weekends, respectively.

The present survey aimed at evaluating the impact of these two single factors (endoscopist's experience and time of endoscopy) on the outcome of patients with non-variceal AUGB presenting at a single large tertiary referral center. We did not include in our study inpatient referrals in order to deal with similar patients and to reduce as much as possible the severe comorbidities usually present in patients during their hospital stay. upper GI haemorrhage while hospitalized for another reason (so-called secondary bleeding) has been associated with an increased risk of rebleeding and death[26].

Findings from our study showed that the endoscopist's experience was an important independent prognostic factor for the outcome of non-variceal AUGB. Indeed, less experienced surgeon resulted in a significantly higher recurrent bleeding rate and transfusion requirement than highly experienced endoscopists, whereas mortality was not influenced probably due to comorbidities.

We found a direct significant association between low experience endoscopists and risk of rebleeding (multivariate OR = 4.47). After a further adjustment for appropriateness, the association disappeared (OR = 1.33), suggesting that if surgeons with lower experience have a rate of appropriateness of treatment similar to that of high experience endoscopists, we could not obtain any significant difference in terms of rebleeding rate between low and high experience endoscopists.

Moreover, the recurrent bleeding of F2A and F2B ulcers was significantly higher among the less experienced endoscopists as compared to the rate of highly skilled surgeons as well as the value reported in the literature[27]. The evaluation of appropriateness of endoscopic therapy by the two consultants who blindly examined the endoscopic reports might help understand the discrepancy. Planned guidelines of hemostasis were not followed entirely by less experienced staff in 36% of cases, particularly in patients with F2B ulcers, where the most frequent protocol violation was the non removal of an adherent clot. The fear of removing an apparently stable clot adherent to the ulcer base as well as the hypothetical difficulties in managing the subsequent hemorrhage under unfavorable circumstances (i.e, without any supervision) is the most frequently reported explanation by the youngest

endoscopists to justify guideline violations. Independently by the experience of endoscopists, fewer protocol violations have been done for patients with lesions classified as both F1A, F1B, and F2C, which might explain at least in part the lower rebleeding rate in these subjects (13.8% and 7.2%, respectively) as compared to those classified as F2A and F2B (40.2%).

Our findings also add some useful information to a still intricate problem that is the optimum treatment for ulcers with no hemorrhage at the time of endoscopy but present with the stigmata of hemorrhage. While there is no doubt that ulcers with actively bleeding vessels are treated immediately with injection and thermocoagulation by both highly experienced and less expert endoscopists, is the management strategy for intermediate findings (Forrest IIa & IIb) which remains less certain, especially when considering the great benefit of acid suppression in this group[28]. If aggressive endoscopic therapy (clot removal+epinephrine injection and thermal therapy) is superior to non endoscopic intervention in patients with adherent clots receiving oral PPI[29], profound in acid suppression without clot removal is more effective than endoscopic intervention alone[30]. A supplemental jog to this matter was provided by our current survey during which all bleeding patients received the same PPI scheme after endoscopy. We adjusted the paradigm to study the value of high dose in PPI alone over endoscopic therapy+ of PPI in F2A and 2B ulcers, showing that combination of appropriate endoscopic therapy with profound acid suppression is better than PPI alone (or PPI plus endoscopic under treatment)[31].

As far as the time of endoscopy is concerned, our findings suggest that emergency endoscopy out of working hours is as safe and effective as endoscopy performed during working hours provided that it is done under optimal conditions as during working hours. The success of procedures out of hours in this study may be related to the existence of a specific bleeding team composed of the endoscopist and two well-trained specialized nurses. It is conceivable that the same results cannot be obtained in other institutions with less endoscopic facilities and untrained assistants (i.e, non-specialized nurses), where examinations may perhaps be delayed by a few hours in order to perform endoscopy more safely during working hours.

In conclusion, endoscopist's experience is an important independent predicting variable for the outcome of non-variceal AUGB, whereas time of endoscopy (working *vs* non working hours) does not make any difference in terms of patients' prognosis. Urgent endoscopy for AUGB should be undertaken by experienced endoscopists because less experienced staff may not achieve hemostasis.

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• *RAPID COMMUNICATION*•

Prevalence of *Helicobacter pylori* infection and intestinal metaplasia in subjects who had undergone surgery for gastric adenocarcinoma in Northwest Italy

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Abstract

AIM: To investigate the seroprevalence of *Helicobacter pylori* (*H pylori*) infection and its more virulent strains as well as the correlation with the histologic features among patients who had undergone surgery for gastric cancer (GC).

METHODS: Samples from 317 (184 males, 133 females, mean age 69±3.4 years) consecutive patients who had undergone surgery for gastric non-cardia adenocarcinoma were included in the study. Five hundred and fifty-five (294 males, 261 females, mean age 57.3±4.1 years) patients consecutively admitted to the Emergency Care Unit served as control. Histological examination of tumor, lymph nodes and other tissues obtained at the time of surgery represented the diagnostic "gold standard". An enzyme immunosorbent assay was used to detect serum anti-*H pylori* (IgG) antibodies and Western blotting technique was utilized to search for anti-CagA protein (IgG).

RESULTS: Two hundred and sixty-one of three hundred and seventeen (82.3%) GC patients and 314/555 (56.5%) controls were seropositive for anti-*H pylori* ($P<0.0001$; OR, 3.58; 95%CI, 2.53-5.07). Out of the 317 cases, 267 (84.2%) were seropositive for anti-CagA antibody *vs* 100 out of 555 (18%) controls ($P<0.0001$; OR, 24.30; 95%CI, 16.5-35.9). There was no difference between the frequency of *H pylori* in intestinal type carcinoma (76.2%) and diffuse type cancer (78.8%). Intestinal metaplasia (IM) was more frequent but not significant in the intestinal type cancer (83.4% *vs* 75.2% in diffuse type and 72.5% in mixed type). Among the patients examined for IM, 39.8% had IM type I, 8.3% type II and 51.9% type III (type III *vs* others, $P = 0.4$).

CONCLUSION: This study confirms a high seroprevalence of *H pylori* infection in patients suffering from gastric adenocarcinoma and provides further evidence that searching for CagA status over *H pylori* infection might confer additional benefit in identifying populations at greater risk for this tumor.

INTRODUCTION

Gastric cancer (GC) is the world's second leading cause of cancer-related mortality[1] but in some countries it represents the most common malignancy in males[2].

GC occurrence in many Italian regions is similar to that in Japan. In Italy, GC is usually discovered at a later stage and therapeutic approaches cannot save a majority of patients. As a consequence, mortality parallels incidence[3].

The most frequent histologic type of GC is adenocarcinoma, which is thought to originate from a continuing and active proliferation of gastric pits following the destruction of glands due to active inflammatory infiltration. The process that has been described by Correa[4] from an inflammatory setting (gastritis) through intestinal metaplasia (IM) and dysplasia, evolves to adenocarcinoma.

In 1994, the International Agency for Research on Cancer defined H $pylori$ as a class I gastric carcinogen[5]. Evidence supporting a causal association has been demonstrated by epidemiological data[6], ecologic studies[1] and in experimental animal models[7]. Regarding the first aspect, in a prospective study including 1 526 Japanese subjects during a mean follow-up of 7.8 years (range 1.0-10.6 years), 2.9% of infected persons developed GC vs none among uninfected subjects[8]. A combined analysis of 12 case-control studies (with 1 228 GC cases considered) nested within prospective cohorts has found an association between non-cardia GC and H $pylori$ infection of 5.9 (95% confidence interval [CI] 3.4-10.3)[9]. A meta-analysis of 21 case-control studies suggested that the risk of GC is increased by threefold in those chronically infected with H $pylori$[10]. More recently, another meta-analysis of case-control studies with age- and sex-matched controls was published by Huang et al[11]. In this work, a comprehensive literature search identified 16 qualified studies with 2 284 cases and 2 770 controls. The authors found that H $pylori$ and CagA (cytotoxin-associated gene A) protein seropositivity significantly increases the risk for GC by 2.28- and 2.87-fold, respectively.

There is still no final conclusion regarding the association between the infection and the malignancy due to marked geographic variations. Some studies have not found any correlation between seropositivity for H $pylori$ antibodies (as an indicator of H $pylori$ infection) and GC[12-14]. For example, in the study performed by Rudi et al[12] in Germany, 58.6% of patients suffering from GC and 50.6% of control subjects have IgG antibodies against H $pylori$. In Gambia, though more virulent strains of H $pylori$ are present, gastric atrophy and IM are rare[15]. Seropositivity for H $pylori$ and the CagA antigen cannot explain the differences in the prevalence of precancerous gastric lesions in two Chinese populations with contrasting GC rates[16]. Recently, Wong et al[17] found that the incidence in GC development is similar between the subjects receiving H $pylori$ eradication treatment and those receiving placebo during a period of 7.5 years in a high-risk region of China. Furthermore, not all the stomach tumors are H $pylori$ positive.

In previous local pilot studies in North Italy, a high prevalence of H $pylori$ infection has been associated to the presence of GC[18,19]. To investigate the correlation in a vast area of Northwest Italy in more detail, we started a research network on gastric cancer and precursor lesions in 1993, which we named Metaplasia H $pylori$ Histology (MHEPHISTO). In this multicenter survey, a prospective case-control study of patients who had undergone surgery for GC in Northwestern Italy was performed. The aim was to ascertain the seroprevalence of H $pylori$ infection and its more virulent strains by searching for antibodies against the CagA protein and to establish the correlation with the subtypes of IM.

MATERIALS AND METHODS

Study population

Specimens from 317 (184 males, 133 females, mean age 69 ± 3.4 years) consecutive patients who had undergone surgery for gastric non-cardia adenocarcinoma were included in the study. Five hundred and fifty-five patients (294 males, 261 females) consecutively admitted to the Emergency Care Unit of S. Giovanni Battista (Molinette) Hospital of Torino served as control with a mean age 57.3 ±4.1 years. Cases and controls came from the geographical area of Northwestern Italy.

Methods

Clinical diagnosis of malignancy was established by standard medical examinations including upper GI endoscopy, diagnostic ultrasound and computed tomography (CT) scan. Endoscopic ultrasound (EUS) served as a part of the routine examination.

Histological examination of tumor, lymph nodes and other tissues obtained at the time of surgery represented the diagnostic "gold standard". Pathologists with special interest and experience in GI pathology reviewed the histological sections. Appropriate forms were used to record the pathological findings. All the diagnostic criteria used for our survey were discussed and sample slides were reviewed by the pathologists before the study to minimize interobserver variations as far as possible.

Surgical specimens were immersed in paraffin for routine pathological examination. Microtome sections (7-8 μm thick) were stained with hematoxylin and eosin as well as high iron diamine/alcian blue to identify sialo-and sulfomucins. Adenocarcinoma was diagnosed when the malignant cells invaded the lamina propria in single cells, glandular or solid nest arrangements, usually accompanied with fibrosis of the surrounding tissue. Carcinomas were classified histologically as either intestinal or diffuse type in accordance with Lauren's classification[20]. Intestinal metaplasia classified according to Filipe et al[21] was defined as metaplastic transformation of gastric glandular and surface epithelium towards intestinal mucosal elements including goblet, absorptive, and Paneth cells.

Personnel not aware of the histological diagnoses performed serological testing. A commercial enzyme

Table 1 Seroprevalence of anti-*H pylori* antibodies among patients with gastric cancer and controls

Parameters	Gastric cancer (%)	Controls (%)
H pylori positive	261 (82.3)	314 (56.5)
H pylori negative	56	241
Anti-CagA positive	267 (84.2)	100 (18)
Anti-CagA negative	50	455

Table 2 Features of patients suffering from gastric cancer based on tumor subtype

Parameters	Intestinal type carcinoma (%)	Diffuse type carcinoma (%)
H pylori positive	138/181 (76.2%)	67/85 (78.8%)
Intestinal metaplasia	151/181 (83.4%)	64/85 (75.2%)

immunosorbent assay (ELISA, Helori-test® Eurospital, Trieste, Italy) was used to detect serum anti-*H pylori* (IgG) antibodies. The assay sensitivity and specificity were 94% and 87%[22]. Briefly, calibrators, positive and negative controls and diluted (1:200) serum samples were added to the wells coated with purified *H pylori* group-specific antigens. Plates were incubated for 60 min at 37 ℃, and then the liquid was removed completely and washed thrice with 200 μL/well of washing solution. One hundred microliters of anti-IgG conjugate was pipetted into each well. The wells were incubated for 60 min at 37 ℃, washed thrice with 200 μL/well of washing solution and 100 μL of chromogenic substrate was added to each well. The wells were again incubated for 30 min at 37 ℃, the reaction was stopped by adding 25 μL of the stopping solution. Reading was performed at 405 nm and the mean optical density was expressed as a percentage of the optical density of positive control serum assayed on the same plate. To detect the presence of serum IgG against *H pylori* CagA protein, Western blotting technique was used. *H pylori* CCUG 17874 (type strain, CagA positive, CagA mass = 128 ku) was cultured in *Brucella* broth containing 0.2% cyclodextrin at 37 ℃ in a microaerobic environment for 48 h. At the end of incubation, to exclude the presence of contaminants, the broth culture was subcultured onto plain blood agar plates and examined under optical microscope after staining with carbol fuchsin. The broth culture was centrifuged and the pellet was washed twice with phosphate buffered saline (PBS, pH 7.4) at 4 ℃. A whole cell suspension containing approximately 10^{10} bacteria was lysed and denatured in Laemmli buffer at 95 ℃ for 5 min, then run electrophoretically on 10% polyacrylamide gel with sodium dodecyl sulfate. Proteins were transferred onto nitrocellulose sheets saturated with 3% defatted milk in PBS with 0.1% Triton X (PBSMT). Strips were cut and serum samples were assayed at the dilution of 1:100 in PBSMT. After overnight incubation with constant agitation at room temperature, strips were washed with PBSMT and then incubated with an anti-human IgG serum conjugated with peroxidase at room temperature for 90 min. After the washings, the reaction was visualized by adding the substrate (H_2O_2 in a solution of 4-chloro-1-naphthol in Tris buffer 0.05 mol/L, pH 6.8). We used serum samples from patients infected respectively with CagA positive and negative *H pylori* strains as positive and negative controls with or without antibodies to CagA. Specific polyclonal antisera against CagA (kindly donated by Biocine-Chiron, Siena) was used as further controls.

The seroprevalence of anti-*H pylori* as well as the distribution of anti-CagA seropositivity in cases and controls were compared using the χ^2 test. Odds ratio (OR) and 95%CI assessing the risk of GC associated with *H pylori* infection were calculated using the Mantel-Haenszel method. $P<0.05$ was considered statistically significant.

RESULTS

All patients selected were suffering from gastric non-cardia adenocarcinoma (intestinal type in 181, diffuse type in 85 and mixed type in 51). Among these, 261 out of 317 (82.3%) were seropositive for IgG anti-*H pylori* compared to 314 out of 555 (56.5%) controls ($P<0.0001$; OR, 3.58; 95%CI, 2.53-5.07) (Table 1). Moreover, out of the 317 cases, 267 (84.2%) were seropositive for anti-CagA antibody *vs* 100 out of 555 (18%) controls ($P<0.0001$; OR, 24.30; 95%CI, 16.5-35.9) (Table 1).

There was no difference between the frequency of *H pylori* in intestinal type and diffuse type carcinoma. Overall, *H pylori* occurred in 138 out of 181 patients (76.2%) suffering from the former compared to 67 out of 85 (78.8%) suffering from the latter (P = NS) (Table 2). Intestinal metaplasia was more frequently seen in the intestinal type cancer (151/181, 83.4% *vs* 64/85, 75.2% in diffuse type and 37/51, 72.5% in mixed type) but the difference was not statistically significant (P = NS, Table 2). Among the patients examined for IM, 72 out of 181 (39.8%) had IM type I, 15 out of 181 (8.3%) type II, and 94 out of 181 (51.9%) type III, (type III *vs* others P = 0.4). Furthermore, among the patients with IM of either body or antral mucosa, 117 out of 151 (77.4%) with intestinal type carcinoma were positive for *H pylori* compared to 59 out of 85 (69.4%) with diffuse type carcinoma (P = 0.17).

DISCUSSION

Gastric carcinogenesis involves a slow but continuous stepwise evolution from superficial gastritis and glandular atrophy to metaplasia and dysplasia and finally to adenocarcinoma[23]. The process of carcinogenesis which may well extend over decades provides an excellent opportunity for early detection and intervention to prevent further progression of the sequence of events preceding the development of the neoplasma. This is especially true because *H pylori* (which can be readily treated) is known to be the main factor though not the only[24] etiological agent

and initiating carcinogen.

On the other hand, the prognosis of GC is poor. In most industrialized countries, only around 10% subjects survive for 5 years. The sole exception is Japan where this malignancy is often identified at an early stage and in younger and fitter patients[26]. In Italy, 10-year survival is 12.1% in all GC patients and 20.8% in resected cases. However, though the survival is good when the diagnosis is performed at an early stage, only a few cases are diagnosed at stages when cure by radical surgery is more probable[3].

Regarding the biological plausibility for a causal role, a higher intragastric pH after the development of atrophic gastritis provoked by *H pylori* may favor the production of carcinogens[26]. The generation of reactive oxygen species and increased level of inducible nitric oxide synthase may in turn cause genetic alterations leading to cancer[27]. Nardone *et al*[28] demonstrated by morphometric and immunohistochemical techniques that *H pylori* infection seems to be responsible for genomic instability in patients with chronic atrophic gastritis and eradication of *H pylori* can reverse inflammation, atrophy, metaplasia, and genomic instability.

CagA gene is situated at the end of the large region of the genome identified to be a pathogenicity island (PAI). The strains of *H pylori* expressing CagA protein are considered more virulent, being linked with an increased risk of duodenal ulcer and GC[23]. Moreover, CagA status is associated with a higher prevalence of p53 mutation in gastric adenocarcinoma[29].

This multicenter study showed a significant association between *H pylori* infection in particular by its more virulent strains and the presence of GC. In addition, the results confirmed that type III IM was most frequently associated with *H pylori* infection. These data suggest that CagA status is a helpful parameter in defining a subgroup of *H pylori*-infected patients at increased risk of developing gastric adenocarcinoma. The difference between the rate of *H pylori* infection and more virulent strains can be explained by the fact that CagA antibodies persist for a longer time than *H pylori* IgG surface antibodies. Hence, relying on *H pylori* IgG antibodies alone might misclassify a significant proportion of patients who once had the infection[11].

The established epidemiological association does not prove that there is a direct causal relationship. Therefore, to further confirm a causal role, we are going to evaluate the effect of *H pylori* on the morphological changes of gastric mucosa in patients with precancerous gastric lesions.

In conclusion, seroprevalence of *H pylori* infection is high in patients suffering from gastric adenocarcinoma, which provides further evidence that searching for CagA status over *H pylori* infection might confer additional benefit for identifying populations at greater risk for this tumor.

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• *RAPID COMMUNICATION*•

Upregulation of 25-hydroxyvitamin D₃-1α-hydroxylase by butyrate in Caco-2 cells

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Abstract

AIM: To investigate the possible involvement of 25-hydroxyvitamin D₃-1α-hydroxylase [1α-25(OH)₂D₃] in butyrate-induced differentiation in human intestinal cell line Caco-2 cells.

METHODS: Caco-2 cells were incubated either with 3 mmol/L butyrate and 1 μmol/L 25(OH)₂D₃ or with 1 μmol/L 1α-25(OH)₂D₃ for various time intervals ranging from 0 to 72 h. Additionally, cells were co-incubated with butyrate and either 25(OH)₂D₃ or 1α-25(OH)₂D₃. 1α-25(OH)₂D₃ mRNA was determined semi-quantitatively using the fluorescent dye PicoGreen. Immunoblotting was used for the detection of 1α-25(OH)₂D₃ protein. Finally, enzymatic activity was measured by ELISA.

RESULTS: Both butyrate and 1α-25(OH)₂D₃ stimulated differentiation of Caco-2 cells after a 48 h incubation period, while 25(OH)₂D₃ had no impact on cell differentiation. Synergistic effects on differentiation were observed when cells were co-incubated with butyrate and vitamin D metabolite. Butyrate transiently upregulated 1α-25(OH)₂D₃ mRNA followed by a timely delayed protein upregulation. Coincidently, enzymatic activity was enhanced significantly. The induction of the enzyme allowed for comparable differentiating effects of both vitamin D metabolites.

CONCLUSION: Our experimental data provide a further mechanism for the involvement of the vitamin D signaling pathway in colonic epithelial cell differentiation by butyrate. The enhancement of 1α-25(OH)₂D₃ followed by antiproliferative effects of the vitamin D prohormone in the Caco-2 cell line suggest that 25(OH)₂D₃ in combination with butyrate may offer a new therapeutic approach for the treatment of colon cancer.

INTRODUCTION

In addition to its well-known role in mineral and skeletal homeostasis, it is widely recognized that the physiologically most active molecular form of vitamin D₃, 1α-25(OH)₂D₃, also re-strains cell proliferation, induces differentiation, and apoptosis in a large variety of normal and tumor cells, including cells of the large intestine[1-3]. Most of the long-term pleiotropic actions of 1α-25(OH)₂D₃ are mediated by binding to a high-affinity vitamin D receptor (VDR)[3-5], which is a member of the nuclear hormone-receptor superfamily. The growth-restraining anticancer effects of vitamin D on colon cells are conveyed through genomic and post-genomic pathways involving a crosstalk with mediator synthesis and signaling, the mediation of a cell-cycle arrest, and finally the induction of apoptosis[6].

1α-25(OH)₂D₃, the active form of vitamin D, is enzymatically formed from its precursor 25(OH)₂D₃. The enzyme was thought to exist only in the kidney, until recently it has been identified in other non-renal tissues including the large intestine[7,8]. Further studies demonstrated that in colorectal cancer expression of 1α-25(OH)₂D₃ and VDR increases in parallel with ongoing proliferation in the early phase of tumorigenesis, whereas in poorly differentiated late-stage carcinomas only low levels of both genes can be detected[9-11]. It is postulated that through this upregulation, colorectal cancer cells become able to increase their potential for an autocrine counter-regulatory response to neoplastic cell growth in the early stages of malignancy. Thus, 1α-25(OH)₂D₃ theoretically represents a suitable target for the prevention of colorectal carcinogenesis.

Short-chain fatty acids, in particular butyrate, have been shown to inhibit growth in colon cancer cell lines. We

have previously shown that butyrate and 1α-25(OH)₂D₃ act synergistically in inducing differentiation of Caco-2 cells[12,13]. Furthermore, at least some of the anti-proliferative and pro-differentiating effects of butyrate are mediated via an upregulation of VDR with subsequent p21^Waf1/Cip1 expression[14]. These data prompted us to investigate a possible effect of butyrate on the expression of 1α-25(OH)₂D₃ in Caco-2 cells.

MATERIALS AND METHODS

Chemicals and supplies

Disposable cell culture was purchased from Nalge Nunc International (Wiesbaden, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), sodium pyruvate, nonessential amino acids, and PBS were obtained from GIBCO BRL (Eggenstein, Germany). Penicillin and streptomycin were obtained from Biochrom (Berlin, Germany). Butyric acid (sodium salt) was purchased from Merck-Schuchardt (Munich, Germany).

25(OH)₂D₃ and 1α-25(OH)₂D₃ were dissolved in ethanol (final maximal concentration of ethanol in medium was 1 mL/L to yield a 10 mmol/L stock solution, which was stored at -20 ℃). Butyric acid was dissolved in PBS (final maximal concentration of PBS in medium was 1 mL/L). Control experiments with either 0.1% ethanol or 0.1% PBS were performed to exclude effects of the solvents on the results of our investigations.

Cell culture

Caco-2 cells were obtained from the German Cancer Research Center (Heidelberg, Germany). The stock was maintained in 175-cm^2 flasks in a humidified incubator at 37 ℃ in an atmosphere of 95% air and 50 mL/L CO_2. The medium consisted of DMEM supplemented with 10% FCS, 1% penicillin/streptomycin, 1% sodium pyruvate, and 1% nonessential amino acids. The cells were passaged weekly using Dulbecco's PBS containing 0.25% trypsin and 1% EDTA. The medium was changed thrice per week. Passages 30-38 were used in all the experiments. Cells were screened routinely for possible contamination with mycoplasma by PCR at monthly intervals using the VenorGem mycoplasma detection kit (Minerva Biolabs, Berlin, Germany). For the experiments, the cells were seeded onto plastic cell culture wells in serum-containing medium and allowed to attach for 24 h. Before the treatment, the cells were synchronized in a medium containing 1% FCS. Cells were treated with 3 mmol/L butyrate, 1 μmol/L 25(OH)D₃, or 1 μmol/L 1α-25(OH)₂D₃ for various time intervals ranging from 0 to 72 h. Additionally, cells were co-incubated with butyrate and either 25(OH)₂D₃ or 1α-25(OH)₂D₃ or solvent.

Cytotoxicity

Cytotoxicity was assayed by measuring lactate dehydrogenase activity in a cell culture medium using a commercially available kit (LDH kit, Merck, Darmstadt, Germany).

Cell differentiation

Alkaline phosphatase activity was used to assess the differentiation of Caco-2 cells. Enzymatic activity was determined as previously described[12] using the Ecoline alkaline phosphatase assay kit (Merck, Darmstadt, Germany). Cellular protein was determined by Coomassie blue assay using a commercial kit (Bio-Rad Laboratories GmbH, Munich, Germany).

Analysis of 1α-25(OH)₂D₃ mRNA by semi-quantitative RT-PCR

RT-PCR was conducted with the Gene Amp/Perkin Elmer RNA PCR kit according to the manufacturer's protocol, starting from 2 μg total RNA. Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as control. From the results of preliminary experiments, 20 PCR cycles for GAPDH and 30 cycles for 1α-25(OH)₂D₃ were selected as optimal amplification conditions to produce a log-linear relationship between the amount of each mRNA and intensity of PCR product. The PCR reaction contained 0.2 mmol/L dNTP and 2.5 U AmpliTaqGold DNA polymerase (Applied Biosystems, NJ, USA) and either 0.1 μmol/L primers for 1α-25(OH)₂D₃ or 0.2 μmol/L primers for GAPDH. The conditions of the reaction for 1α-25(OH)₂D₃ were: initial denaturation at 94 ℃ for 15 s followed by annealing at 59 ℃ for 30 s, extension at 72 ℃ for 60 s and a final extension at 72 ℃ for 7 min after the last cycle. The respective annealing temperature for GAPDH was 45 ℃. Primers for amplification were as follows: 1α-25(OH)₂D₃ sense 5' -CAGAGGCAGCCATGAGGAAC-3', 1α-25(OH)₂D₃ antisense 5'-GGGTCCCTTGAAGTGGCATAG-3; GAPDH sense 5'-GCACCGTCAAGGCTGAGAAC-3', GAPDH antisense 5'-CCACCACCCTGTTGCTGTAG-3'. The expected sizes of 1α-25(OH)₂D₃ and GAPDH were 440 and 803 base pairs (bp), respectively. Aliquots of the PCR mixtures (10 μL) were analyzed by electrophoresis using a 1% agarose gel containing 0.5 μg/mL ethidium bromide.

For semi-quantitative analysis of amplified PCR products, the fluorescent dye PicoGreen (Molecular Probes, Goettingen, Germany) was used according to the manufacturer's instructions[15,16]. Two microliters of amplified dsDNA in 100 μL TE buffer was mixed with an equal volume of diluted PicoGreen reagent (5 mL/L in TE buffer). Samples were incubated for 5 min at room temperature and protected from light in a microtiter plate. The fluorescence was measured (λ_{ex} = 485 nm; λ_{em} = 538 nm) in a Spectra Fluor Plus fluorometer from Tecan (Crailsheim, Germany). The standard curve for the quantitative analysis was obtained with λ DNA standard in TE buffer, being linear from 1 to 128 ng/well.

Immunoblot analysis of 1α-25(OH)₂D₃

For the analysis of 1α-25(OH)₂D₃ protein expression levels, an equal number of cells was directly lysed in cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA) and aliquots of 5 μg protein in the loading buffer were separated by SDS/PAGE through a 12.5% Tris-HCl-precasted linear gradient gel (Bio-Rad GmbH, Munich,
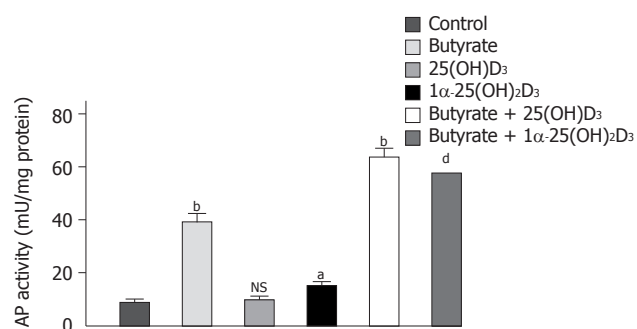
Figure 1 Effects of 1α-25(OH)$_2$D$_3$, 25(OH)$_2$D$_3$ and butyrate on differentiation of Caco-2 cells as assessed by AP activity. Cells were treated for 48 h with a medium supplemented with 10^{-6} mol/L 1α-25(OH)$_2$D$_3$, 10^{-6} mol/L 25(OH)$_2$D$_3$, 3 mmol/L butyrate, or with one of the combinations of butyrate (3 mmol/L) and 1α-25(OH)$_2$D$_3$ (10^{-6} mol/L) or butyrate (3 mmol/L) and 25(OH)$_2$D$_3$, (10^{-6} mol/L). Values are expressed as milliunits of AP activity per milligram cellular protein. [a]$P<0.05$; [b]$P<0.01$; [d]$P<0.001$; NS: non significant.

Germany) and electroblotted onto protran nitrocellulose transfer membranes (Schleicher & Schuell Bioscience, Dassel, Germany). The transfer efficiency was visualized using prestained molecular weight protein standards (Bio-Rad GmbH, Munich, Germany). Membranes were then soaked for 1 h at 25 ℃ in Tris-buffered saline (100 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl) containing 1 mL/L Tween 20 (0.1% TTBS) and 50 g/L dried nonfat milk. The membranes were subsequently washed (2×5 min) in 0.1% TTBS followed by an incubation for 90 min at 25 °C with a commercial sheep anti-murine 1α-25(OH)$_2$D$_3$ antibody (Binding Site Limited, Birmingham, UK) at a dilution of 1:500. After washing, the blot was incubated for 1 h at 25 ℃ with a horseradish peroxidase-linked donkey anti-rabbit antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at a dilution of 1:1 000 in 0.1% TTBS and 20 g/L dried nonfat milk. The washing steps were repeated and subsequently enhanced chemiluminescence detection was performed according to the manufacturer's instructions (ECL Plus, Amersham Biosciences, UK). SDS-PAGE immunoblots were quantitated using a luminescent image scanner Desaga CabUVIS and ProViDoc software (Desaga, Wiesloch, Germany).

1α-25(OH)$_2$D$_3$ activity

Cells were seeded (10^6 cells/well/mL) in RPMI medium in a six-well plate. After incubation for 24 h with 5×10^{-8} mol/L 25(OH)$_2$D$_3$, the supernatant and cells were harvested. 1α-25(OH)$_2$D$_3$ was separated from other vitamin D metabolites by extraction columns (J.T. Baker, Phillipsburg, NJ, USA) and determined with a commercially available enzyme-linked immunosorbent assay (Immundiagnostik, Bensheim, Germany), being specific for 1α-25(OH)$_2$D$_3$ and unable to recognize other vitamin D metabolites.

Statistical analysis

All data were expressed as mean±SD. One-way ANOVA was used to compare means. $P<0.05$ was considered statistically significant.

Figure 2 Time-dependent effect of butyrate on 1α-25(OH)$_2$D$_3$ expression. **A**: Caco-2 cells were grown for 36 h in serum-free medium in the absence (lane 1) or presence of butyrate (3 mmol/L) (lanes 2-4). 1α-25(OH)$_2$D$_3$ mRNA was analyzed by semiquantitative RT-PCR with the fluorescent dye PicoGreen. **B**: Caco-2 cells were treated for 72 h in the absence (lane 1) or presence of butyrate (3 mmol/L) (lanes 2-4) and then harvested for immunoblot analysis. The band at approximately 55 ku corresponds to 1α-25(OH)$_2$D$_3$ protein. Quantitation of protein was performed using a luminescent image scanner. [a]$P<0,05$ vs control; NS: non significant.

RESULTS

Influence of active vitamin D forms on butyrate-induced cell differentiation

After any cytotoxicity of either substrate was ruled out (data not shown), the influence of the two active vitamin D forms, 25(OH)$_2$D$_3$ and 1α-25(OH)$_2$D$_3$, on butyrate-induced
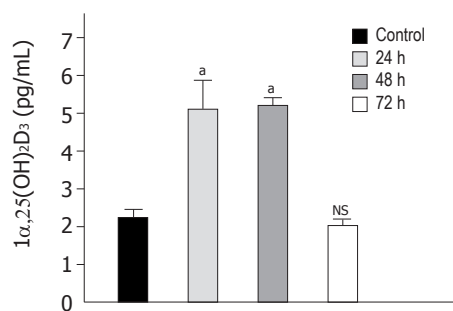
Figure 3 Time-dependent effect of butyrate on 1α-25(OH)$_2$D$_3$ activity. Caco-2 cells were treated for 72 h in the absence (lane 1) or presence of butyrate (3 mmol/L) (lanes 2-4) and then harvested for ELISA. Values are expressed as pg/mL of 1α-25(OH)2D3. $^aP<0,05$ *vs* control; NS, non significant.

cell differentiation was determined. Figure 1 displays the individual and combined effect of butyrate, 25(OH)$_2$D$_3$ and 1α-25(OH)$_2$D$_3$ on AP activity. As compared to control, butyrate and 1α-25(OH)$_2$D$_3$ significantly stimulated differentiation of Caco-2 cells after a 48-h incubation period, the effect of butyrate being much more prominent (436±27% *vs* 70±7%, respectively). In contrast, 25(OH)$_2$D$_3$ had no impact on cell differentiation. Synergistic effects on differentiation were observed when Caco-2 cells were co-incubated with butyrate and either vitamin D metabolite. In this case, AP activity increased to 633± 7% (butyrate+25(OH)$_2$D$_3$) and 702±107% (butyrate+1α-25(OH)$_2$D$_3$) as compared to control.

Effect of butyrate on 1α-25(OH)$_2$D$_3$ expression
The above mentioned data suggested a possible impact of 1α-25(OH)$_2$D$_3$ mRNA on the synergistic potential of butyrate and vitamin D forms in inducing colonic epithelial cell differentiation. We therefore investigated 1α-25(OH)$_2$D$_3$ mRNA expression in Caco-2 cells treated with butyrate. As shown in Figure 2A, butyrate led to a transient increase of 1α-25(OH)$_2$D$_3$ mRNA by 67.3± 27% as compared to control after 24 h, which returned to basal values after 36 h. Induction of gene expression was increased after 24 h and gradually decreased thereafter (Figure 2B).

Effect of butyrate on 1α-25(OH)2D3 enzymatic activity
We finally assessed whether induction of 1α-25(OH)$_2$D$_3$ mRNA and protein was accompanied with an increased enzymatic capacity of Caco-2 cells. Enzymatic conversion into the active metabolite 1α-25(OH)$_2$D$_3$ was enhanced by a factor of 2.3±0.6 after 24 and 48 h of treatment with 3 mmol/L butyrate as compared to control (Figure 3).

DISCUSSION
The present report provides further experimental evidence that butyrate-induced colonic epithelial cell differentiation at least in part is mediated by the vitamin D signaling pathway. Our data demonstrate that butyrate treatment of the Caco-2 cell line results in an upregulation of gene

and protein expression of 1α-25(OH)$_2$D$_3$, followed by an increased enzymatic activity with conversion of 25(OH)$_2$D$_3$ to 1α-25(OH)$_2$D$_3$. The induction of this enzyme then allowed for the matchable differentiating effects of 25(OH)$_2$D$_3$ in comparison to 1α-25(OH)$_2$D$_3$, thereby presenting a possible new chemopreventive mechanism in colon cancer.

We and others have previously shown that some of the antineoplastic effects of the short-chain fatty acid butyrate in colonic epithelial cells are operated by synergistic actions with 1α-25(OH)$_2$D$_3$. It has been demonstrated in various colonic cancer cell lines that butyrate and 1α-25(OH)$_2$D$_3$ act synergistically in reducing proliferation and enhancing differentiation[13,14,17,18]. One molecular mechanism participating in this interplay represents the upregulation of VDR expression, followed by a stimulation of the negative cell cycle regulator p21[Waf1/Cip1] and a downregulation of cyclins and cdks[12]. The alteration of the expression of a variety of genes as well as by inhibiting histone acetylase[19,20] and phosphorylation by enhancing nonhistone protein phosphorylation[21] and causing hypo- or hypermethylation of DNA[22,23] is the other known molecular mechanism by which butyrate mediates its antitumor effects. Similar additive antiproliferative effects of vitamin D with other histone deacetylation inhibitors such as TSA have also been described in a non-malignant epithelial breast cell line[24].

Evidence from a variety of experimental studies, epidemiological findings and a few human clinical trials indicate that vitamin D can modulate and inhibit carcinogenesis[6]. Vitamin D activity in epithelial cells involves the conversion of 25(OH)$_2$D$_3$ to 1α-25(OH)$_2$D$_3$ during the interaction of 1α-25(OH)$_2$D$_3$ with VDR. Subsequently, activation of VDR leads to repression or activation of specific proto-oncogenes or tumor-suppressor genes that are related to proliferation and differentiation in a large variety of normal and tumor tissues including the small and large intestine[1–5,25]. In addition, post-genomic pathways also seem to be involved in growth regulation. VDR seems to undergo specific alterations in expression during the development and progression of colon cancer. Compared to normal colon mucosa, VDR density is increased in hyperplastic polyps and in early stages of tumorigenesis, but declines in late-stage neoplasia[9-11]. As a further support to this stage-dependent expression, Belleli *et al*[26] found that the number of 1α-25(OH)$_2$D$_3$-binding sites is significantly decreased in the colon of carcinogen-treated rats.

1α-25(OH)$_2$D$_3$ is believed to exist solely in the kidney and it has also been identified in other non-renal tissues including the large intestine[7,8].

Due to its higher affinity to VDR, 1α-25(OH)$_2$D$_3$, the active form of vitamin D, is about 500-1 000-fold more active than its precursor 25(OH)$_2$D$_3$. Though there exists a large body of evidence, that 1α-25(OH)$_2$D$_3$ exerts pleiotropic antitumor effects against several malignancies *in vitro*, its clinical use as a chemopreventive agent is limited by hypercalcemia. For example, the concentrations of

1α-$25(OH)_2D_3$ required to inhibit colon cancer in tissue culture are in the range of 10^{-6}-10^{-9} mol/L[27-29]. Therefore, several groups have searched for other vitamin D-related anti-neoplastic therapeutic strategies. Recently, Schwartz et al[30] have demonstrated not only the expression of 1α-$25(OH)_2D_3$ and VDR in normal and malignant pancreatic tissue but also antiproliferative effects of the prohormone $25(OH)D_3$[30]. Furthermore, prostatic 1α-$25(OH)_2D_3$ has been found to be largely unregulated by serum levels of parathyroid hormone and calcium[31]. Due to the large therapeutic window of $25(OH)_2D_3$ compared to 1α-$25(OH)_2D_3$, the authors concluded that $25(OH)_2D_3$ could play a role in the prevention of both pancreatic and prostate cancers. In our experimental setting, $25(OH)_2D_3$ was not able to exert antiproliferative effects in Caco-2 cells as a result of the weak expression of 1α-$25(OH)_2D_3$. However, the induction of this enzyme by butyrate provoked the same impact on the differentiation of this colon cancer cell line as 1α-$25(OH)_2D_3$. Whether these activities are specific for Caco-2 cells or represent tissue-specific peculiarities of colonic epithelium has to be determined.

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ELSEVIER

• *RAPID COMMUNICATION*•

Using the polymerase chain reaction coupled with denaturing gradient gel electrophoresis to investigate the association between bacterial translocation and systemic inflammatory response syndrome in predicted acute severe pancreatitis

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Abstract

AIM: To investigate the use of PCR and DGGE to investigate the association between bacterial translocation and systemic inflammatory response syndrome in predicted severe AP.

METHODS: Patients with biochemical and clinical evidence of acute pancreatitis and an APACHE II score ≥ 8 were enrolled. PCR and DGGE were employed to detect bacterial translocation in blood samples collected on d 1, 3, and 8 after the admission. Standard microbial blood cultures were taken when there was clinical evidence of sepsis or when felt to be clinically indicated by the supervising team.

RESULTS: Six patients were included. Of all the patients investigated, only one developed septic complications; the others had uneventful illness. Bacteria were detected using PCR in 4 of the 17 collected blood samples. The patient with sepsis was PCR-positive in two samples (taken on d 1 and 3), despite three negative blood cultures. Using DGGE and specific primers, the bacteria in all blood specimens which tested positive for the presence of bacterial DNA were identified as *E coli.*

CONCLUSION: Our study confirmed that unlike traditional microbiological techniques, PCR can detect the presence of bacteria in the blood of patients with severe AP. Therefore, this latter method in conjunction with DGGE is potentially an extremely useful tool in predicting septic morbidity and evaluating patients with the disease. Further research using increased numbers

of patients, in particular those patients with necrosis and sepsis, is required to assess the reliability of PCR and DGGE in the rapid diagnosis of infection in AP.

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INTRODUCTION

Infection and septic complications are the major factors contributing to the poor outcome in acute severe pancreatitis. They cause up to 80% of deaths and occur in 5-10% of patients[1-3]. It is thought that in the majority of cases infection is caused by bacterial translocation from the gut lumen[4-7], a hypothesis which animal experiments have generally supported[8-10]. Unfortunately attempts to confirm the link between bacterial translocation and morbidity and mortality in acute pancreatitis in human beings have been largely unsuccessful[11,12].

Conventional microbiological blood culture methods are currently used widely[13-15], but may fail to yield positive results, if the causative organism is fastidious in nature, cell dependent or has a fungal etiology. It is thought that 60-70% of the bacteria in the human intestinal tract cannot be cultured[16,17]. Molecular-based diagnostic approaches are therefore being increasingly employed, especially when a quick diagnosis is required[18-21].

The use of the polymerase chain reaction (PCR) to identify microbial DNA in clinical specimens has been described by many investigators[22-25]. PCR using 16S rRNA-specific primers has identified bacterial DNA in blood after the surgery[26]. 16S rRNA is a highly conserved region of bacterial DNA, found in all Gram-positive and Gram-negative bacteria[27]; if these primers are used, the majority of pathogenic bacteria can theoretically

Table 1 Oligonucleotide primers used for amplification of total DNA extracted from human blood samples and total chromosomal DNA recovered from selected bacterial strains

Primer designate	Sequences of (+) and (-) primers (nucleotide)	Gene target	Size of amplicon (bp)
BG-1 (+ strand)	5′CTT TGC CTG GTT TCC GGC ACC AGA A- 3′ (201-225)	b-Galactosidase gene of *E coli*	762
BG-4 (- strand)	5′AAC CAC CGC ACG ATA GAG ATT CGG G- 3′ (983-939)		
BD-1 (+ strand)	5′AGT TTG ATC CTG GCT GAG- 3′ (8-27)	DNA coding for 16S rRNA	798
BD-2 (- strand)	5′GGA CTA CCA GGG TAT CTA AT- 3′ (805-787)		
BFR-1 (+ strand)	5′ACT CTT TGT ATC CCG ACG ATT-3′ (484-504)	Glutamine synthase gene of *Bacteroides* spp.	581
BFR-2 (- strand)	5′GAG GTT GAT GCC TGT ATC GGT-3′ (1 065-1 045)		
F3 (+ strand)	5′CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCAC-GGGGGGGCCTACGGGAGGCAGCAG-3′	Variable V3 region of 16S rRNA	233
Rev-2 (- strand)	5′ATTACCGCGGCTGCTGG-3′		

be detected and identified by subsequent cloning and sequencing. From a practical point of view blood cultures can take days to yield a result, whereas PCR can produce results within hours.

PCR without subsequent cloning can identify the bacterial genus leaving the species undefined, which may cause difficulties, for instance, if the therapeutic guidelines for the species are different. Polymicrobial infections can also be problematic due to the inability of PCR to identify several microorganisms in a single specimen[28]. These problems can be solved by denaturing gradient gel electrophoresis (DGGE)[27], which uses the presence of unique heteroduplexes in DNA affecting migration to separate different DNA fragments[29].

Our investigation was carried out to assess the potential of PCR and DGGE as rapid tools for the detection and identification of systemic bacterial translocation in blood samples of patients with acute severe pancreatitis. The study also aimed to elucidate the relationship between the presence or absence of clinical infection; manifested as either sepsis or infected necrosis, and the detection of translocated bacterial DNA.

MATERIALS AND METHODS

Sample collection and subjects
Patients who were admitted with a diagnosis of acute pancreatitis predicted to have severe disease during the study period were enrolled. Patients were only considered if their duration of symptoms was 48 h or less on admission. Pancreatitis was defined as appropriate clinical signs and symptoms with hyperamylasemia of more than three times the upper limit of normal. Patients predicted to have severe disease were identified by an APACHE II score of eight or more on admission[30].

Blood samples were taken on d 1, 3, and 8 of admission for examination by PCR and DGGE techniques. Standard microbial blood cultures were taken only when there was a clinical evidence of sepsis or when felt to be clinically indicated by the supervising team to limit the number of venesections patients enrolled in the study would need to volunteer. The supervising teams were not aware of the PCR results.

Blood samples
Blood was transferred from Na2 EDTA tubes (Sigma, UK) to sterile 1.5 mL Eppendorf tubes (Sigma, UK), and purified using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Whole blood samples were processed in aliquots of 400 μL for DNA extraction.

DNA extraction
The DNA from human blood samples and from different bacterial strains was purified, and bacterial DNA was amplified using PCR with primers specific for (i) the bacterial 16S rRNA region, (ii) *E coli,* and (iii) *Bacteroides* spp., as outlined below. Enzymatic amplification for DGGE was performed on human blood samples tested PCR positive for the presence of bacterial DNA.

Bacterial cells
Prior to DNA extraction, bacterial cells were cultured in LB medium (10 mL) overnight at +37 ℃ inside an incubator shaker (New Brunswick Scientific). Chromosomal DNA was extracted using the QIAGEN Genomic DNA Purification Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions.

Oligonucleotide primers for PCR
The oligonucleotide primers were synthesized by Sigma Aldrich Co. The primer pairs, sequences, gene targets and size of the product amplified after PCR are listed in Table 1. The primer pair designated BD-1 and BD-2 is specific for a highly conserved region of different bacterial DNA coding for 16S ribosomal RNA. The second primer pair BG-1 and BG-4 was derived from the β-galactosidase gene of *E coli*, which is found in most *E coli* strains. The third primer pair used BFR-1 and BFR-2 targets specifically for the ubiquitous glutamine synthase gene found in many *Bacteroides* spp[26]. The fourth primer pair F3 and Rev 2 was used for amplification of variable V3 region of 16S rDNA gene[29].

Microbial DNA amplification by PCR
PCR was performed with minor modification following the protocol of Kane *et al*[26]. Extracted DNA (20-30 μL) was placed in 0.5 mL sterile Eppendorf tube to which the

Table 2 A summary of the results obtained from testing of blood samples from six patients; CRP and SIRS evaluation, blood culture testing using selective growth media and PCR analysis

Patient no.	Day	1	2	3	4	8	Complications
1	CRP	135	158	91	70	40	None
	SIRS	Y	Y	N	N	N	
	PCR	Negative		Negative		Positive	
2	CRP	55	83	108	105	94	None
	SIRS	N	N	N	N	N	
	PCR	Negative		Negative		Negative	
3	CRP	345	301	289	234	142	None
	SIRS	Y	Y	Y	N	N	
	PCR	Negative		Negative		Negative	
4	CRP	301	284	248	169	122	None
	SIRS	Y	Y	Y	Y	N	
	PCR	Positive		Negative		Negative	
5	CRP	59	201	193	155	114	Pneumonia, sepsis
	SIRS	Y	Y	Y	Y	N	(-ve blood culture)
	PCR	Positive		Positive		Negative	
6	CRP	326	209	150	128		None
	SIRS	Y	Y	Y	Y		
	PCR	Negative		Negative			

NB: patient no. 6 was discharged before d 8. Patient no. 5 was treated with antibiotics on d 2.

PCR reaction mixture was added to make a final volume of 50 μL per tube. The mixture consisted of the following: 5 μL of 10× PCR reaction buffer, 1 μL each of (10 μmol/L) designated primers, 1 μL of a mixture of 10 mmol/L deoxynucleotide triphosphate (dNTP), 0.2 μL of 1 unit/μL Super Taq DNA polymerase (HT Biotechnology Ltd) and nuclease free dH2O (Sigma Chemical Ltd) up to the final volume.

PCR amplification was carried out using a Techne (Progene) machine through the cycles as follows: an initial cycle of 95 ℃/3 min, 60 ℃/45 s, and 72 ℃/10 min was followed by 35 cycles of 95 ℃/45 s, 60 ℃/45 s, and 72 ℃/1 min; an extension period of 72 ℃/10 min completed the cycling sequence.

PCR amplification of 16S rDNA fragments for DGGE

Enzymatic amplification for DGGE was performed on samples positive for bacterial DNA. PCR reaction quantifications were outlined as above. The amplification was performed as follows: an initial cycle of 95 ℃ for 15 min was followed by 35 cycles each of 1 min at 94, 60, and 70 ℃, respectively; an extension period of 72 ℃ for 5 min completed the cycling sequence.

Agarose gel electrophoresis

All PCR products were separated by electrophoresis on 1% agarose gel in 1× TAE buffer (0.04 mol/L Tris-acetate, 0.002 mol/L EDTA). The gels were stained with ethidium bromide (0.5 μg/mL) for 30 min, washed twice with distilled H2O and photographed under UV light using a UVP gel documentation system. A 1-kbp DNA ladder (Sigma) was used as a molecular weight marker. All reagents were purchased from Sigma, UK.

DGGE analysis

DGGE was performed using Ingeny Phos system (Leiden, The Netherlands). A 10 μL volume of each PCR product was applied directly onto 9% (wt/vol) polyacrylamide gels in 0.5× TAE (20 mmol/L Tris-acetate, 10 mmol/L sodium acetate, 0.5 mmol/L Na2-EDTA) with gradients which were formed with 9% (wt/vol) acrylamide, 37:1) and which contained 0 and 100% denaturant [7 mol/L urea (GIBCO BRL)] and 40% (wt/vol) formamide. The gel was subjected to 200 V for 10 min at 60 ℃ and 85 V for 16 h. After electrophoresis, the gel was stained in ethidium bromide solution (0.5 μg/mL) for 30 min, washed twice by distilled H2O and analyzed under UV light using UVP gel documentation system.

Blood cultures

Conventional blood cultures were processed using the BacT/ALERT® system manufactured by BioMerieux©. BacT/ALERT® aerobic (SA) and anaerobic (SN) culture media bottles were taken peripherally from patients under aseptic conditions and a BacT/ALERT® 3D analyzer was used to process the samples. Positive samples were Gram stained, and subcultured onto appropriate solid culture media, which was examined at 24 and 48 h.

Ethics

The study was performed as part of a larger study into enteral nutrition in acute pancreatitis. Written informed consent was a condition of entry into the study. The local ethics committee (Portsmouth, Hampshire, UK) approved the study.

RESULTS

Of the six patients tested one developed septic complications; pneumonia, respiratory failure and severe systemic inflammatory response syndrome, although this patient subsequently made a full recovery (patient
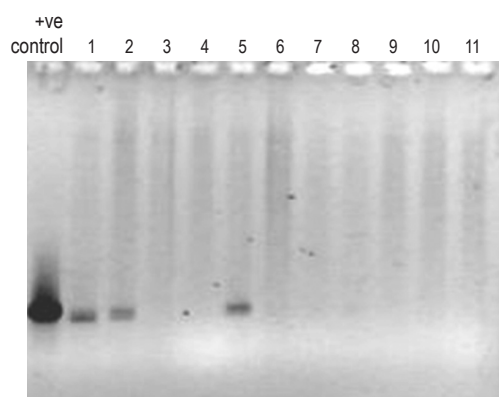
Figure 1 PCR profiles of total DNA recovered from human blood samples and of *E coli* DNA (control) obtained using primers BD1 and BD2 specific for bacterial 16S rRNA region. Positive control: Chromosomal DNA from *E coli*. Lanes 1-11: total DNA extracted from human blood samples.
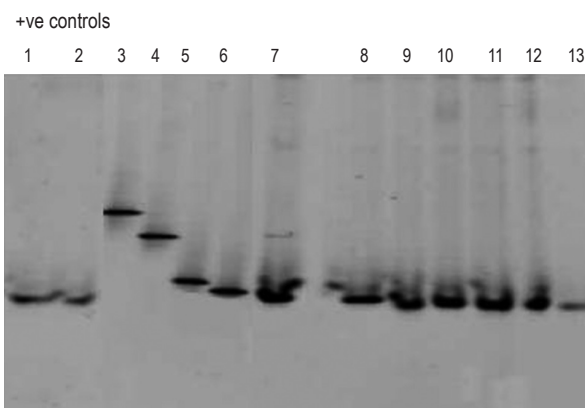

Figure 3 DGGE profiles of the amplified region of bacterial DNA coding for 16S rRNA using primers F3 and Rev 2. Positive controls: Lane 3. *Staphylococcus aureus*; Lane 4. *Pseudomonas aeruginosa*; Lane 5. *Bacillus cereus*; *Lane* 6. *E coli*; Lanes 1-2 and 7-13: bacterial DNA present in human blood samples.
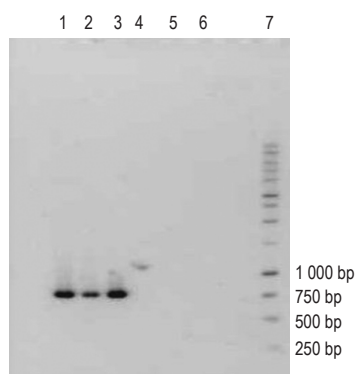

Figure 2 PCR profile of *E coli* chromosomal DNA. Lanes 1-3: DNA amplified using primers BG1 and BG4 specific for *E coli*. Lanes 4-6: DNA amplified using primers BFR1 and BFR2 specific for *Bacteroides* spp. Lane 7: 1 kb standard DNA ladder.

no. 5, Table 2). This was the only patient in the group that retrospectively developed severe acute pancreatitis according to the Santorini Consensus definitions[31]. This patient did not, however, require ventilatory support, and did not develop necrosis, infected or otherwise. The other patients had uneventful illness.

PCR detected the presence of bacteria in 4 of the 17 samples (23.6%). The blood samples from the patient with sepsis tested PCR-positive for bacterial DNA in specimens collected on d 1 and 3. This patient had three sets of standard blood cultures taken on d 1, 3, and 5, all of which were negative; he/she was the only patient in our study in whom standard blood cultures were taken.

When the incidence of the systemic inflammatory response syndrome (SIRS), C-reactive protein (CRP) levels and septic complications were compared with the PCR results there was no observed correlation between the presence of bacterial DNA in the blood, as confirmed by PCR, and SIRS or CRP data (Table 2).

The PCR profiles of DNA recovered from human blood samples and of *E coli* DNA (positive control), treated with general bacterial primers (Table 1) are depicted in Figure 1 (lanes 1 and 2-13, respectively). The appearance

of characteristic bands indicative of the presence of bacterial DNA (lane marked positive control) can be seen in human blood samples (lanes 1-11). Figure 2 (lanes 1-6) illustrates the profile of *E coli* DNA obtained using two different pairs of primers (Table 1); one pair specific only for *E coli* (lanes 1-3) and another pair for *Bacteroides* spp. (lanes 4-6, negative control). The treatment of *E coli* DNA with *Bacteroides* specific primers resulted in a blank PCR profile (i.e. no bands were observed in lanes 4-6), while the use of *E coli* specific primers resulted in the appearance of characteristic PCR product (lanes 1-3).

Human blood samples treated with *Bacteroides* specific primers did not reveal any bands when subjected to PCR (data not shown).

Subsequent analysis employing DGGE (Figure 3, lanes 1-13) revealed that the blood samples which tested PCR positive for bacterial DNA (lanes 1-2 and 7-13) contained DNA fragments of the identical size as the one characteristic for *E coli* DNA (lane 6). DGGE profiles of DNA from bacterial strains, such as *Staphylococcus aureus* (lane 3), *Pseudomonas aeruginosa* (lane 4) and *Bacillus cereus* (lane 5) served as negative controls.

DISCUSSION

These results from our preliminary investigation reveal that although conventional blood cultures techniques fail to demonstrate bacterial infection, using PCR and DGGE it is possible to detect bacterial DNA in the blood in patients with acute severe pancreatitis and also to identify the species present. The number of patients tested in the study is inadequate to make inferences regarding the connection between the presence of bacteria, systemic inflammatory response syndrome and morbidity.

Based on the obtained results it seems plausible to propose that the bacteria detected on d 1 and 3 in patient no. 5 were related to the infectious complications and sepsis. It is unlikely that the pneumonia was caused by *E coli* (sputum cultures were negative). The detection of bacterial DNA was probably indicative of a higher degree

of bacterial translocation, which led to other infections as well as the possible *E coli* sepsis. The positive PCR profile of blood samples taken on admission from patient no. 4 is also not surprising. This patient represented a case of relatively severe disease with a high CRP and probably also had bacterial translocation.

The DGGE profile of the samples from all the patients was exactly the same, i.e. positive exclusively for *E coli*. Interpretation of these results in view of the low numbers of subjects has to be treated with great caution, and it is not until more samples are taken from a larger cohort of patients that statistically valid interpretations can be offered.

To the best of our knowledge, prior to our study, reports on PCR detection of bacterial DNA in patients with acute pancreatitis are limited to two publications[11,12]; neither of these investigations used DGGE as a method of identifying bacteria.

Zhang et al[12] performed a PCR on blood specimens on patients only with acute necrotizing pancreatitis. They reported a PCR detection of bacterial DNA in 8 out of 22 tested samples (33.35%), but the samples were taken exclusively during periods of likely sepsis. The lower PCR positive rate of 23.6% in our study probably reflects the fact that blood specimens from patients were collected in a sequential fashion i.e. from the time of admission onwards, and from patients with predicted severe pancreatitis, rather than diagnosed necrotizing disease. Similarly to our study all of the positive blood samples tested were found to contain *E coli*.

Ammori et al[11] failed to find any bacterial DNA by performing PCR on blood samples from 26 patients with acute pancreatitis. Blood cultures that tested positive for bacterial infection revealed the presence of *E coli* and *Enterococcus* in blood of one patient, and coagulase negative *Staphylococcus* in the blood of another. The reasons for the negative PCR results reported by Ammori et al. are not clear, but may include contamination by substances which inhibit the polymerase reaction.

In acute pancreatitis it is perhaps what separates necrotizing from non-necrotizing disease and what differentiates infected necrosis from sterile necrosis which is of most interest. The controversy surrounding when to operate on necrotizing pancreatitis illustrates this[32-34]; a debate which centers on whether it is necessary to prove infection before operative therapy. The diagnosis of infection is not straightforward, and the currently recommended method of diagnosis, i.e. needle aspiration[34], carries its own risk due to the inherent interventional nature of the procedure. It is possible that more sensitive non-invasive methods of detecting infection such as PCR could improve diagnostic accuracy.

The presence of *E coli* DNA in blood specimens does not necessarily mean that the intestine is the source of infection. It is conceivable that these organisms arose from, for example, the biliary tract due to cholangitis. Moreover, the PCR detection method simply demonstrates the presence of bacterial DNA, and does not specify genera and species. DNA extraction methods provide total DNA that could originate from dead or living microorganisms in the blood or microbes that have been engulfed, or subsequently released by the phagocytes. This approach cannot differentiate between controlled and invasive infections.

The importance of PCR techniques with respect to clinical application will depend on establishing a relationship between the presence or absence of clinical infection and the presence of bacterial DNA in the blood. The PCR detection of bacterial DNA may provide information about the nature of the inflammatory response in acute pancreatitis when traditional methods fail to detect bacteria, even in the absence of culture-positive complications. This type of research may also reveal more about the nature of susceptibility towards infective complications in pancreatitis. In addition, the sensitivity and accuracy of PCR could help target antibiotic therapy in the future.

It is anticipated that in a cohort study including patients with sepsis or septic necrosis, and with the development of more sophisticated quantitative PCR techniques, the proposed approach could offer considerable diagnostic potential.

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Gastro esophageal reflux disease is associated with absence from work: Results from a prospective cohort study

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Abstract

AIM: To study the association of gastro-esophageal reflux disease (GERD) with the absence from work and to estimate the extent of loss in gross domestic product due to inability to work.

METHODS: Analysis was based on the prospectively gathered data of a large European cohort study involving 6 215 symptomatic GERD patients (ProGERD). Among these patients, 2 871 were initially employed. The calculation of the loss of gross domestic product was based on the assumption that the prevalence of GERD was about 15% in Germany. According to the German Federal Statistical Office, the mean gross wage of employees was 150 €/d in 2002.

RESULTS: The data of 2 078 employed patients who were prospectively followed up for over 2 years were analyzed. At study entry, the patients reported a mean of 1.8 d per year of inability to work. During the prospective follow-up under routine clinical care, the proportion of patients reporting days with inability to work decreased from 14% to 6% and the mean number of days per year with inability to work decreased to 0.9 d. Assuming a prevalence of troublesome GERD of 15% in the employed German population, the loss of gross domestic product amounted to 668 million €/year in Germany.

CONCLUSION: GERD causes a relevant impairment on the national economics by absence from work. The presented data demonstrate the importance of GERD, not only for patients and health insurance companies, but also for the community at large.

INTRODUCTION

With a prevalence of 15-40%, gastro-esophageal reflux disease (GERD) is a common adult condition in Western countries[1-3]. GERD may be associated with a marked decrease in quality of life, a loss of productivity and impairment of daily activities[4]. In some patients symptoms may be severe enough to prevent the patient from going to work. The resulting decrease in working time is associated with a significant loss in productivity. In North America, up to 10% of GERD patients have to be absent from work[5,6] and the indirect costs in the USA caused only by time away from paid labor resulting from consumption of health care were 470 million US$ in 1998[7]. The complete costs of labor lost have to be borne by employers and/or health insurance companies, and there is an accompanying decrease in the gross domestic product. The aim of the present investigation was to estimate the extent of such losses on the basis of the data of a large prospective European trial in patients with symptomatic GERD.

MATERIALS AND METHODS

The ProGERD study is an ongoing prospective, multicenter cohort study comprising an initial treatment phase and a 5-year follow-up phase. Patients ($n = 6\ 215$) were recruited from centers ($n = 1\ 253$) in Germany (more than 90%), Austria and Switzerland. The majority of these patients were recruited by gastrointestinal specialists in private practice. The protocol of the study was designed to include half of the patients with erosive reflux disease (ERD) and half with non-erosive reflux disease (NERD). Barrett's esophagus was not an exclusion criterion and in 702 patients this condition was diagnosed. The patients were treated with esomeprazole for up to 8 wk, and in

the follow-up period at the discretion of their physicians. After 2 years of follow-up, 64% of the patients were still on proton pump inhibitor (PPI) medication (continuous PPI or on demand treatment).

At study entry and every year, the patients completed a standardized questionnaire that assessed demographic, medical, and social characteristics and quality of life. One section of the questionnaires dealt with the patient's inability to work (IW) caused by reflux symptoms in the year prior to inclusion in the study and to the last year before the study visit. The patients were carefully instructed about the aims and the methods of the study, including repetition of the initial questionnaire. Further details of the ProGERD trial have been published elsewhere[8].

The most reliable assumption of the frequency of GERD in Europe was available from a large population-based telephone survey performed in 5 046 randomly chosen persons[1]. More than 1 000 persons per country (Germany, France, Italy, Sweden, and UK) were interviewed. Reflux symptoms occurred in 28% of German adults with about 58% of them claiming impaired quality of life. This means that the overall frequency of GERD is 16% in the entire adult population of Germany[9].

The number of overall IW days reported in the ProGERD trial was projected on the overall German employed population, based on the data from the German Federal Statistical Office. Germany has 82.5 million inhabitants and the labor force is composed of 36.5 millions, of whom some 33 millions are compulsorily insured. The mean gross wage of employees was 150 €/d in 2002.

In this study, the human capital method was used to calculate the indirect costs of GERD, a method that uses the full replacement costs independent of whether the worker is replaced or not[10]. We assumed a loss of the gross domestic product in the same amount of the whole mean gross wage of employees. The basis for the calculation of the decrease of gross domestic product was the number of IW days caused by GERD symptoms reported in the ProGERD trial.

We conducted an explorative analysis of potential patient-associated predictors (gender, age, BMI, consumption of alcohol, smoking, and severity of the GERD disease) of IW days using univariate statistics as appropriate. The level of significance was 5%.

RESULTS

Data about employment were available from 5 965 out of 6 215 patients (96.0%). At the beginning of the trial, 2 871 out of 5 965 (48.1%) patients were gainfully employed, 2 103 retired (35.3%), and 991 without employment (16.6%). After a 2-year follow-up period, the data about employment of 5 286 patients were available (2 312 employed, 2 097 retired, 877 without employment). We included the data of 2 078 patients who were gainfully employed and answered the respective questions about

inability to work due to GERD at the initial visit and during the 2-year follow-up period. This excluded the data of those who lost their work or retired in the follow-up period or did not answer the questions at both time points of investigation.

Initially 14% of the employed patients were admitted retrospectively to IW due to reflux symptoms during the past year. During the 2-year follow-up period, the percentage decreased to 6% under routine clinical care. The mean number of IW days was 2.5/yr prior to inclusion in the study (mean value 2.5 d in 12 mo and adjusted with the exclusion of the weekend period: 1.8 d), and decreased to 1.2/yr during the prospective follow-up period (mean value 1.2 d in 12 mo and adjusted with the exclusion of the weekend period: 0.9 d). There was no significant relationship between IW and gender, age, BMI, consumption of alcohol, smoking, and severity of GERD. In Germany, the labor force is composed of 36.5 millions, of whom some 33 millions are compulsorily insured. The mean gross wage of employees is 150 €/d (source: German Federal Statistical Office 2002). Based on the human capital method for calculating costs of illness, the total loss of gross domestic product is shown in Tables 1 and 2 assuming different frequencies of GERD in the labor force. Table 1 shows the retrospective data before entering into the follow-up period, and Table 2 lists the prospective data after the 2-year follow-up period.

Table 1 Loss of gross domestic product due to inability to work (IW) in patients with untreated GERD in Germany on the basis of three different frequency assumptions

Prevalence of GERD in the adult population	10%	15%	20 %
Total days of inability to work	5.9 million	8.9 million	11.9 million
Loss of gross domestic product	891 million €	1.34 billion €	1.78 billion €

Table 2 Loss of gross domestic product due to inability to work (IW) in patients with GERD under routine clinical care in Germany on the basis of three different frequency assumptions

Prevalence of GERD in the adult population	10%	15%	20 %
Total days of inability to work	2.97 million	4.46 million	5.94 million
Loss of gross domestic product	446 million €	668 million €	891 million €

DISCUSSION

ProGERD is a large prospective cohort study designed to examine the endoscopic and symptomatic progression of GERD in routine care as well as the economic burden of the disease. With more than 6 000 included patients, the study is the largest prospective clinical trial in this field and could answer several questions on a high level of confidence. The influence of GERD on the gross

domestic product, being part of the indirect costs of the disease, was estimated in this analysis. On the basis of these data, the total costs of the economy of GERD, due to loss of gross domestic product were reliably assessable. Assuming a prevalence of 15% of the disease in the employed German population, costs amounted to 668 million €/yr under routine clinical care. The costs in the same population were twice as high in the year before entering the trial. These calculations highlight the great economic importance of GERD, which is not only a problem for patients and health insurance companies, but also has a major impact on the community at large. The total loss of gross domestic product estimated by our analysis was, for example, close to the total yearly PPI sales of 842 million € in Germany in 2003[11].

Our calculation was based on the human capital method for calculating the costs of illness. Another approach to calculating indirect costs is the friction cost method, which measures the amount of production lost due to the disease based on the time span organizations needed to restore the initial production, so that production losses are assumed to be confined to the period needed to replace a sick worker[10]. A stated advantage of the friction cost method is that, unlike the human capital method, it takes into account the fact that employees with long-term illness or disabilities can be replaced in markets with less than full employment. There is no general agreement on whether the human capital or friction cost method is more valid for measuring the productivity costs of illness. Long-term sick leave due to GERD symptoms is probably rare-none of the patients included in the ProGERD study reported long-term sick leave because of GERD symptoms during the prospective follow-up period-and replacement of the worker is an exceptional event. Therefore, the human capital method might be the more reliable approach. Even assuming more conservative estimates of indirect costs based on the friction cost method, GERD is certainly still associated with a considerable social and economic burden.

As expected, only a limited portion of about 14% of the GERD patients even got admitted to IW caused by reflux symptoms. The symptoms are mostly mild to moderate, though the quality of life in patients with GERD is markedly decreased[12], the level is comparable to other chronic or even life-threatening diseases[13]. In the follow-up period, the portion of patients with IW declined to 6%. Other trials from the USA have reported partially a lower portion of 2-10%[5,6], suggesting that socioethical factors might influence the willingness to be absent from work.

The follow-up data indicated the effectiveness of the treatment of GERD in reducing the IW days. The mean number of IW days decreased from 2.5 to 1.2 in the 2-year observation period. The patients were treated in the follow-up period at the discretion of their physicians, and around two out of the three patients were still on PPI treatment after the 2-year follow-up period.

GERD is nowadays the disease with the highest direct costs in the USA (9.3 billion US$)[7]. European data

are available from Sweden that includes the costs for dyspepsia and peptic ulcer disease. Therefore, it is difficult to compare them with our findings[14].

Our calculation of the loss of gross domestic product was based on the statement of the patients using a long recall period of one year, and it might result in an underestimation of disease-related absence from work. A recall bias on self-reported work productivity occurs more often already after a 4-wk recall period compared with a 2- or 1-wk recall period[15]. Therefore, our calculation can be considered as conservative, and the loss might be significantly higher. A recent study showed that the impact of GERD on the costs of reduced productivity is even higher compared to that on the costs of absence from work[16]. Therefore, it is highly possible that our calculation might further underestimate the overall loss of gross domestic product due to GERD, because we only referred to the IW days instead of estimating the reduced productivity.

The exact calculation of the indirect costs is somehow difficult. Typically indirect costs include costs associated with the lost or impaired ability to work or to enjoy leisure activities because of morbidity, the time required by the patient's family to receive medical care, and loss of future earning potential owing to premature death. Data about impaired ability to work due to GERD are insufficient. In a small cross-sectional study from the USA, about 41% of GERD patients reported that they have lost some work productivity[6]. The authors have reported here in addition that the time off for physician visits and reduced productivity are the most costly losses from GERD. Our main focus was on the additional part of costs due to absence from work.

The impact of GERD on the economy differs widely between different countries depending on several factors (like cultural, socioeconomic, and economical status). Nevertheless, our data clearly suggest that the loss of gross domestic product due to GERD-related IW days might be higher compared to the total sale volume of PPI in Western countries in general. Definite data on this topic are lacking. It would, therefore, be interesting to study the impact of effective pharmacotherapy not only on clinical symptoms and healing of lesions but also on the loss of productivity.

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• *RAPID COMMUNICATION* •

Internet-based data inclusion in a population-based European collaborative follow-up study of inflammatory bowel disease patients: Description of methods used and analysis of factors influencing response rates

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Abstract

AIM: To describe an Internet-based data acquisition facility for a European 10-year clinical follow-up study project of a population-based cohort of inflammatory bowel disease (IBD) patients and to investigate the influence of demographic and disease related patient characteristics on response rates.

METHODS: Thirteen years ago, the European Collaborative study group of IBD (EC-IBD) initiated a population-based prospective inception cohort of 2 201 uniformly diagnosed IBD patients within 20 well-described geographical areas in 11 European countries and Israel. For the 10-year follow-up of this cohort, an electronic patient questionnaire (ePQ) and electronic physician per patient follow-up form (ePpPFU) were designed as two separate data collecting instruments and made available through an Internet-based website. Independent demographic and clinical determinants of ePQ participation were analyzed using multivariate logistic regression.

RESULTS: In 958 (316 CD and 642 UC) out of a total number of 1 505 (64%) available IBD patients, originating from 13 participating centers from nine different countries, both ePQ and ePpPFU were completed. Patients older than 40 years at ePQ completion (OR: 1.53 (95%CI: 1.14-2.05)) and those with active disease during the 3 mo previous to ePQ completion (OR: 3.32 (95%CI: 1.57-7.03)) were significantly more likely to respond.

CONCLUSION: An Internet-based data acquisition tool appeared successful in sustaining a unique Western-European and Israelian multi-center 10-year clinical follow-up study project in patients afflicted with IBD.

INTRODUCTION

Data acquisition in multi-center epidemiological follow-up studies traditionally has been dependent on postal paper form questionnaires filled out by study subjects and on paper form extraction files for relevant medical information completed by physicians and/or research assistants. Great advantages of the use of the Internet as a platform for health status measurements have been reported since the mid-1990s[1]. Internet-based facilities supporting data acquisition in studies concerning patients with inflammatory bowel disease (IBD) have been performed from then onwards[2-4]. Traditionally, in multi-center studies, questionnaires in paper format are forwarded from patients and centers to central database-hosting facilities, where data are manually transformed

into electronic databases. This multi-step process is time- and labor-consuming and prone to errors. Paper form data acquisition utilities normally contain question flow instructions for study subjects and for physicians that can easily be misinterpreted resulting in missing or wrong answers. Furthermore, the physical transformation of large numbers of paper format data into an electronic database adds to the risk of human errors.

For the purpose of a large multi-center follow-up study of a European population-based cohort of patients with IBD[5], we created an Internet-based data acquisition facility, in order to avoid the above-mentioned methodological imperfections and to assure data quality. The design of the mentioned study as well as practical and technical details and implications of the new electronic instrument are described and discussed. The influence of demographic and disease-related patient characteristics on the patient questionnaire response rates is also analyzed.

History of the cohort

After 3 years of preparation, between October 1991 and September 1993, the European Collaborative study group of IBD (EC-IBD) gathered a population-based prospective inception cohort of 2 201 uniformly diagnosed IBD patients within 20 well-described geographical areas in 12 European countries[6]. Initially it was hypothesized that a North-South incidence gradient in IBD existed, which could be proved[5]. Later, in the context of this registry, a 1- and 4-year clinical follow-up[7,8] were studied, as well as the occurrence of rheumatologic manifestations[9] and the relationship between quality of care and quality of life[10].

The currently executed 10-year clinical follow-up study project was planned since 1998, granted by the European Commission, and was started in 2001.

MATERIALS AND METHODS

Materials

In order to optimize data acquisition in this European multi-center study project, electronic data acquisition tools were designed for Internet-based access. The development of the tools and the subsequent data management leading to an analyzable dataset involved a five-step process; (1) a "patient questionnaire" (PQ) and "physician per patient follow-up form" (PpPFU) were prepared as two separate data collecting instruments and originally constructed for the purpose of this study. The information obtained by the PQ was derived from direct questioning of the patients, whereas physicians and research assistants extracted data from patient files into the PpPFU. PQ items were demographics, life style factors, disease activity and use of medication, family history, data on pregnancy and fertility, health care consumption, disability, and quality of life (SF-36 was used). PpPFU items, as observed during the 10-year follow-up period, were vital status, cause of death, disease activity (change of immunosuppressive medication and surgical events were used as indicators due to the retrospective nature of data acquisition), disease location

and behavior, use of medication, surgery, health care consumption, colorectal cancer, and dysplasia. Members of the original study group and additional investigators formed "working groups" to deal with various research topics of the follow-up, including genotype-phenotype, course of disease, pregnancy and fertility, cancer, health care consumption and costs, and dissemination of messages to the medical and lay public. A central body of senior representatives from the EC-IBD group constructed final comprehensive versions of both instruments based on the input of all the members of the working groups. The electronic patient questionnaire (ePQ) questions were translated into the nine languages of the participating countries by a professional translation agency and subsequently checked for correctness by a representative from every participating country from within the EC-IBD study group. For the PpPFU, the language used was English; (2) a tailor-made innovative high performance data entry application was designed, assuring maximum flexibility for programmers and users. The ePQ was designed by the construction of a questionnaire design module, where questions and answers could be directly introduced in different languages with needed routings. The final ePQ contained 747 questions and could be used in nine different languages including Hebrew. The electronic physician per patient follow-up form (ePpPFU) was designed to receive an unlimited number of entries on multiple topics on a 10-year time scale. Graphic design using simple colors in order to visually discriminate users' choices improved user friendliness; (3) the PQ questions and the PpPFU items, originally constructed on paper, were introduced into the electronic facilities. A pre-designed compulsive question flow was implemented in the ePQ. This implicated that patients filling in the ePQ were automatically guided towards a subsequent question, based on a given previous answer thus skipping irrelevant questions. Electronic guidance facilities concerning mandatory and optional fields were embedded in the ePpPFU. In this way, physicians and/or research assistants completing the ePpPFU of a particular patient were not allowed to close and finalize the document concerning an individual patient before having collected all the minimally required information; (4) the electronic data acquisition facilities were made available to all data collecting centers through a username-password secured and firewall protected Internet-based website. Links to ePQ statistics and ePpPFU statistic pages displayed real time counts of both instruments for all centers together showing instantaneous, comparative and cumulative progress of data inclusion, accessible to every member involved in the data inclusion of this project; (5) after completion of the data acquisition phase, export files from the Internet questionnaire program were imported into the database environment. The database was situated in Maastricht. Database validation took place at this center before the distribution of the final database to all working groups. The validation was done by logical comparison of the crude database with existing databases of previous EC-

Table 1 Cumulative number of IBD (CD+UC) patients per center followed-up or lost to follow-up for ePQ and ePpPFU. Centers indicated in gray had not reached the 60% response rate threshold

Center	Total number of CD+ CU patients	EPQ response (%)	EPQ response without deaths and No IBD %	Reason for non response EPQ					PpPFU response (%)	PpPFU response without No IBD %	Reason for non response PpPFU				EPQ ànd PpPFU (%)
				Not willing	Untrace-able	No IBD	Death	Not started			Not willing	Untrace-able	No IBD	Not started	
Beer Sheeva	60	41 (68)	76	5	8	3	3	0	46 (77)	77	3	11	0	0	42 (70)
Copenhagen	147	103 (70)	84	12	7	0	25	0	132 (90)	90	6	9	0	0	120 (82)
Cremona	51	35 (68)	76	4	7	0	5	0	43 (84)	84	1	7	0	0	40 (78)
Heraklion	62	38 (61)	68	2	13	0	5	4	49 (79)	79	3	17	0	0	40 (65)
Ioannina	43	36 (84)	88	2	3	0	2	0	39 (91)	91	0	4	0	0	38 (88)
Oslo	378	226 (60)	69	65	34	6	45	2	332 (88)	89	21	25	1	0	271 (72)
Reggio Emilia	84	61 (73)	79	4	12	0	7	0	69 (82)	82	5	11	0	0	68 (81)
S-Limburg	216	156 (72)	76	28	20	0	11	1	194 (90)	90	0	23	0	0	159 (74)
Vigo	100	73 (73)	77	4	18	0	5	0	81 (81)	81	3	16	0	0	77 (77)
SUBTOTAL	1 141	769 (67)	75	126	122	9	108	7	985 (86)	86	42	123	1	0	855 (75)
Almada	21	11 (52)	58	1	7	0	2	0	12 (57)	57	0	9	0	0	11 (52)
Dublin	186	57 (30)	34	10	58	3	17	41	80 (43)	43	1	26	2	78	56 (30)
Firenze	115	48 (42)	42	0	0	0	0	67	39 (34)	34	0	4	0	72	36 (31)
Milano	42	19 (45)	58	1	6	0	9	7	0 (0)	0	0	0	0	42	0 (0)
SUBTOTAL	364	135 (37)	41	12	71	3	28	115	131 (36)	36	1	39	2	192	103 (28)
Total	1 505	904 (60)	67	138	193	12	136	122	1 116 (74)	74	43	162	3	192	958 (64)

IBD projects in terms of numbers of patients per center with known diagnosis, sex, age, and disease behavioral characteristics. The results of this data validation effort were summarized in a logbook that was distributed to all the project members for possible revisions that subsequently were implemented as changes in the database. Final data sets were available for analysis promptly after closing the data acquisition phase.

Methods

Before the start of the data collection, a minimally required response rate per center was set at 60% identification of the patients to have both ePQ and ePpPFU completed. Centers that did not comply with this minimum response rate were analyzed separately from those that did.

UC patients were grouped for disease location at diagnosis as: (1) rectum only; (2) rectum and sigmoid and/or descending colon; and (3) disease location beyond the splenic flexure. The CD patients were classified for disease location at diagnosis, according to the Vienna classification[11], into: (1) upper gastrointestinal (esophagus and/or stomach and/or duodenum and/or jejunum); (2) (terminal) ileum; (3) ileocolonic; and (4) colon only.

Independently, both demographic and clinical determinants of ePQ participation (diagnosis, gender, center, disease location at diagnosis, age at ePQ completion, and disease activity during 3 mo period previous of ePQ completion) were analyzed using univariate and multivariate logistic regression models. All potential predictors were included in both models. A $P \leqslant 0.05$ was considered to be statistically significant in the multivariate analysis. The statistical package for the social sciences (SPSS 11.5.1 for Windows; SPSS Inc., Chicago, IL, USA) was used for the analysis.

The Ethics Committees of all the participating centers approved the study protocol, and all subjects gave their informed consent before the start of the study.

RESULTS

During the data collection period from August 1st 2002 to January 1st 2004, the electronic data entry facilities appeared to be well-equipped and flexible tools for data entry and storage. However, the data export facility used in order to enable fast provision of stored data into formats prepared for analysis showed some initial deficiencies. Questions, answer categories and formal control rules, used for feedback during insertion, were not always automatically incorporated in the export process. This could, however, be repaired by the information management team.

All centers that originally participated in the EC-IBD cohort had been approached to take part in the follow-up study. Thirteen out of the original twenty centers distributed over nine countries participated. In 958 (316 CD and 642 UC) out of a total number of 1 505 IBD patients eligible for follow-up (64%), both ePQ and ePpPFU were completed (Table 1). When considering the entire cohort of both CD and UC patients, nine centers complied with the minimal 60% response threshold rendering 855 out of 1 141 patients (75%). Table 1 summarizes the details of the total number of IBD (both CD and UC) patients in the cohort. In the CD category, 10 of the participating 13 centers complied with the minimal 60% response threshold leaving 378 patients of whom 288 (76%)had completed both ePQ and ePpPFU (Table 2). In the UC category, nine centers complied leaving 776 patients of whom 575 (74%) had completed both instruments (Table 3).

Table 2 shows two Beer Sheeva patients indicated as "no IBD" in the ePQ, who were not classified as such in the ePpPFU. Review of available clinical data revealed that both patients have CD. An identical discrepancy concerning one Dublin patient in the "no IBD" category could not be clarified. This patient was considered to have CD. In the UC category (Table 3) divergences between ePQ and ePpPFU for patients indicated as "no IBD"

Table 2 Number of CD patients per center followed-up or lost to follow-up for ePQ and ePpPFU. Centers indicated in gray had not reached the 60% response rate threshold

Center	Total number of CD patients	EPQ response (%)	EPQ response without deaths and No IBD %	Reason for non response EPQ					PpPFU response (%)	PpPFU response without No IBD %	Reason for non response PpPFU				EPQ ànd PpPFU (%)
				Not willing	Untrace-able	No IBD	Death	Not started			Not willing	Untrace-able	No IBD	Not started	
Almada	13	8 (62)	67	0	4	0	1	0	8 (62)	62	0	5	0	0	8 (62)
Beer Sheeva	21	13 (62)	72	2	3	2	1	0	15 (71)	71	1	5	0	0	14 (67)
Copenhagen	58	41 (71)	85	5	2	0	10	0	52 (90)	90	6	0	0	0	47 (81)
Cremona	10	7 (70)	78	2	0	0	1	0	9 (90)	90	1	0	0	0	8 (80)
Heraklion	14	7 (50)	70	0	3	0	3	1	11 (79)	79	0	5	0	0	9 (64)
Ioannina	6	4 (67)	80	0	1	0	1	0	5 (83)	83	0	1	0	0	5 (83)
Oslo	110	69 (63)	70	15	13	1	11	1	97 (88)	89	4	8	1	0	79 (73)
Reggio Emilia	34	24 (71)	77	0	7	0	3	0	27 (79)	79	1	6	0	0	27 (79)
S-Limburg	77	60 (78)	82	8	5	0	4	0	69 (90)	90	0	8	0	0	61 (79)
Vigo	35	27 (77)	84	1	4	0	3	0	30 (86)	86	1	4	0	0	30 (86)
SUBTOTAL	378	260 (69)	77	33	42	3	38	2	323 (85)	85	14	42	1	0	288 (76)
Dublin	63	17 (27)	30	2	25	2	5	12	25 (40)	40	0	6	1	31	17 (27)
Firenze	29	13 (45)	45	0	0	0	0	16	13 (45)	45	0	1	0	15	11 (38)
Milano	13	4 (31)	50	0	2	0	5	2	0 (0)	0	0	0	0	13	0 (0)
SUBTOTAL	105	34 (32)	36	2	27	2	10	30	38 (36)	37	0	7	1	59	28 (27)
TOTAL	483	294 (61)	69	35	69	5	48	32	361 (75)	75	14	49	2	59	316 (65)

Table 3 Number of UC patients per center followed-up or lost to follow-up for ePQ and ePpPFU. Centers indicated in gray had not reached the 60% response rate threshold

Center	Total number of CU patients	EPQ Response (%)	EPQ response without deaths and No IBD %	Reason for non response EPQ					PpPFU response (%)	PpPFU response without No IBD %	Reason for non response PpPFU				EPQ ànd PpPFU (%)
				Not willing	Untrace-able	No IBD	Death	Not started			Not willing	Untrace-able	No IBD	Not started	
Beer Sheeva	39	28 (72)	78	3	5	1	2	0	31 (79)	79	2	6	0	0	28 (72)
Copenhagen	89	62 (70)	84	7	5	0	15	0	80 (90)	90	0	9	0	0	73 (82)
Cremona	41	28 (68)	76	2	7	0	4	0	34 (83)	83	0	7	0	0	32 (78)
Heraklion	48	31 (65)	67	2	10	0	2	3	38 (79)	79	3	12	0	0	31 (65)
Ioannina	37	32 (86)	89	2	2	0	1	0	34 (92)	92	0	3	0	0	33 (89)
Oslo	268	157 (59)	69	50	21	5	34	1	235 (88)	88	17	17	0	0	192 (72)
Reggio Emilia	50	37 (74)	80	4	5	0	4	0	42 (84)	84	4	5	0	0	41 (82)
S-Limburg	139	96 (69)	72	20	15	0	7	1	125 (90)	90	0	15	0	0	98 (71)
Vigo	65	46 (70)	73	3	14	0	2	0	51 (78)	78	2	12	0	0	47 (72)
SUBTOTAL	776	517 (67)	74	93	84	6	71	5	670 (86)	86	28	86	0	0	575 (74)
Almada	8	3 (38)	50	1	3	0	1	0	4 (50)	50	0	4	0	0	3 (38)
Dublin	123	40 (33)	36	8	33	1	12	29	55 (45)	45	1	20	1	47	39 (32)
Firenze	86	35 (41)	41	0	0	0	0	51	26 (30)	30	0	3	0	57	25 (29)
Milano	29	15 (51)	60	1	4	0	4	5	0 (0)	0	0	0	0	29	0 (0)
SUBTOTAL	246	93 (38)	41	10	40	1	17	85	85 (35)	35	1	27	1	133	67 (27)
TOTAL	1 022	610 (60)	67	103	124	7	88	90	755 (74)	74	29	113	1	133	642 (63)

regarding one patient from Beer Sheeva and five patients from Oslo could not be clarified. These six patients were considered to have UC. Discrepancies between total numbers of patients for ePQ and ePpPFU also occurred because of Internet disruptions during the insertion process in the CD category regarding two patients from Heraklion (Table 2), and in the UC category regarding five patients from Heraklion, one from Oslo, one from Reggio Emilia, one from South-Limburg and one from Dublin (Table 4). The answers given by these patients were lost and these patients were excluded from the database. Disease location at diagnosis was known for 739 of 766 UC patients and 363 of 378 CD patients.

In the 60% of complying centers, 126 of 1 141 patients [11%] refused to participate without a large difference between the age groups (79/707 [11.2%] in patients older than 40 years at ePQ completion and 47/434 [10.8%] in patients equal to or younger than 40 years at ePQ completion). 10.7% of the patients lost to follow-up for the ePQ were indicated as untraceable. Of patients aged >40 years at ePQ completion, 56/707 [7.9%] could not be traced. In those ≤40 years at ePQ completion, 66/434 [15.2%] of patients could not be traced.

In the UC patient group, age >40 years and in the combined CD and UC patient groups active disease recorded within 3 mo previous to ePQ completion were significant positive predictors of ePQ response. Gender, center, diagnosis, age at diagnosis and disease location showed no significant differences in ePQ response in centers that had complied with the 60% minimal ePQ

Table 4 Results of univariate and multivariate logistic regression analyses for identification of predictors of ePQ completion in the entire cohort

CD+UC					
N total = 1 141	*n*	Response (%)	Unadjusted Odds Ratio (95%CI)	Adjusted Odds Ratio (95%CI)	*P*
CD/UC	365/776	289 (79)/588 (76)	1.22 (0.90-1.64)	1.24 (0.91-1.70)	0.171
Female/male	552/589	429 (77)/448 (76)	1.10 (0.84-1.44)	1.09 (0.82-1.44)	0.564
Age at ePQ completion >40/≤40 yr	707/434	559 (79)/318 (73)	1.38 (1.04-1.82)	1.53 (1.14-2.05)	0.004
Flare/no flare[1]	88/1 053	80 (91)/797 (76)	3.21 (1.53-6.73)	3.32 (1.57- 7.03)	0.002

[1]Flare within 3 mo period previous of the moment of ePQ completion. *N*: number of patients; CI: confidence interval.

Table 5 Results of univariate and multivariate logistic regression analyses for identification of predictors of ePQ completion in the UC population separately

UC					
N total = 776	*n*	Response (%)	Unadjusted Odds Ratio (95%CI)	Adjusted Odds Ratio (95%CI)	*P*
Female/male	370/406	280 (76)/308 (76)	0.99 (0.71-1.38)	1.01 (0.72-1.42)	0.956
Age at ePQ completion >40/≤40 yr	520/256	409 (78)/179 (70)	1.59 (1.13-2. 23)	1.70 (1.19-2.41)	0.003
Flare/no flare[1]	55/721	48 (87)/540 (75)	2.30 (1.02-5.17)	2.35 (1.03-5.37)	0.043
Left sided/rectum	333/221	252 (76)/169 (77)	0.96 (0.64-1.43)	0.99 (0.65-1.51)	0.965
Pancolitis/rectum	185/221	140 (76)/169 (77)	0.96 (0.61-1.51)	1.04 (0.65- 1.67)	0.875
Unknown/rectum	37/221	27 (73)/169 (77)	0.83 (0.38-1.83)	0.71 (0.31-1.64)	0.426

[1]Flare within 3 mo period previous of the moment of ePQ completion. N: number of patients; CI: confidence interval; Left sided: rectum and sigmoid and/or descending colon; Unknown: unknown disease location.

Table 6 Results of univariate and multivariate logistic regression analyses for identification of predictors of ePQ completion in the CD population separately

CD					
N total = 378	*n*	Response (%)	Unadjusted Odds Ratio (95%CI)	Adjusted Odds Ratio (95%CI)	*P*
Female/male	190/188	154 (81)/144 (77)	1.31 (0.80-2.15)	1.35 (0.79-2.30)	0.270
Age at ePQ completion >40/≤40 yr	196/182	156 (80)/142 (78)	1.10 (0.67-1.80)	1.34 (0.79-2.30)	0.281
Flare/no flare[1]	34/344	33 (97)/265 (77)	9.84 (1.32-73.1)	10.9 (1.43-82.4)	0.021
Ileo colon/upper	136/20	110 (81)/16 (80)	1.06 (0.33-3.42)	0.91 (0.26-3.19)	0.879
Ileum/upper	54/20	41 (76)/16 (80)	0.79 (0.22-2.78)	0.95 (0.27-3.35)	0.631
Colon/upper	153/20	122 (80)/16 (80)	0.98 (0.31-3.15)	0.71 (0.18-2.77)	0.622
Unknown/upper	15/20	9 (60)/16 (80)	0.38 (0.083-1.69)	0.38 (0.075-1.90)	0.237

[1]Flare within 3 mo period previous of the moment of ePQ completion. *n*: number of patients; CI: confidence interval; Upper: esophagus and/or stomach and/or duodenum and/or jejunum; Unknown: unknown disease location.

response in neither the uni- nor multivariate analyses. Details of the results of these analyses are displayed in Tables 4, 5, and 6.

Results of identical analyses performed in the patients of non-complying centers revealed no different viewpoints.

DISCUSSION

An Internet-based data acquisition tool sustaining a unique European multinational multi-center 10-year clinical follow-up study in patients afflicted with IBD appeared successful. User-friendly tailor-made software applications facilitated remote data inclusion, making it a one-step process, thereby minimizing the risk of human error, optimizing efficiency and convenience of the study effort and rendering continuous transparency of the project progress. The data management procedure made

a reliable analyzable data set available promptly after the completion of the data acquisition phase. Overall 67% and 74% follow-up rates were observed for ePQ and ePpPFU, respectively. UC patients older than 40 years at the moment of ePQ completion and UC and CD patients with active disease in the 3 mo period previous to ePQ completion were significantly more likely to respond.

Does the use of Internet-based data acquisition instruments compare to or even outweigh the traditionally available methods? Some comparative studies are available in this field. In one study comprising UC patients, it appeared possible to use direct electronic mail contact to conduct follow-up research; response rates appeared to be related to the number of messages sent, the age of the recipients and the time since the initial contact[12]. The use of Internet tools in psychological research and Web-based assessments of personality constructs have proven to render outcome scores comparable to those of traditional

methods[13,14]. In one study the traditional paper-and-pencil questionnaire resulted in a higher number of errors compared with the Web-based questionnaire[15]. Two other studies compared several student populations that were similar in terms of age, gender, and racial backgrounds with regard to differences in outcome for Web-based and traditional questionnaires[16,17]. In these studies outcome scores of the Internet-based questionnaire and the traditional paper-and-pencil based questionnaires were not different. The time- and cost-effectiveness, convenience for both patients and researchers, the possibilities of immediate availability of data for analysis, and the structural prevention of missing data and incomplete responses by a priori programmed configuration, are important advantages related to the use of Internet-based data inclusion instruments as already discussed elsewhere[18,19].

The type of survey population addressed is of importance in this context. In open Web-based surveys, selection bias occurs due to the non-representative nature of the Internet population, and - more importantly - through self-selection of participants, also called as the 'volunteer effect'[18]. Furthermore, exact measurement of response rates is not possible, because the original size of the patient population is unknown. In quantitative epidemiological research, averages are measured of pre-defined patient populations, and therefore representative patient samples and adequate response rates are needed[20]. In the present study a finite 'closed' patient sample with known socio-demographic details was approached for follow-up for the fourth time in its disease history. In this way the exact response rate could easily be calculated. The patients could not log on to the username-password secured web site by themselves, but were guided by a project representative who assisted with the insertion procedure according to the patients' individual needs.

The patient group under study, being a population-based cohort, was representative by its nature. High follow-up rates were observed in the majority of the centers complying with the minimally required 60% response rate. This reduced selection bias. Thirteen of the original twenty EC-IBD centers participated in this FU study. The remaining seven had to refrain because of technical and/or logistic reasons. This did not jeopardize the population-based character of the study, since all participating centers had individually met the criteria for population based patient inclusion when the cohort was formed in the period of 1991-1993. In the present study, centers not reaching the minimally required 60% follow-up rate were analyzed separately acknowledging their effort and also securing the intent of overall population based methodology. The original population size of almost 930 000 inhabitants negatively influenced the follow-up rates in Dublin. The magnitude of the Irish project covering six university and six private hospitals made sufficient follow-up percentages, 10 years after the diagnosis, unrealistic. Most areas that reached the 60% follow-up rate threshold had original population sizes between 300 000 and 500 000 inhabitants, suggesting that this size enables

successful long-term follow-up. Considering the broad European multi-center nature of this study and the fact that the entire patient population had been diagnosed 10 years earlier, the high response rates must be regarded as excellent.

UC patients who responded to the ePQ were significantly older than those who did not. This reflects most probably the higher dedication and availability of this age group compared to the younger patients. In the CD group, there was no difference between the age groups. The expected drop of response of the elderly because of supposed computer anxiety could not be confirmed in this study. Eleven percent of the patients indicated to have been lost to follow-up for the ePQ were in fact traced, but refused to participate without a difference between the age groups. The need of physical presence to complete the ePQ could explain at least part of the observed refusal rate. Furthermore, active disease within 3 mo period from ePQ completion was shown to be a clear positive predictor of ePQ participation in both UC and CD, and the majority of patients (1 073/1 162 [92.3%]) were in clinical remission during this period. Most probably, disease activity accompanies increased concern and increases motivation to participate. This observation could be an important factor of bias that has to be controlled for in the quality of life analyses planned in this project. 10.7% of the patients lost to follow-up for the ePQ were indicated to be untraceable. Patients equal to or younger than 40 years at ePQ completion were less likely to be traced, compared to those patients being older than 40 years at ePQ completion. This difference could be explained by the high mobility **characteristic of younger** people.

Apart from 11 occasions of system failure and some initial inconsistencies of the data export facility, this electronic facility appeared to be a well-equipped flexible tool for data entry and storage. Possibilities for an efficiency gain in terms of a better fit between Internet questionnaire application and the data management process had been described before[21] and could be confirmed in this study.

In conclusion, an Internet-based electronic data acquisition application successfully sustained a unique multinational multi-center study project in patients afflicted with IBD. The application has high potential for future use in follow-up studies of this cohort and could serve as a template for other multi-national follow-up studies.

ACKNOWLEDGMENTS

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• *RAPID COMMUNICATION* •

Serum insulin, insulin resistance, β-cell dysfunction, and gallstone disease among type 2 diabetics in Chinese population: A community-based study in Kinmen, Taiwan

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Abstract

AIM: To explore the association of serum insulin, insulin resistance, and β-cell dysfunction with gallstone disease (GSD) in type 2 diabetics.

METHODS: We used a community-based study conducted between 1991 and 1993 in Kinmen, Taiwan to identify type 2 diabetics. A screening program for GSD was performed in 2001 by a panel of specialists who employed real-time ultrasound sonography to examine the abdominal region after the patient had fasted for at least 8 h. Screening was conducted in 2001 on 848 patients diagnosed with type 2 diabetes. The HOMA method was used to compare the profile differences for insulin resistance (HOMA IR) and β-cell dysfunction (HOMA β-cell).

RESULTS: We studied 440 type 2 diabetics who attended sonography check-ups. After excluding eight insulin-treated diabetics, the prevalence of GSD among the remaining 432 was 13.9% (26/187) among males and 14.7% (36/245) among females. After adjustment for other GSD-associated risk factors in addition to age and obesity, GSD risk increased among females with levels of serum insulin [4th *vs* 1st quartile odds ratios (OR) = 4.46 (95%CI: 1.71-11.66)] and HOMA IR [4th *vs* 1st quartile OR = 4.46 (95%CI: 1.71-11.66)]. Better HOMA β-cell function was significantly related to decreased risk of GSD [4th *vs* 1st quartile OR = 0.16 (95%CI: 0.03-1.70)]. Among males, age and central obesity were the most significant risk factors for GSD. No association of GSD with serum insulin, HOMA IR, and HOMA β-cell was observed among males.

CONCLUSION: Serum insulin, insulin resistance, and β-cell dysfunction are risk factors for GSD in females, but not males with type 2 diabetes.

INTRODUCTION

Clinically, type 2 diabetes with concomitant gallstone disease (GSD) increases the incidence of acute cholecystitis and has a higher probability for progression to septicemia. It is generally believed that subjects with diabetes secrete more lithogenic bile than non-diabetics; i.e., diabetes and GSD might be viewed as closely linked diseases[1]. Indeed, several community-based epidemiologic studies have demonstrated that not only diabetics have an increase in GSD-related morbidity[2-4], but also, our previous report showed that the prevalence of overall GSD among type 2 diabetics was higher as compared with

other general Chinese populations when using the same methods of GSD assessment[5]. In order to reduce health-care expenditures caused by GSD, organized preventive strategies are recommended.

Insulin resistance syndrome, initially described by Reaven[6], is a cluster of risk factors for coronary artery disease. This pathological condition is characterized by an inadequate physiological response of peripheral tissues to circulating insulin, and results in metabolic and hemodynamic disturbances[7]. Hyperinsulinemia has also characteristically been found in subjects with type 2 diabetes as a result of insulin resistance, which is considered to be of primary importance in the pathogenesis of diabetes. In addition, impairment of β-cell function, which becomes most evident during insulin resistance due to increased demands for insulin, is also well known now as a major factor influencing the progression from normal glucose tolerance, through impaired glucose tolerance to frank type 2 diabetes[8,9]. Results of the epidemiological studies have shown that increased insulin resistance and decreased insulin secretion are strongly related to the risk of developing type 2 diabetes[10-12].

The insulin-related factors responsible for the development of GSD remain to be clarified. Although it has been suggested that serum insulin affects the development of this co-disease in the general population[13,14], few community-based studies have been conducted to explore the direct effect of serum insulin, insulin resistance, and β-cell dysfunction to GSD among the diabetics. Additionally, other factors, including diabetic duration and glucose toxicity, have been proposed to affect the development of GSD in type 2 diabetics[3]. Using the community-based study in Kinmen, Taiwan, we aimed to determine whether serum insulin, insulin resistance, and β-cell dysfunction, were independently related to GSD among type 2 diabetics.

MATERIALS AND METHODS

Organization of community-based gallstone disease screening for type 2 diabetes

We used baseline data from a community-based screening for type 2 diabetes targeted to subjects aged 30 years or more in Kinmen, Taiwan, between January 1991 and December 1993. The details of the study design and execution have been described in detail elsewhere[15]. The identification of type 2 diabetes was based on the WHO definition in 1985[16]: subjects with fasting plasma glucose (FPG) $\geqslant$7.8 mmol/L ($\geqslant$140 mg/dL) or 2 h postload $\geqslant$ 11.1 mmol/L ($\geqslant$200 mg/dL) were defined as individuals with type 2 diabetes. Subjects with a history of type 2 diabetes and who received medication were defined as known cases. However, in the GSD screening done in 2001, the patients that fulfilled the criteria of the revised WHO criteria (revised in 1999) were enrolled. Patients with FPG $\geqslant$7.0 and <7.8 mmol/L in 1991 to 1993 were also recruited[17]. A total of 1 123 type 2 diabetics aged 30 and above were identified based on the population survey carried out by the Yang-Ming Crusade in Kinmen. After

excluding those who migrated or died, the remaining 858 type 2 diabetics formed a cohort who were eligible for abdominal ultrasound sonography. These 858 subjects were invited by telephone calls or invitation letters in 2001 to be screened for GSD. Informed consent was obtained from all participants before the survey[5].

Information concerning biochemical markers and diagnosis of gallstone disease

The details of the data collection have also been described in detail elsewhere[5]. In brief, baseline information including demographic and biochemical variables was collected in the period 1991-93. Face to face interviews were conducted by the Yang-Ming Crusade, a volunteer organization of well-trained medical students of National Yang-Ming University. Fasting blood samples were drawn by public health nurses. Overnight fasting serum and plasma samples (preserved with EDTA and NaF) were collected and kept frozen (-20 ℃) until analysis. FPG concentrations were determined using the hexokinase-glucose-6-phosphate dehydrogenase method with a glucose (HK) reagent ldt (Gilford, Oberlin, OH, USA). Higher systolic blood pressure (SBP) was defined as SBP $\geqslant$140 mmHg, and higher diastolic blood pressure (DBP) was defined as DBP $\geqslant$90 mmHg. Obesity was defined by a body mass index (BMI) $\geqslant$25 kg/m^2, an abnormal total cholesterol was defined as $\geqslant$5.17 mmol/L ($\geqslant$200 mg/dL), a raised triglyceride as $\geqslant$2.24 mmol/L ($\geqslant$200 mg/dL), a raised blood urea nitrogen (BUN) as $\geqslant$7.14 mmol/L ($\geqslant$20 mg/dL), a high level of uric acid was defined for males as $\geqslant$0.42 mmol/L ($\geqslant$7 mg/dL) and for females as $\geqslant$0.36 mmol/L ($\geqslant$6 mg/dL), and central obesity as a waist circumference $\geqslant$90 cm in males or $\geqslant$80 cm in females[18].
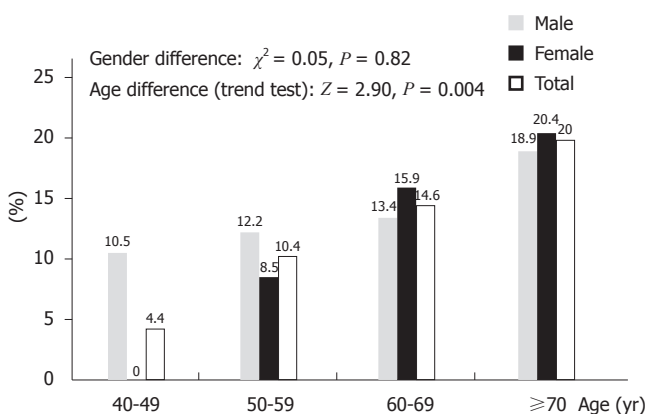
The follow-up screening regime for GSD was performed in 2001. GSD was diagnosed by a panel of specialists using real-time ultrasound sonography to examine the abdominal region, after fasting for at least 8 h, for the presence of "movable hyperechoic material with acoustic shadow". Cases of GSD were classified as follows: single gallbladder stone; multiple gallbladder stones; and cholecystectomy, excluding gallbladder polyps. Cases were identified as any type of GSD among type 2 diabetics. Furthermore, in order to ensure consistency of GSD diagnosis among the specialists, the Kappa statistic was used to assess the agreement of interobserver reliability among study specialists. A pilot study was performed with 50 randomly selected type 2 diabetics who were not study subjects. For interobserver reliability, the Kappa value for diagnosis of GSD was 0.77 (95%CI: 0.50-0.96).

Assessment of insulin resistance and β-cell dysfunction

The HOMA method was used to compare differences in the profiles for insulin resistance (HOMA IR) and for β-cell dysfunction (HOMA β-cell) in subjects with and without GSD[19]. The HOMA model assumes that the control of FPG and fasting insulin (FI) is governed by a self-contained feedback loop between the pancreas, liver, and insulin-sensitive and insulin-insensitive glucose

Table 1 Crude and age-adjusted odds ratios of baseline-associated factors of GSD among type 2 diabetics in Kinmen

	GSD (yes *vs* no)						
	Male				Female		
	Crude OR (95%CI)		Age-adjusted OR (95%CI)		Crude OR (95%CI)		Age-adjusted OR (95%CI)	
Age (≥65 *vs* <65 yr)	3.14	(1.32-7.47)[a]	-	-	2.25	(1.04-4.90)[a]	-	-
Type 2 diabetes (known *vs* new cases)	0.90	(0.34-2.39)	0.87	(0.32-2.34)	1.44	(0.68-3.02)	1.46	(0.68-3.13)
FPG (≥11.1 *vs* <11.1 mmol/L)	2.12	(0.70-6.37)	1.95	(0.64-5.94)	1.24	(0.50-3.07)	1.49	(0.59-3.79)
SBP (≥140 *vs* <140 mmHg)	1.70	(0.73-3.97)	1.50	(0.63-3.56)	2.59	(1.23-5.46)[a]	1.99	(0.92-4.31)
DBP (≥90 *vs* <90 mmHg)	1.44	(0.62-3.35)	1.57	(0.67-3.73)	1.12	(0.52-2.42)	1.21	(0.55-2.67)
Obesity (yes *vs* no)	2.04	(0.85-4.88)	2.00	(0.83-4.83)	2.97	(1.30-6.83)[a]	2.88	(1.24-6.70)[a]
Central obesity (yes *vs* no)	2.76	(1.13-6.77)[a]	2.36	(1.01-5.97)	1.85	(0.68-5.02)	1.47	(0.53-4.09)
Cholesterol (≥5.17 *vs* <5.17 mmol/L)	1.02	(0.19-1.91)	0.88	(0.18-1.09)	0.92	(0.45-1.88)	0.85	(0.41-1.77)
Triglyceride (≥2.24 *vs* <2.24 mmol/L)	1.92	(0.12-2.36)	1.61	(0.13-2.84)	1.43	(0.20-2.46)	1.35	(0.15-1.96)
BUN (≥7.14 *vs* <7.14 mmol/L)	0.44	(0.16-1.25)	0.38	(0.13-1.09)	1.65	(0.75-3.61)	1.30	(0.58-2.93)
Uric acid (high *vs* normal)	0.30	(0.09-1.06)	0.29	(0.08-1.03)	1.61	(0.77-3.38)	1.12	(0.51-2.48)
AST (≥40 *vs* <40 U/L)	1.05	(0.08-5.72)	0.60	(0.07-5.31)	3.68	(1.01-9.32)[a]	3.93	(1.04-10.89)[a]
ALT (≥40 *vs* <40 U/L)	1.09	(0.19-4.33)	1.09	(0.21-5.61)	2.00	(0.67-6.02)	2.20	(0.71-6.85)
Serum insulin (2nd *vs* 1st quartile)	0.29	(0.06-1.33)	0.30	(0.06-1.39)	1.39	(0.44-4.40)	1.68	(0.52-5.45)
(μU/mL) (3rd *vs* 1st quartile)	0.61	(0.19-2.00)	0.56	(0.17-1.86)	2.05	(0.71-5.88)	2.20	(0.75-6.45)
(4th *vs* 1st quartile)	0.61	(0.18-2.03)	0.65	(0.20-2.17)	5.38	(2.14-13.48)[a]	5.32	(2.08-13.64)[a]
Log HOMA IR (2nd *vs* 1st quartile)	0.61	(0.19-2.00)	0.60	(0.18-1.99)	1.17	(0.38-3.57)	1.65	(0.52-5.29)
(3rd *vs* 1st quartile)	0.58	(0.06-1.29)	0.49	(0.06-1.36)	2.60	(1.02-6.64)[a]	2.66	(1.02-6.94)[a]
(4th *vs* 1st quartile)	0.63	(0.19-2.08)	0.60	(0.18-2.01)	2.60	(1.01-6.66)[a]	2.59	(1.01-6.76)[a]
Log HOMA β-cell (2nd *vs* 1st quartile)	1.16	(0.35-3.80)	1.27	(0.38-4.25)	0.35	(0.08-1.55)	0.45	(0.10-2.03)
(3rd *vs* 1st quartile)	0.52	(0.11-2.43)	0.51	(0.11-2.39)	0.16	(0.02-1.23)	0.17	(0.02-1.35)
(4th *vs* 1st quartile)	0.25	(0.03-2.97)	0.22	(0.03-1.73)	0.17	(0.02-1.28)	0.16	(0.02-1.21)

[a]P<0.05.

Figure 1 Sex and age-specific prevalence of gallstone diseases among type 2 diabetics in Kinmen.

metabolizing tissues[19]. The formulas were developed and validated against the hyperinsulinemic-euglycemic clamp for insulin resistance and the hyperglycemic clamp for insulin secretion[19]. The details are as follows:

$$\text{Insulin resistance(HOMA IR)} = \frac{FI \times FPG}{22.5}$$

$$\text{β-cell function(HOMA β-cell)} = \frac{20 \cdot FI}{FPG - 3.5}$$

Because the HOMA model is not applicable to type 2 diabetics treated with insulin, eight insulin-treated subjects were excluded from the HOMA analysis. In addition, due to skewed distributions, the values of the HOMA model were subjected to log transformation in further analysis and re-transformed for tabulations.

Statistical analysis

Statistical analysis was performed using SAS version 9.0 (SAS Institute Inc., Cary, NC, USA). In the univariate analysis, crude and adjusted OR (adjustment for sex and age) were estimated and 95% confidence intervals (CI) were used. Multiple logistic regression was used to investigate whether insulin resistance and β-cell dysfunction were independently associated with GSD after adjustment for confounding factors. A P value <0.05 was considered statistically significant. In addition, HOMA IR, and HOMA β-cell scores were divided into quartiles after log transformation.

RESULTS

In our earlier study in 2001, 440 of 858 type 2 diabetic subjects (51.3%) had received real-time ultrasound sonography of the abdomen[5]. Using this cohort in the present study, and after excluding eight type 2 diabetics who had been treated with insulin, we determined the distribution of GSD among the remaining 432 participants. Figure 1 shows the sex- and age-specific prevalence of all types of GSD among type 2 diabetics. The sex-specific prevalence values [male: 13.9% (26/187); female: 14.7% (36/245)] were not significantly different (χ^2 = 0.05, P = 0.82). The prevalence of all types of GSD was significantly increased with age [Z (trend) = 2.90, P = 0.004]. Among the subjects aged 40-59 years, males had a higher prevalence of GSD than females. Conversely, among subjects aged 60 and above, females had a higher prevalence of GSD than males.

Table 1 presents the crude and age-adjusted OR for the association between certain relevant baseline-associated risk factors and GSD. Among males, baseline factors that were significantly related to GSD included age (≥65 *vs* <65 years, crude OR = 3.14, 95%CI: 1.32-7.47) and central obesity (yes *vs* no, age-adjusted OR = 2.36, 95%CI:

Table 2 Multiple logistic regression of association between baseline serum insulin, insulin resistance, β-cell dysfunction and GSD among type 2 diabetics in Kinmen

| | GSD (yes vs no) | | | | | |
| | Male | | | Female | | |
Variables	OR	(95%CI)	P for trend test	OR	(95%CI)	P for trend test
Model I						
Age ($\geqslant$65 vs <65 years)	1.04	(1.00-1.09)	-	1.05	(1.02-1.09)	-
Obesity (yes vs no)	-	-	-	2.35	(1.04-5.62)	-
Central obesity(yes vs no)	2.66	(1.01-6.99)	-	-	-	-
Serum insulin (μU/mL)						
2nd vs 1st quartile	0.39	(0.08-1.99)		1.47	(0.44-4.87)	
3rd vs 1st quartile	0.52	(0.14-1.95)	0.51	2.01	(0.68-5.96)	<0.0001
4th vs 1st quartile	0.56	(0.16-2.04)		4.46	(1.71-11.66)	
Model II						
Age ($\geqslant$65 vs <65 years)	1.04	(1.02-1.09)	-	1.06	(1.02-1.10)	-
Obesity (yes vs no)	-	-	-	2.25	(1.02-5.52)	-
Central obesity(yes vs no)	2.68	(1.03-6.97)	-	-	-	-
Log HOMA IR						
2nd vs 1st quartile	0.79	(0.22-2.78)		1.63	(0.49-5.43)	
3rd vs 1st quartile	0.53	(0.07-1.51)	0.18	1.63	(0.55-4.82)	0.02
4th vs 1st quartile	0.68	(0.20-2.36)		2.81	(1.03-7.91)	
Model III						
Age ($\geqslant$65 vs <65 years)	1.05	(1.00-1.10)	-	1.05	(1.01-1.09)	-
Obesity (yes vs no)	-	-	-	2.58	(1.09-6.13)	-
Central obesity(yes vs no)	2.82	(1.03-7.69)	-	-	-	-
Log HOMA β-cell						
2nd vs 1st quartile	0.94	(0.60-9.97)		0.45	(0.10-2.09)	
3rd vs 1st quartile	0.78	(0.15-4.09)	0.14	0.17	(0.02-1.31)	0.01
4th vs 1 quartile	0.32	(0.04-2.89)		0.16	(0.03-1.70)	

Other independent variables controlled in model included age, type of cases, SBP, DBP, cholesterol, triglyceride, BUN, uric acid, AST, and ALT.

1.01-5.97). According to univariate analysis in which the highest quartile was compared with the lowest, the female diabetics with GSD (compared to those without GSD) were older ($\geqslant$65 vs <65 years, crude OR = 2.25, 95%CI: 1.04-4.90), were more obese (yes vs no, age-adjusted OR = 2.88, 95%CI: 1.24-6.70), had higher AST values ($\geqslant$40 vs <40 U/L, age-adjusted OR = 3.93, 95%CI: 1.04-10.89), had greater serum insulin values (4th vs 1st quartile, age-adjusted OR = 5.32, 95%CI: 2.08-13.64) and had higher log HOMA IR values (4th vs 1st quartile, age-adjusted OR = 2.59, 95%CI: 1.01-6.76).

Associations between baseline serum insulin, insulin resistance, β-cell dysfunction, and GSD in each gender after adjustment for confounding factors were examined using a multiple logistic regression model. Fasting insulin was correlated with HOMA IR and HOMA β-cell dysfunction. These correlations were shown to be independent through the use of separate models. Furthermore, because the level of significance was very similar for raw (non-transformed) and log transformed data, in order to interpret the results more easily, the results for serum insulin were calculated for raw values. As shown in Table 2, older age was significantly associated with GSD in both males and females. Among males, there was a statistically significant association of GSD with central obesity, but no association of GSD with serum insulin, log HOMA IR, or log HOMA β-cell. Among females, not only obesity, but also serum insulin (P<0.0001 for trend test) and log HOMA IR (P = 0.02 for trend test) were significantly related to GSD (after adjustment

for confounding variables). The association of GSD with serum insulin (4th vs 1st quartile, OR = 4.46, 95%CI: 1.71-11.66) and log HOMA IR (4th vs 1st quartile, OR = 2.81, 95%CI: 1.03-7.91) was revealed when the highest quartile was compared with the lowest. Although an association between log HOMA β-cell dysfunction and GSD was not found in the multivariate model, the test for trend was still strongly significant (P = 0.01).

DISCUSSION

Consistent with other epidemiologic studies[3,5], our study also revealed that age is a significant risk factor for GSD among type 2 diabetics (after adjustment for confounding factors). Larger amounts of cholesterol secreted by the liver and a decrease in the catabolism of cholesterol to bile acid were observed in the elderly population[20]. Long-term exposure, such as longer duration of type 2 diabetes, also might account for the increased risk of developing GSD among the elderly population[21]. In addition, the present study also showed the interaction between sex and age to prevalence of GSD. Further epidemiological and etiological investigations are clearly needed in order to clarify the pathophysiological mechanisms of interactive effects between sex, age, and GSD among type 2 diabetics.

Obesity might raise the saturation of bile by increasing biliary secretion of cholesterol, with the latter probably depending on a higher synthesis of cholesterol in obese subjects[5,22]. Our data demonstrated a significant association between obesity and GSD among females.

As in other studies, both univariate and multivariate analyses showed significantly higher BMI among females when compared to controls[23,24]. However, among males, the present study failed to find a positive association between higher BMI and GSD, whereas central obesity was significantly associated with GSD. These results are consistent with those of other population-based studies[25,26]. It has been suggested that BMI might not be as good an indictor of obesity among males as among females[27]. Some studies have also suggested that failing to find a relationship between BMI and GSD among males reveals a tendency for a positive association with other indices of obesity, such as slimming treatment and skinfold thickness[27,28]. Another possible reason is that loss of muscle bulk might be associated with GSD among males, i.e., males with GSD had gained less weight during adult life than non-GSD males despite having more abdominal fat, suggesting that they had lost more lean body mass[26]. Because central obesity was shown to be an important predictor of type 2 diabetes and various metabolic abnormalities, further study is needed to determine the contribution to and pathophysiological mechanisms of the formation of GSD in type 2 diabetics.

The results of this community-based study provide further support for an association between hyperinsulinemia and insulin resistance with GSD among females. Female diabetics with GSD had significantly higher baseline serum insulin, higher HOMA IR and lower HOMA β-cell function in comparison to controls without GSD pathology. This relationship persisted even after controlling the most common confounding factor of the association, such as central obesity, which was related to GSD and serum insulin[3]. Males showed no significant differences from controls regarding insulin resistance or its surrogates, lending support to a proposed interaction of these factors and sex with regard to lithogenesis. Previous clinical case-control studies and epidemiologic studies also showed similar results in the general population and in the diabetic population[3,13,29]. The mechanism underlying the interaction between insulin and gallstone formation is undetermined, though it has been speculated that decreased GSD motility and increased cholesterol saturation of bile are involved[29]. Gallstone motility had been demonstrated to be impaired in diabetics, favoring cholesterol gallstone crystal formation and growth[30]. Insulin may inhibit basal and cholecystokinin-stimulated gallbladder motility[31]. Another possible reason for the lack of an association between serum insulin and GSD among males is abdominal adiposity, which probably explains the hyperinsulinemia as the association of serum insulin with the disappearance of GSD. In addition, the fact that the HOMA model was used to estimate insulin resistance could be viewed as showing an interaction between serum insulin and FPG. The higher insulin levels mean the greater insulin resistance. Insulin resistance related to GSD with response to the duration of type 2 diabetes would also increase the risk of prevalent GSD[3].

Among females, a positive relationship between serum C-peptide and GSD was obtained in previous studies[13]. Measurement of C-peptide under standardized conditions provides a sensitive, well accepted and clinically validated assessment of β-cell function[32]. The intravenous glucose tolerance test (IVGTT) is widely used to test pancreatic β-cell function. Plasma C-peptide concentration is measured and used to infer insulin secretion since it is secreted in equimolar amounts with insulin, and its extraction by the liver is negligible[33]. In the present study, HOMA β-cell dysfunction was strongly related to GSD among females but not among males, implying that insulin secretion might act differently in relation to GSD formation in males and females, even among those with type 2 diabetes.

The present study took advantage of several important and valuable methods. First, most previous studies were hospital-based, and Berkson's bias (selection bias) was inevitable. A community-based study design, such as the one we used, could diminish this kind of selection bias. Second, potential information bias was reduced because GSD screening for type 2 diabetics in 2001 was collected without the knowledge by the specialists of results of the baseline biochemical factors in 1991-1993. Third, we used ultrasound examination instead of clinical history of GSD as used in the previous study[3]. Because of this, we should have improved diagnostic accuracy and diminished underestimation and misclassification of GSD. Nevertheless, associated biochemical markers of GSD were obtained retrospectively, and the causal relationship between serum insulin, insulin resistance, β-cell dysfunction and GSD could not be clarified in our study. Baseline characteristics pertinent to the risk of type 2 diabetes for attendants were not significantly different from those for non-attendants except for age, which means that the 51.3% who participated may also be representative of those refusing to join our study (after adjustment for age)[5]. But the relatively lower response rate still might be caused by decreased statistical power. In addition, due to the complications and expense associated with the clamp technique (which prohibited its use in our community-based epidemiologic studies that had substantial sample sizes), the measurements of insulin resistance and β-cell dysfunction were based on an indirect method (the HOMA model). However, previous studies confirmed that the HOMA method and clamp-measured insulin resistance were highly correlated, implying that we could also obtain unbiased results using less sophisticated methods[34,35].

In conclusion, the present study shows that there are gender differences with regard to the relationships between serum insulin, insulin resistance, β-cell dysfunction and GSD among type 2 diabetics (after adjustment for confounding factors). These observations suggest that factors related to type 2 diabetes might need to be considered separately in male and female diabetic patients in the pathogenic mechanism of increased risk of GSD.

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• *RAPID COMMUNICATION* •

Evaluation of immunogenicity and reactogenicity of recombinant DNA hepatitis B vaccine produced in India

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Abstract

AIM: (1) To gain information on immune responses to an accelerated schedule of 0, 1, and 2 mo in paramedical staff and BDS students who are at an increased risk of getting hepatitis B infection and come under high risk groups. (2) To assess the efficacy and safety of *Enivac-HB* in different age groups, using genetically modified yeast strain *Pichia pastoris,* a new recombinant hepatitis B vaccine developed and manufactured in India.

METHODS: A prospective, comparative, and single blinded trial of rapid (0, 1, and 2 mo) hepatitis B immunization schedulewas reported. A total of three hundred and seven (212 females and 95 males) healthy volunteers divided into three age groups (18-29, 30-39, and 40-49) were enrolled after screening for markers of hepatitis B. All the volunteers received 20 mg of the vaccine intramuscularly at 0, 1, and 2 mo.

RESULTS: Geometric mean titers were calculated pre and post vaccination. Before immunization the GMT was 0.0124 mIU/mL. One month after the administration of the third dose of recombinant vaccine 296/307 (96.5%) subjects achieved seroprotective levels of anti-HBs. The geometric mean anti-HBs titers achieved after one month of the third dose was 2 560.0 mIU/mL. The geometric mean anti-HBs titer of males was 2 029.0 mIU/mL, while that of the females was 2 759.0 mIU/mL. In the age group of 18-29 years, anti-HBs titer was 3 025.0 mIU/mL, while that in the age group of 30-39 years was 2 096.0 mIU/mL. In third age group of 40-49 years, anti-HBs titer was 1 592.0 mIU/mL. Hyper-responses (anti-HBs$\geq$100 mIU/mL) were shown in 88.0% (271/307) of subjects. Eleven (3.5%) subjects responded poorly to the vaccine in the age group of 40-49 years. There was only mild pain at the site of injection otherwise there were no other adverse drug reactions (ADRs).

CONCLUSION: This vaccine (*Enivac-HB*) is safe and efficacious, providing significant protection after the third dose and rapid hepatitis B immunization schedule of 0, 1, and 2 mo can be recommended whenever rapid protection is the goal.

INTRODUCTION

Hepatitis B virus (HBV) infection is responsible for a high proportion of the worlds cases of cirrhosis, and is the cause of up to 80% of all cases of hepatocellular carcinoma (HCC)[1,2]. The situation is grim in developing countries like India, where blood bank infrastructure is non-existent outside the major metropolitan cities and safe blood handling practice standards are low[3]. The weighed prevalence of hepatitis B in India has been estimated to be 4.7%[4,5], which makes this an intermediate prevalence country.

Hepatitis B vaccination has been one of the success stories of the 20th century and has been extensively used in a wide range of groups throughout the world. Hepatitis B vaccination program have successfully reduced the prevalence of hepatitis B, in Taiwan[6] where universal HBV vaccination has led to a significant reduction of hepatitis B prevalence and incidence of hepatocellular carcinoma in children. The immunogenicity, efficiency, and safety profile of hepatitis B vaccine has been well established. More than 90% seroconversion has been achieved in adult populations consistently[7-10]. The safety profile of the recombinant hepatitis B vaccine has been very good[11].

Numerous genetically engineered hepatitis B vaccines are available in India today. However, vaccines do differ in terms of latency (time taken for the production of effective antibodies), reactogenicity and price. Newer technologies involving more advanced yeast strains like *Pichia pastoris*[12] used for manufacturing the vaccines are today revolutionizing genetically engineered hepatitis B vaccines.

The aim of the study was (1) to gain information on immune responses to an accelerated schedule of 0, 1, and 2 mo in paramedical staff and BDS students who are at an increased risk of getting hepatitis B infection and come under high risk groups. (2) To assess the efficacy and safety of *Enivac-HB* in different age groups, using genetically modified yeast strain *P pastoris,* a new recombinant hepatitis B vaccine developed and manufactured in India.

MATERIALS AND METHODS

Blood samples

The study was conducted in Maulana Azad Medical College, New Delhi for over the period of 6 mo (October 2002-April 2003). Five milliliters of blood sample was taken by venipuncture with the informed consent of all the 317 volunteers. Serum sample was separated and stored at -20 ℃ to perform various serological tests.

Clinical protocol

Permission to conduct this study on adult human volunteers was obtained from Drugs Controller of India. The Institute Ethics Committee approved the study protocol. The study population consisted of 317 volunteers. The following inclusion criteria had to be met. The subjects had to be tested negative for hepatitis B surface antigen (HBsAg), anti-hepatitis B surface antibody (anti-HBs), and anti-hepatitis B core antibody, IgM anti-HBe, IgG anti-HBe and subjects had to be at least 18 years of age and had to sign an informed consent. Subjects were excluded from the study if they had been previously vaccinated with HBV vaccine; or if they were presently taking immunosuppressive drugs; or had a history of hypersensitivity to yeast; or had to receive immunoglobulins, blood or blood products within the previous 6 mo. Subjects who were pregnant, who had significant hematological, hepatic, renal, cardiac or respiratory diseases or who had participated in any other trial 30 d before or during the present study were excluded. Physical examination such as pulse, blood pressure, temperature, and edema were checked. All the 307 healthy seronegative volunteers of the age ranging between 18 and 49 years were recruited for participating in the vaccination schedule of 0, 1, and 2 mo.

HBV vaccination

Of the 317 volunteers, 307 volunteers, who were negative for all the serological markers of hepatitis B infection, completed the hepatitis B vaccination program. Seven were positive for HBV markers and three volunteers had high anti-HBs titers. All the 307 volunteers received the three doses of HBV vaccination. The subjects were administered 1 mL (20 mg of recombinant DNA) of hepatitis B (*Enivac-HB*) vaccine intramuscularly in the deltoid muscle according to the following schedule: 1st dose-0 d, 2nd dose -30 d after the 1st dose, 3rd dose-60 d after the 1st dose. Vaccinees were monitored for adverse events closely for 3 d after each dose, i.e. fever, pain at the site of injection, erythema, and swelling, nausea, rash, fatigue, bodyache, and scored as absent, mild, moderate or severe. Samples for anti-HBs antibody titers were determined at 0 and 90 d. Anti-HBs antibodies were done using a commercially available quantitative ELISA kit (AUSAB-EIA, Abbot Labs, USA). Protection with hepatitis B vaccination was considered to be achieved when the concentration of anti-HBs antibody titers was ≥10 mIU/mL. A non-response was defined as anti-HBs antibody titers ≤10 mIU/mL[13], responders were those with titer levels ≥10 mIU/mL and ≤100 mIU/mL, high responders were those with anti-HBs titers ≥100 mIU/mL, and those with titers ≥1 000 mIU/mL was hyper-responder[13].

Vaccine

A new recombinant DNA hepatitis B vaccine (*Enivac-HB*, Panacea Biotec, India) containing hepatitis B surface antigen (s-gene) produced in genetically engineered yeast *P pastoris* cells[12] was evaluated. A derivative of the plasmid V322 carrying hepatitis B viral DNA was used to amplify the HBsAg. The s-gene isolated from hepatitis B was placed in *P pastoris* cell. The HBsAg nucleotide sequence[14] was purified to >95% by adsorption by the antigen in the cellular extract to colloidal silica, followed by its desorption, ion exchange chromatography and ultracentrifugation. The vaccine was prepared by adsorbing the purified HBsAg onto aluminum hydroxide gel (0.5 mg aluminum per dose) and thiomersal was added as a preservative to a final concentration of 0.05 mg/dL. *P pastoris* is the most advance second generation yeast strain with proven advantages over other yeast strains like *Saccharomyces cerevisiae*[12] which is used in the manufacturing process of many commercially available genetically engineered hepatitis B vaccines in India. The yeast strain *P pastoris* can grow at a highly acidic pH that becomes a barrier for growth of many contaminating microbes during the process of fermentation[12]. In short, the manufacturing process itself is a purification procedure. The yield of hepatitis B surface antigen obtained with this strain of *P pastoris* is higher and it means per liter more surface antigen is obtained with *P pastoris* than with many other strains[12]. *Enivac-HB* is manufactured using a technology that is free from toxic substances like cesium chloride[12]. Some commercially available vaccines in India are known to use technology that involves the usage of cesium chloride[12]. This advantage automatically translates into lesser contamination and hence, lesser chances of reactogenicity[12].

Statistical analysis

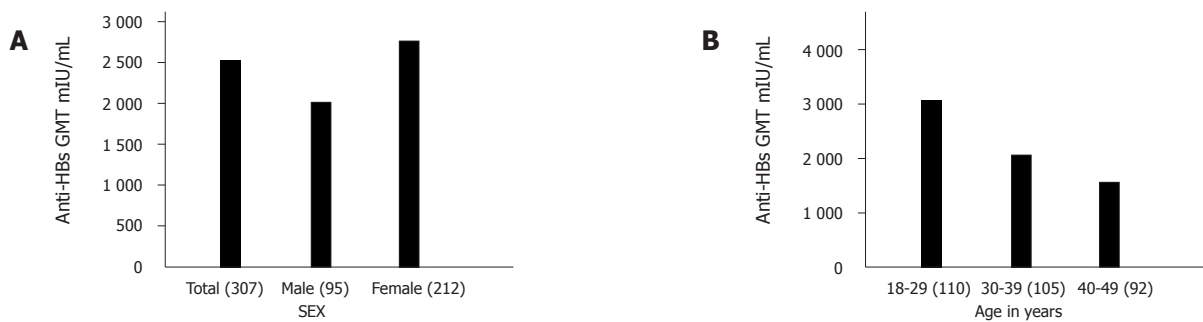Seroconversion and seroprotection were compared by

Figure 1 Efficacy of *Enivac-HB* in study participants by gender variation (**A**) and age (**B**) on the 90th d evaluation after the initial vaccination. The anti-HBs titer is expressed in milli-international unit per milliliter.

descriptive statistics. Geometric mean titers (GMTs) pre and post-vaccinations were compared by Student's *t* test. $P<0.05$ was compared as statistically significant (95%CI).

RESULTS

A total of 317 subjects were screened, 307 subjects negative for all the serological markers of hepatitis B were enrolled in the study. The mean age of the study group was 33.5±9.4 years, with 95 male subjects and 212 female subjects. Dose schedules were 0, 1, and 2 mo. Adverse events were recorded by specifically interviewing each subject during the entire duration of the study. The adverse drug reactions (ADRs) were assessed closely for 3 d after each dose and that no vaccinee had fever or any other designated systemic complaint with only mild pain at the site in 79%, 84.9%, and 75.4% of the vaccinees following doses 1, 2, and 3, respectively. Hematological and biochemical parameters were normal in all the subjects at the end of the study compared to the baseline value. None of the patients became HBsAg and/or anti HBe positive during the follow-up period. "The geometric mean anti-HBs titer" at the initiation of the vaccination program was 0.124 mIU/mL. One month after the administration of the third dose of recombinant vaccine, 296/307 (96.5%) subjects achieved seroprotective levels of anti-HBs antibodies. "The geometric mean anti-HBs titer" achieved after one month of the third dose was 2 560.0 mIU/mL geometric mean titers. "The geometric mean anti-HBs titer" of males was 2 029.0 mIU/mL, while that of females was 2 759.0 mIU/mL as shown in Figure 1A. In the age group of 18-29 years, anti-HBs titer was 3 025.0 mIU/mL, while that of 30-39 years was 2 096.0 mIU/mL. In 40-49 years of age, anti-HBs titer was 1 592.0 as shown in Figure 1B. The above value is statistically significant with $P<0.05$, and had 95%CI. Hyper-responses (anti-HBs ≥100 mIU/mL) was shown by 88.0% (271/307; $P<0.05$) of subjects. Twenty subjects all in the age group of 40-49 were (11/307; 3.5%) non-responders, with the geometric mean anti-HBs titers 8.60 mIU/mL. The mean age of the 11 non-responders was 46.4±6.9 years, 1.3% (4) males and 2.2% (7) females were non-responders but the difference was not statistically significant.

DISCUSSION

Accelerating the vaccination schedule against hepatitis B is appealing because it may increase patient compliance and provide earlier protection for the people who are already in a high risk group or environment. A comparative Indian study of HBV vaccine in three age groups, 18-29, 30-39, and 40-49 years, was done. The experimental data obtained during the course of the trial indicated that seroprotection one month[15] after the third dose was (96.5%) with mean geometric anti-HBs titers 2 560.0 mIU/mL. Twenty subjects all in the age group of 40-49 years (11/307; 3.5%) showed low response to the vaccine and demonstrated an antibody titer of 8.60 mIU/mL. While all the subjects in rest of the age group achieved 100% seroconversion. Risk factors that have been associated with non-response to hepatitis B vaccine include increasing age, male gender, obesity, history of smoking, administration of vaccine in buttock rather than deltoid[3]. The relationship of hepatitis B vaccination response with age is controversial. Our study suggests that seroconversion in age group >40 years is 79% which is considered high compared to most other studies[16,17] where seroconversion rate of 60% has been reported. However, as all the above studies, we too found a decreasing seroconversion rate with increasing age. These findings favor the hypothesis that increasing age decreases seroprotective antibody formation after vaccination.

In the present clinical study, it was observed that female volunteers showed a better response in comparison to male volunteers ($P<0.001$). Jilg *et al*[18] reported a slightly lower response in males and Dentico *et al*[19] also reported that the sex factor is one of the parameters influencing the response following vaccination. Whether or not there exists a sex related variation of the immunogenic response is still controversial.

The vaccination schedule (0, 1, and 2 mo) employed in the present study has been well studied in the other trials[15-19]. Marsano *et al*[20] have established that the 0, 1, and 2 mo schedule of vaccination gives a rate that is quicker than and identical to the rate of seroprotection of the standard schedule of vaccination of 0, 1, and 6 mo and is much quicker. The recombinant yeast-derived vaccine evaluated did not produce any severe adverse reactions. There was only mild pain at the site of injection otherwise

it was completely safe with no adverse drug reactions.

From the present data, we can confirm that the *Enivac-HB* is highly immunogenic. This new indigenously manufactured vaccine using *P pastoris* as yeast strain is safe and provides effective titers against hepatitis B. In a country with an estimated 40 million or more carriers of the hepatitis B virus and an estimated 18 million newborns each year, the availability of an indigenously manufactured vaccine would probably make it easier to include the vaccine in a community-based program.

The study conclusively proves that the recombinant DNA hepatitis B vaccine (*Enivac-HB*) produced using genetically engineered yeast cell *P pastoris* appears to be highly immunogenic and safe and confers a seroprotection of 96.5% of the subjects with 88.0% showing hyper-response. The study suggests that the vaccine appears to be well tolerated and the rapid vaccination schedule of 0, 1, and 2 mo can be recommended whenever rapid protection is the goal.

ACKNOWLEDGMENTS

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• *RAPID COMMUNICATION*•

Bone disorders in experimentally induced liver disease in growing rats

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the data of other studies using different animal models. A novel finding is the transiently accelerated skeletal growth and bone strength after a 8-wk long phenobarbital treatment following diethylnitrosamine injection.

Key words: Bone disorder; Bone mineral content; Mechanical resistance; Experimental liver cirrhosis; Growing rat

Abstract

AIM: To investigate the change of bone parameters in a new model of experimentally induced liver cirrhosis and hepatocellular carcinoma (HCC) in growing rats.

METHODS: Fischer-344 rats (n = 55) were used. Carbon tetrachloride (CCl_4), phenobarbital (PB), and a single diethylnitrosamine (DEN) injection were used. Animals were killed at wk 8 and 16. Bone mineral content, femoral length, cortical index (quotient of cortical thickness and whole diameter) and ultimate bending load (F_{max}) of the femora were determined. The results in animals treated with DEN+PB+CCl_4 (DPC, n = 21) were compared to those in untreated animals (UNT, n = 14) and in control group treated only with DEN+PB (DP, n = 20).

RESULTS: Fatty liver and cirrhosis developed in each DPC-treated rat at wk 8 and HCC was presented at wk 16. No skeletal changes were found in this group at wk 8, but each parameter was lower ($P<0.05$ for each) at wk 16 in comparison to the control group. Neither fatty liver nor cirrhosis was observed in DP-treated animals at any time point. Femoral length and F_{max} values were higher ($P<0.05$ for both) in DP-treated animals at wk 8 compared to the UNT controls. However, no difference was found at wk 16.

CONCLUSION: Experimental liver cirrhosis and HCC are accompanied with inhibited skeletal growth, reduced bone mass, and decreased mechanical resistance in growing rats. Our results are in concordance with

INTRODUCTION

Metabolic bone disease is a common complication of chronic liver diseases[1-4]. However, the pathogenesis is not fully understood and is scarcely investigated in an animal model. Both increased bone resorption[5-7] and decreased bone formation[8-11] have been described in chronic liver diseases. Decreased bone density[10] and impaired microarchitecture[12] are associated with increased fracture risk[13]. Though several methods are available to examine different aspects of bone metabolism, there is not a single test to define the quality of bones. We have investigated how the bone mineral content (BMC), cortical thickness, bone length, and mechanical loading capacity of femora were changed during the development of experimentally induced fatty liver, liver cirrhosis, and HCC in growing rats. No published data are available on simultaneous measurements of these parameters in growing rats. We used a new cirrhosis model in this study. Our results are in concordance with those of other studies. Novel finding is the transiently accelerated skeletal growth and bone strength after a 8-wk long phenobarbital treatment following diethylnitrosamine injection.

MATERIALS AND METHODS

Animals and induction of liver diseases

A total of 60 male Fisher-344 rats, weighing 273.3±38.7 g, were included and 55 rats survived the study protocol.

Table 1 Serum parameters and body weight in UNT, DP-treated control and DPC-treated rats at wk 8 and 16 (mean±SD)

Parameter	UNT control (wk 8) $n = 8$	UNT control (wk 16) $n = 6$	DP control (wk 8) $n = 10$	DP control (wk 16) $n = 10$	DPC (wk 8) $n = 10$	DPC (wk 16) $n = 11$
Bilirubin (mmol/L)	1.8±1.2	2.1±1.3	1.85±0.9	0.88±0.3	48.14±66[a]	1.31±0.5
ALP (U/L)	735.7±89.3	619.5±96.3	493.9±52.3	402.8±80.2	1 354±1 108[a]	437.8±116.2
AST (U/L)	112.7±13.3	158.8±40.9	157.3±52.9	127.5±43.3	7 095±5 483[a]	115.8±15
ALT (U/L)	69.42±8.9	107.8±12.1	158.5±78.4	93.1±71.1	5 175±3 675[a]	81.54±17.9
Albumin (g/L)	32.85±0.4	34.66±1	33.6±2.2	37.4±1.2	27.8±3[a]	35.36±1.6
Body weight (g)	240.1±14.3	321.8±48.9	269.4±12.1	307.7±11.3	227.4±16.4	284.2±25.2

[a]$P<0.05$ vs for comparison between DPC and both UNT and DP controls at wk 8.

The animals were housed in steel cages (5 rats/cage) in an air-conditioned room with a relative humidity of 55%. Tap water and pelleted rodent chow (CRLT/N, Farmer Product Ltd, Hungary) were available *ad libitum*. The Animal Care and Use Committee of Semmelweis University, Faculty of Medicine approved all the animal procedures.

Fatty liver and liver cirrhosis were induced by the administration of CCl_4 (0.5 mL/kg body weight, dissolved in sunflower oil, administered thrice a week by intragastric tube) and phenobarbital (PB, phenobarbital-Na; Sigma, St. Louis, MO, USA, tap water was replaced by 0.05% of PB-solution after a 12-h fasting, daily) throughout the 8-wk treatment following a single injection of diethylnitrosamine (DEN; Serva, Heidelberg, at a dose of 200 mg/kg body weight, dissolved in 0.9% NaCl solution). HCC developed at wk 16 as described earlier[14]. Animals were killed at wk 8 and 16 under light ether-anesthesia (all animals were fasted for 24 h prior to killing).

Results in animals treated with DEN+PB+CCl_4 (group DPC, $n = 21$) were compared to those in untreated animals (group UNT, $n = 14$) and in control group treated only with DEN+PB (group DP, $n = 20$).

Biochemical tests

Serum bilirubin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) as well as albumin were measured using the Olympus AU600 autoanalyzer (Olympus Ltd, Japan).

Pathological investigation of liver

Livers were macroscopically and histologically investigated. Slices from the removed liver were fixed in 8% buffered formalin and embedded in paraplast. Picrosirius red, H&E, and PAS staining were used for histological examination[14]. Hepatic fibrosis, cirrhosis[15], and lesions[16] were classified according to the international recommendations.

Bone investigation

Partial carcass preparation was performed. The left rear limb was carefully dissected free from the iliac acetabulum with the head of the femur preserved. Then, the isolated femur was prepared by dissecting from the tibia along with the remaining muscles and ligaments and stored in saline-soaked gauze at -20 ℃ until further testing.

Radiological studies

Soft radiographs were performed using an X-ray imaging system (Siemens Mammomat 3000, Germany) with exposure at 25 kVp, 10 mAs on the femur. Femora were carefully positioned anteroposteriorly. Exposure period was 0.3 s. Exposed plates (Agfa, Germany) were analyzed using OSIRIS imaging software. Femoral length (mm) was measured and the values were validated by an aluminum phantom (1 mm in diameter). Each exposure included both the phantoms and the investigated bone. Cortical index was calculated: cortical thickness divided by the whole diameter at the mid-point of medial diaphysis.

Bone densitometry

Using an I-125 source (1 000 MBq initial activity from Nordion), a water-bath system (single photon absorption, SPA) device (NK364/A, Gamma Technical Corporation, Budapest, Hungary) was used to determine the BMC in mg/cm. A precise, replaceable and narrow collimator was made for the source holder for the *in vitro* animal experiments. Aluminum phantoms (Gamma Technical Corporation, Budapest, Hungary) were used for the calibration. The femora were fixed by sellotape on a water equivalent plastic sheet and then placed on the attenuation block. The attenuation block was 2 cm wide. BMC value was measured at the mid-point of the medial diaphysis.

Biomechanical testing

Biomechanical tests were carried out by a computer-controlled testing device (Zwick-020, Zwick, Ulm, Germany) using the whole bone specimens. The anteroposterior three-point bending test of the femoral shaft was performed as previously described[17]. The femur was placed on a testing bearer, and the distance between the supporting rods was fixed at 15.7 mm. Force was elicited by a rod at a constant deformation rate of 2 mm/min at a right angle to the anterior midpoint of the femoral midshaft. We determined the ultimate bending load of the whole bone specimen (F_{max}, N). The F_{max} was corrected by BMC (load/density, N/mg/cm) and cortical index (described above, load/cortical index, N/mm/mm) of femoral midshafts avoiding the impact of BMC and cortical index on bone strength.

Statistical analysis

Mann-Whitney test was used for intergroup comparisons.

Table 2 Femur length, cortical index, BMC, and ultimate bending load of femoral mid-diaphysis and corrected loading data for cortical index and BMC in UNT, DP-treated control and DPC-treated rats at wk 8 and 16 (mean±SD)

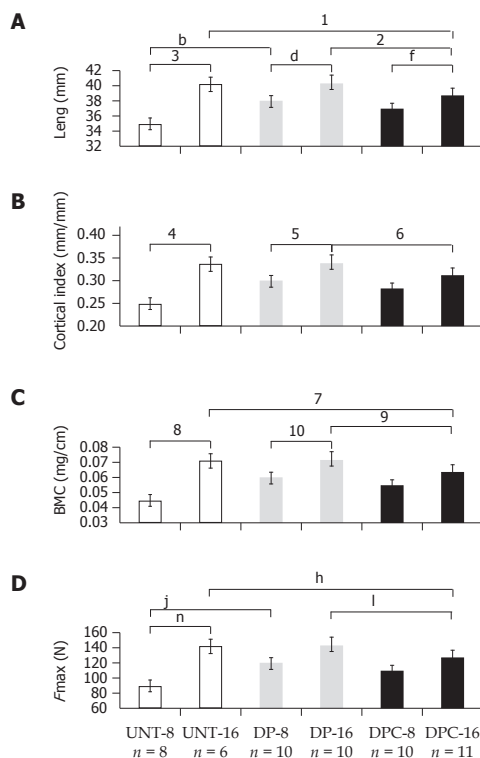| Parameter	UNT control (wk 8) $n = 8$	UNT control (wk 16) $n = 6$	DP control (wk 8) $n = 10$	DP control (wk 16) $n = 10$	DPC (wk 8) $n = 10$	DPC (wk 16) $n = 11$
Femur length(mm)	34.8±1.0	40.1±1.2	37.9±0.6	40.2±0.8	36.8±0.8	38.6±0.9
Cortical index (mm/mm)	0.277±0.026	0.325±0.029	0.287±0.015	0.33±0.018	0.285±0.021	0.286±0.036
BMC (mg/cm)	0.049±0.005	0.06±0.008	0.056±0.003	0.057±0.002	0.047±0.006	0.049±0.004
Femur F_{max}(N)	88.3±14.3	123.2±13.0	111.8±7.9	118.5±6.02	93.8±5.41	96.5±12.6
F_{max}/Cortical index (N/mm/mm)	328.7±53.4	382.6±62.5	390.4±32.4	359.8±24.25	331.1±35.4	339.9±46.5
F_{max}/BMC (N/mg/cm)	1 796±239	2 049±95.2	2 000±124	2 075±114.6	2 019±195	1 961±115.9

Figure 1 Mean±SD of femur length (**A**), cortical index of femoral midshaft (**B**), BMC of femur in mid-point of medial diaphysis (**C**), and ultimate bending load of femoral midshaft (**D**) in UNT, DP-treated control and DPC-treated rats at wk 8 and 16. A: [1]$P = 0.020$, [2]$P = 0.002$ *vs* DPC-16, [3]$P = 0.001$ *vs* UNT-8, [b]$P<0.001$ *vs* UNT-8, [d]$P<0.001$ *vs* DP-8, [f]$P<0.001$ *vs* DPC-8; B: [4]$P = 0.018$ *vs* UNT-8, [5]$P = 0.013$ *vs* DP-8, [6]$P = 0.004$ *vs* DPC-16; C:[7]$P = 0.001$ *vs* DPC-16, [8]$P = 0.002$ *vs* UNT-8, [9]$P = 0.016$ *vs* DPC-16, [10]$P = 0.003$ *vs* DP-8; D: [h]$P<0.001$ *vs* DPC-16, [j]$P<0.001$ *vs* UNT-8, [l]$P<0.001$ *vs* DPC-16, [n]$P<0.001$ *vs* UNT-8.

Pearson's parametric correlation test was applied for measuring the strength of a relationship between the two variables. $P<0.05$ was considered statistically significant. All statistical analyses were performed using SPSS version 10.0 for Windows (Chicago, IL, USA). Results were expressed as mean±SD.

RESULTS

Biochemical results, body weight, and liver histology
Serum parameters and body weight of UNT, DP- and DPC-treated rats are shown in Table 1. DPC-treated rats had elevated bilirubin, ALP, AST, and ALT ($P<0.001$

for each parameter) compared to UNT and DP-treated rats at wk 8. Serum albumin was decreased in DPC-treated animals at wk 8 compared to the other two groups ($P<0.001$). There were no differences in the laboratory values between the study groups at wk 16. No differences were found between DP-treated and UNT groups neither at wk 8 nor at wk 16.

Increased body weight was observed in UNT and DP-treated controls and DPC-treated rats from wk 8 to 16 ($P<0.001$). However, weight was reduced in DPC-treated in comparison to DP-treated group at wk 8 ($P<0.001$) and 16 ($P = 0.016$). DP-treated group had significantly higher body weight at wk 8 than UNT controls ($P<0.001$).

Liver histology showed inflammatory cell infiltrates, necrosis, fatty changes, regenerating nodules and cirrhosis in DPC-treated rats at wk 8 (10 out of 10 rats). Neoplastic nodules were found in five samples. Cirrhosis and HCC (11 out of 11) developed in DPC-treated group at wk 16. Normal hepatic histology was found in all UNT and DP-treated control rats.

Bone length, cortical index, bone mineral content and ultimate bending load of the femora
The femoral length increased in each group from wk 8 to 16 (Table 2, Figure 1A). However, animals treated with DPC had shorter femora than controls at wk 16. Femora were longer in DP-treated rats than in UNT controls at wk 8. Femoral length correlated with body weight ($r = 0.824$, $P<0.001$).

Significant increases of cortical index were seen in both UNT and DP-treated control rats (Table 2, Figure 1B). At wk 16, cortical index was smaller in DPC-treated animals than in DP-treated rats. Poor but significant correlation was measured between cortical index and BMC ($r = 0.362$, $P = 0.008$).

BMC increased only in UNT control rats from wk 8 to 16, indicating the physiological bone mineralization (Table 2, Figure 1C). DPC-treated rats had lower BMC than DP-treated rats at wk 8. BMC was lower in DPC-treated animals than in UNT and DP-treated control rats at wk 16. Body weight strongly correlated with BMC ($r = 0.748$, $P<0.001$).

Ultimate bending load increased from wk 8 to 16 in UNT controls (Table 2, Figure 1D). F_{max} in DP-treated rats was greater than that in UNT rats at wk 8. At wk 16,

F_{max} was smaller in DPC-treated rats than in both UNT and DP-treated controls. However, when we corrected the ultimate bending load by BMC or cortical index, no differences were found between the groups. Strong correlation was found between BMC and ultimate bending load ($r = 0.842$, $P<0.001$). Relationship was also found between F_{max} and cortical index ($r = 0.548$, $P<0.001$). F_{max} correlated with body weight ($r = 0.758$, $P<0.001$) and femoral length ($r = 0.751$, $P<0.001$).

DISCUSSION

Using a new model, we have found that bone disorders due to diethylnitrosamine, phenobarbital, and CCl$_4$ administration were characterized by decreased femoral length, reduced bone mass, and increased fragility after 16-wk long treatment in growing rats. Our findings on cortical bone mass are in concordance with other studies using different models of experimentally induced liver cirrhosis[18-21]. van der Merwe et al[22] found that the femoral length, the bone mass and the midshaft thickness were lower in CCl$_4$-treated rats after portosystemic shunting. Muguerza et al[23] found that the femoral density, the anteroposterior and lateromedial diameters and the ultimate bending load decreased in CCl$_4$-induced cirrhotic rats, but no significant differences were observed in the bone length. In our experiment, the age-dependent growth of femoral length, BMC and ultimate bending load were arrested as cirrhosis progressed and HCC developed. Tanaka et al[24] also found that longitudinal bone growth is reduced on the femora of rats with CCl$_4$-induced liver cirrhosis. Decreased bone mineral density has been observed in growing rats with liver cirrhosis induced by the administration of CCl$_4$ and phenobarbital[20].

In our study, DP-treated rats had longer femora and increased ultimate bending load than untreated control rats at wk 8, nevertheless BMC and cortical index did not differ from untreated controls. The stimulating skeletal effect of DP treatment at wk 8 might be caused by the enzyme inductor effect of phenobarbital. It is well known that phenobarbital induces not only xenobiotic-metabolizing cytochrome P450 (e.g. CYP2B1)[25] but also estrogen and vitamin D$_3$ synthesizer cytochrome P450 enzymes (CYP19A1, CYP27A1 CYP27B1, CYP3A4, CYP2D)[26-28]. Therefore, the increased longitudinal bone growth and ultimate bending load in DP-treated rats may be the consequence of enhanced estrogen and vitamin D activation. However, the increase in ultimate bending load was stunted after 8 wk in DP-treated rats. It is possible that bone maturation has become adapted to the accelerated skeletal growth and mineralization after 8 wk. In DPC-treated rats, only a tendency towards both longitudinal bone growth and ultimate bending load was observed in comparison to untreated controls at wk 8, suggesting that the adverse skeletal effect of CCl$_4$ does not allow to reveal stimulating effect of phenobarbital.

The biochemical parameters of liver function showed serious inflammation in DPC-treated rats at wk 8 corresponding to the histology showing fatty liver and inflammatory cell accumulation beyond the regenerating nodules. The decrease in serum albumin level at wk 8 in DPC-treated rats indicates the reduced synthetic capacity of the liver. Though biochemical and histological symptoms of steatohepatitis were present, it had no detectable negative effect on bone mass, bone length, cortical index and ultimate bending load at this time point, which is in concordance with results of van der Merwe et al[22] who did not find detectable bone disease in early stage liver dysfunction. Similar to human liver diseases, longer time is needed for the development of metabolic bone disease. Biochemical and histological signs of inflammation ceased in DPC-treated rats at wk 16, while fibrosis, cirrhosis with regenerative nodules and HCC foci dominated the histological picture. However, femoral length, BMC and ultimate bending load were stunted at this time point, which is in concordance with results of Figueiredo et al[29]. The mechanism is complex. The high level of hyperbilirubinemia detected at wk 8 could be one of the factors, since bilirubin has a direct inhibitory effect on osteoblast proliferation[30].

Bone fractures depend not only on BMC but also on bone quality, size and shape[31-33]. The decrease of F_{max} at wk 16 clearly showed the altered bone metabolism. Since correlation was found between F_{max} and cortical index, femoral length and BMC and there was no difference in F_{max}/cortical index and F_{max}/BMC ratios between the different groups, the increased fragility should be the consequence of stunted longitudinal and cortical growth and arrested mineralization in growing rats with liver cirrhosis and HCC.

The mechanism by which liver cirrhosis arrests skeletal growth and influences other features of bone metabolism is not fully understood. Whether the bone alteration is caused by a direct toxic effect of the applied agents on bones or it is a secondary consequence of the experimentally induced liver cirrhosis remains unknown.

In conclusion, experimentally induced liver cirrhosis and HCC are accompanied with inhibited growth, reduced BMC and cortical index and decreased mechanical resistance in growing rats. Our results are in concordance with the data of other studies using different animal models. A novel finding is the transiently accelerated skeletal growth and bone strength after a 8-wk long phenobarbital treatment following diethylnitrosamine injection.

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• *RAPID COMMUNICATION* •

Retinol-binding protein, acute phase reactants and *Helicobacter pylori* infection in patients with gastric adenocarcinoma

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Abstract

AIM: To determine the serum levels of c-reactive protein (CRP), transferrin (TRF), a2-macroglobulin (A2M), ceruloplasmin (CER), a1-acid glycoprotein (AAG), pre-albumin (P-ALB) and retinol-binding protein (RBP) in gastric carcinoma patients and to explore their possible correlation with underlying *Helicobacter pylori* (*H pylori*) infection.

METHODS: We measured the serum levels of CRP, TRF, A2M, CER, AAG, P-ALB, and RBP in 153 preoperative patients (93 males; mean age: 63.1 ± 11.3 years) with non-cardia gastric adenocarcinoma and 19 healthy subjects.

RESULTS: The levels of CRP, CER, RBP, and AAG in cancer patients were significantly higher than those in healthy controls ($P < 0.0001$), while no difference was found regarding the TRF, P-ALB, and A2M levels. Cancer patients with *H pylori* infection had significantly lower RBP values compared to non-infected ones ($P < 0.0001$) and also higher values of CRP and AAG ($P = 0.09$ and $P = 0.08$, respectively).

CONCLUSION: High serum levels of CRP, CER and AAG in cancer patients do not seem to be related to *H pylori* infection. Retinol-binding protein seems to discriminate between infected and non-infected patients with gastric carcinoma. Further studies are needed to explore if it is directly involved in the pathogenesis of the disease or is merely an epiphenomenon.

INTRODUCTION

Though the incidence of gastric adenocarcinoma has decreased during the last 50 years, it still remains one of the most common cancers worldwide. Even though the exact molecular mechanisms leading to gastric carcinogenesis are incompletely understood, the current school of thought accepts a multifactorial model in which various dietary and non-dietary factors (i.e. *Helicobacter pylori* infection) operate at different steps in the process.

Epidemiological studies in the early 1990s demonstrated an up to sixfold increased risk of developing gastric adenocarcinoma in patients infected with *H pylori*[1-4]. More than 10 years ago, *H pylori* was classified as a type I carcinogen for human beings by the World Health Organization, being recognized in this way as a crucial player in the gastric carcinogenesis. Increased epithelial cell proliferation and oxidative damage of the gastric mucosa are the two main mechanisms that seem to operate as a result of *H pylori* infection[5].

Table 1 Characteristics of studied populations and demographic data

Group	Number of subjects	Sex (M/F)	Mean age(yr)	Tumor location (antrum/body)
Gastric cancer	153	93/60	63.1	125/28
Infected	82	46/36	64.2	64/18
Non-infected	71	42/29	62.4	56/15
Healthy controls	19	10/9	62.2	

M, male; F, female.

The other environmental factor that has been shown to play a significant role in gastric carcinogenesis is diet. Diets with low intake of fruits, vegetables and milk and high intake of smoked or salted foods, dried fish, cooking oil, and complex carbohydrates have been shown to increase the risk for gastric cancer[6,7]. Contrary to that, intake of antioxidants has been associated with decreased risk of gastric cancer[8].

The search for useful biomarkers that can help in the diagnosis of cancer and add prognostic information to that already provided by the tumor staging is a field of active research. Even though numerous studies have been published, no single substance has been found to be clinically useful in the management of patients with gastric carcinoma. Immunohistochemical analysis of gastric tumors has shown that they contain protease inhibitors such as a2-macroglobulin[9,10] or a1-acid glycoprotein[11-13] and suggests that they may serve as markers of tumor aggressiveness. Some data also suggest that the acute phase reactants CRP and AAG may have prognostic value[13,14]. Stored sera from patients that later develop gastric cancer contain lower values of ferritin, while transferrin values were not different[15]. Ceruloplasmin has been shown to be useful in some studies[16] but not in others[15-17]. Serum interleukin-6 (IL-6) level correlates with the disease status of gastric cancer, suggesting that it may be used as a new tumor marker for monitoring the response to treatment[18]. Other studies even suggested that IL-6 might have a direct role in the pathogenesis[19,20]. A recent study[21] confirmed that IL-6 is a useful parameter for the diagnosis and grading of gastric cancer, but also suggested a role for malonyldialdehyde (MDA), nitric oxide (NO) and, especially vascular endothelial growth factor (VEGF). Finally, inconclusive data surround the usefulness of the so-called negative acute phase reactants, pre-albumin, and retinol-binding protein, in the assessment of cancer patients[22-26]. Retinol-binding protein was also included in our analysis because retinol and its ligand has been associated with lower risk of gastric cancer in some studies[27] but not in others[28].

The aim of this prospective, observational study was to measure the serum levels of the acute phase reactants a1-acid glycoprotein, transferrin, a2-macroglobulin, ceruloplasmin, retinol-binding protein, pre-albumin, and c-reactive protein in patients with gastric adenocarcinoma and to explore their correlation with *H pylori* infection.

MATERIALS AND METHODS

Patients
One hundred and fifty-three patients (93 men, 60 women) with a mean age of 63.1 ± 11.3 years and histologically confirmed non-cardia gastric cancer were included in the study. In each case we recorded the location of the tumor, the histological type and the lymph node involvement. One hundred and twenty-five malignant tumors were located in the antrum and 28 in the body of the stomach. None of the patients had any gross metastatic disease as determined by chest and abdomen CT scans and received chemotherapy or radiation therapy prior to surgery. The histological diagnosis was based on morphologic examination of hematoxylin/eosin-stained specimens. Table 1 summarizes the demographic data and characteristics of the studied populations. Nineteen healthy volunteers (10 men, 9 women) with a mean age of 62.2 ± 13.1 years were used as controls. We defined "healthy" status as the absence of a cardiovascular disorder, malignancy, gastrointestinal pathology, and *H pylori* infection. Neither patients nor controls had any evidence of infection or received antibiotics for at least 2 mo prior to serum collection with only the use of antacids. Sera from 48 patients were prospectively collected, the rest of the sera were collected in the previous 17 mo and kept frozen at -70 ℃.

The study was approved by the Institutional Review Boards of the participating hospitals and all study individuals gave their informed consent.

Determination of H pylori serology
All enrolled subjects (cancer patients and controls) underwent an enzyme-linked immunosorbent assay (ELISA) IgG serologic test for *H pylori* (Allergy Immunotechnologies Inc., Newport Beach, CA, USA) in accordance with the manufacturer's instructions. The specificity and sensitivity of the serology test validated in our local population were 95% and 90%, respectively. *H pylori* antibody titers higher than 155 mU/L were considered positive and lower than 155 mU/L negative.

Determination of acute phase proteins
Concentrations of the specific acute-phase proteins (c-reactive protein, a1-acid glycoprotein, ceruloplasmin, transferrin, a2-macroglobulin, prealbumin, and retinol-binding protein) were measured by nephelometric method on a Dade Behring nephelometer BNII (Dade Behring, USA), using Dade Behring antibodies and standard reagents. The intra- and inter-assay coefficients of variation were in the range of 2% and 5%, respectively.

Examined parameters
Serum levels of the acute phase reactants were recorded from healthy controls and patients suffering from gastric cancer. These patients were grouped according to whether they were infected with *H pylori* or not. Normal values for the examined parameters were as follows: c-reactive protein (CRP) <50 mg/L, A1-acid glycoprotein (AAG) <1

Table 2 Mean levels of TRF, A2M, AAG, CER, RBP, P-ALB, and CRP in cancer patients and controls

Parameter (mg/dL)	Cancer patients (n = 153)	Controls (n = 19)	P
TRF	265,43	274,95	NS
A2M	263,25	233,11	NS
AAG	245,48	94,79b	<0.001
CER	53,26	41,74b	<0.001
RBP	23,78	3,77b	<0.001
P-ALB	23,04	17,74	NS
CRP	9,32	2,92b	<0.001

[b]P<0.001 *vs* controls, NS: not significant.

Table 3 Mean levels of TRF, A2M, AAG, CER, RBP, P-ALB, and CRP between infected and non-infected cancer patients

Parameter (mg/dL)	Non-infected (n = 71)	Infected (n = 82)	P
TRF	276.46	255.88	NS
A2M	281.49	247.46	NS
AAG	238.21	251.77	NS
CER	52.03	54.32	NS
RBP	31.89	16.75[b]	<0.001
P-ALB	22.35	23.64	NS
CRP	7.99	10.47	NS

[b]P<0.001 *vs* infected, NS: not significant.

200 mg/L, ceruloplasmin (CER) <550 mg/L, transferrin (TRF) <4 300 mg/L, A2-macroglobulin (A2M) <3 200 mg/L, pre-albumin (P-ALB) <450 mg/L and retinol-binding protein (RBP) <60 mg/L.

Statistical analysis

The nonparametric Mann-Whitney *U*-test was employed to study the differences in values between cancer patients and controls and to compare the significance of values' differences between infected and non-infected patients. P<0.05 was considered statistically significant. All analyses were completed using the Statistical Package for Social Sciences (SPSS 11.5).

RESULTS

The assessed parameters found between cancer patients and healthy controls and between infection and non-infection groups of cancer patients are shown in Tables 2 and 3, respectively. Out of a total of 153 cancer patients participating in the study, 82 (53.6%) were infected with *H pylori*. The levels of CRP, CER, RBP, and AAG in cancer patients were significantly higher than those in healthy controls (P<0.0001), while no difference was found regarding the TRF, P-ALB, and A2M levels. Cancer patients with *H pylori* infection had significantly lower RBP (P<0.0001) and higher CRP and AAG (P = 0.09 and P = 0.08, respectively) than those without *H pylori* infection.

DISCUSSION

In the current study, we have measured the serum levels of a group of acute phase reactants in patients with adenocarcinoma localized in the non-cardia part of the stomach. We separated these patients to either antibody-positive or antibody-negative to *H pylori*. From the examined acute phase reactants, we found that the levels of c-reactive protein, ceruloplasmin, a1-acid glycoprotein and retinol-binding protein in cancer patients were significantly higher than those in healthy controls, while no difference was found regarding the transferrin, prealbumin and a2-macroglobulin levels. However, only retinol-binding protein was significantly related to *H pylori* infection as its values were significantly lower in infected patients than

in non-infected ones. The etiology of these biochemical aberrations is probably multifactorial.

Numerous studies have shown that gastric cancer patients have significantly higher levels of CRP[14,21] than healthy controls. A previous study[13] has even showed that elevated CRP levels have a prognostic significance and a recent study suggested that CRP levels contribute to the diagnosis of infection in cancer patients[21]. Our data confirm that CRP is elevated in cancer patients, but we cannot comment on its usefulness in the diagnosis of infection in cancer patients, as this was not the aim of our study. Though it was not different between infected and non-infected patients with *H pylori* (P = 0.09), one possible explanation is that chronic *H pylori* infection did not cause the elevation of CRP with acute infections as pneumonia or bacteremia. The other explanation is that our study did not have the statistical power to detect any difference.

Immunohistochemical analysis of the tumor epithelium showed that the protease inhibitor a2-macroglobulin is related to the invasive growth of gastric cancers[9,10]. Similar preliminary data exist for a1-acid glycoprotein[11-13]. Our data do not support routine measurement of A2M, but confirmed that AAG is a potentially useful marker as it was consistently higher in cancer patients and showed a trend to reach statistical significance in *H pylori*-infected patients (P = 0.08).

The role of iron-carrying protein transferrin is undetermined. Studies showed that there is no difference in the mean TRF values between controls and patients while ferritin values are significantly different[15,29], suggesting that TRF may not be a prognostic factor for future gastric carcinogenesis but its role remains uncertain. In accordance with previous studies, our data did not require measuring transferrin in gastric cancer patients whether they were infected with *H pylori* or not.

Finally, we decided to include the so-called nutritional indices (prealbumin and retinol-binding protein) in our analysis to explore their relation to cancer or *H pylori* infection. In patients with colon cancer, the levels of serum prealbumin, retinol-binding protein, transferrin, and albumin were interrelated and tended to show a similar pattern of change. More specifically, in metastatic colon cancer, prealbumin was the most sensitive indicator of nutritional status and its levels and rates of change

had a prognostic significance. A rapid fall of prealbumin often occurs 2-3 mo prior to death of the patients and this preterminal phase is also frequently heralded by a progressive rise in the CRP level in the absence of any obvious infection[30]. Prealbumin concentration has a prognostic importance in women with epithelial ovarian carcinoma[24] and a general cancer population as well[23]. We cannot confirm the data on the usefulness of prealbumin testing because we did not perform any formal assessment of nutritional status in our study population.

Serum ceruloplasmin levels are higher in gastric and lung cancer[16] and our data have confirmed this finding. We did not find any correlation of *H pylori* infection with ceruloplasmin levels and cannot suggest a pathogenetic role for this copper-chelating protein apart from being a marker of systemic inflammation.

The most interesting findings of our study are the significantly higher levels of retinol-binding protein in gastric cancer patients than healthy controls, the only marker being statistically different between infected and non-infected patients with *H pylori* (it was lower in *H pylori*-infected patients). Our findings are novel and in contrast with previous studies showing decreased RBP levels in the lung[25] and colorectal cancer[17]. Retinol, the ligand of retinol-binding protein, is required to maintain immunity and epithelial turnover and is a key micronutrient needed for combating infection. Studies have shown a good correlation between RBP and retinol even in the context of infection and protein malnutrition[31]. Retinol deficiency could either directly disrupt epithelial integrity or indirectly increase susceptibility to the damaging factors contained in either tobacco smoke (in the case of lung cancer) or diet (in the case of gastric and colorectal cancer). One would expect a lower and not a higher retinol-binding protein value in gastric cancer patients.

A fundamental difference between stomach cancer and other types of cancer is the involvement of *H pylori* in the former. There is evidence that *H pylori* infection *per se* is associated with the abnormalities of the nutritional markers even in the absence of malignancy. Aguilera *et al*[32] studied the relationship between *H pylori* infection, anorexia and malnutrition in 48 peritoneal dialysis patients and found that infected patients with anorexia have lower lymphocyte counts, pre-albumin, transferrin, serum albumin, normalized equivalent of protein-nitrogen appearance and residual renal function. Eradication of *H pylori* could significantly improve the clinical syndrome and the biochemical abnormalities implying a causative role, but unfortunately retinol-binding protein was not measured in that study. The significant decrease in the activity of class IV alcohol dehydrogenase (ADH) in the antrum and corpus of stomachs of men and women infected with *H pylori* may be one of the underlying mechanisms as class IV ADH is the major isoenzyme controlling the production of retinoic acid from retinol and its supply to the human gastric mucosa[33,34]. It has been shown in animal models that the inflammatory response to infection and tissue injury is associated with low concentrations of plasma retinol and its specific transport proteins, retinol-binding protein and pre-albumin[35], suggesting that inflammation-induced hyporetinemia is attributed to a reduction in the hepatic synthesis of RBP and secretion of the retinol-RBP complex. The marked depressing effect of infections on serum retinol and retinol-binding protein has also been shown in human beings[36,37]. We speculate that *H pylori* infection in gastric carcinoma patients can lead to a decrease of retinol-binding protein by one of the above mechanisms but it remains unclear why uninfected patients with gastric cancer still have higher RBP levels than healthy controls.

The role of retinoids in gastric carcinogenesis has been studied in epidemiologic studies with conflicting results[27,28], but the molecular mechanisms that operate at either healthy or disease states have begun to be elucidated. Retinol has been shown to enhance differentiation of the gastric cell lineage in developing rabbits[38] and the reduction of retinoic acid signal has been implicated in the development and evolution of pre-malignant lesions of the human gastric mucosa[39]. The activation of the retinoic acid receptor induces cell differentiation and may antagonize cancer progression. Cellular retinol-binding protein I (CRBP-I) functions in retinol storage and its expression is lower in human cancers than in normal cells. A very recent study[40] showed that CRBP-I downregulation in human mammary epithelial cells chronically compromises retinoic acid receptor activity, leading to the loss of cell differentiation and tumor progression. The fact that class IV ADH is the major isoenzyme responsible for retinoic acid production from retinol and the more significantly decreased enzyme in the presence of *H pylori* infection associated with morphologic changes in the human gastric mucosa is intriguing. The retinoic acid pathway may be one of the missing links in the interplay of *H pylori* infection with gastric carcinogenesis.

In conclusion, gastric cancer patients with *H pylori* infection have significantly lower retinol-binding protein values than non-infected ones and both infected and non-infected groups have higher retinol-binding protein values than healthy controls. This finding may add to our understanding and management of this dreadful disease.

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Histological outcome of chronic hepatitis B in children treated with interferon alpha

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Abstract

AIM: To evaluate the effect of interferon alpha (IFN-α) treatment on the liver histology in children with chronic hepatitis B and to evaluate the usefulness of various histological scoring systems of liver histology in this group of patients.

METHODS: Fibrosis stage and inflammation grade were assessed according to Batts and Ludwig, Ishak *et al*, and METAVIR (only fibrosis stage) before and 12 mo after IFN-α treatment termination in 93 children aged 2-16 years with chronic hepatitis B.

RESULTS: None of the three numerical scoring systems for liver fibrosis showed statistically significant differences in liver fibrosis, while evolution of inflammatory activity revealed statistically significant improvement in the whole group of children with chronic hepatitis B treated with IFN-α and in responders. Significantly positive correlations were found between fibrosis stage and inflammation grade in the respective scoring systems.

CONCLUSION: Treatment with IFN-α did not improve histological fibrosis but decreased inflammatory activity in children with chronic hepatitis B. The three semiquantitative scoring systems seem to be comparable in the estimation of the inflammation grade and fibrosis stage in this group of children.

INTRODUCTION

Interferon alpha (IFN-α) is currently used as a standard treatment for chronic hepatitis B in children. According to Bortolotti[1], the clearance of HBeAg and HBV DNA from serum, normalization of serum ALT activity and improvement of liver histology within 12 mo after treatment discontinuation are considered as the most important endpoints in therapy efficacy evaluation.

Several semiquantitative histological scoring systems have been used in the morphological evaluation of chronic viral hepatitis. The most widely used are the scoring systems according to Ishak *et al*[2], Batts and Ludwig[3], Desmet *et al*[4] and METAVIR[5], which is contrary to the scale proposed by Knodell *et al*[6], definitely separate inflammation grade from fibrosis stage.

The aim of the study was to evaluate the effect of IFN-α treatment on liver histology in children with chronic hepatitis B and to assess the usefulness of different scoring systems of liver histology in pediatric patients. This is the first morphological analysis using different histological semiquantitative scoring systems performed on such a large material obtained from children.

MATERIALS AND METHODS

Patients

The study was carried out prospectively on 93 consecutive children (range 2-16 years, mean age 7.1 years; 69 boys and 24 girls) with biopsy-verified chronic hepatitis B (HBs/+/, HBe/+/, DNA/+/ for at least 12 mo; mean 39 ± 27 mo). Mean ALT activity was 95 ± 79 IU/L. Patients with diagnosed autoimmune hepatitis, liver cirrhosis or HCV coinfection were excluded from the study. None of the children were treated with antiviral or immunomodulating drugs during the 12-mo period before entering the study. IFN-α was applied in the dose of 3 MU thrice a week subcutaneously for 20 wk (this schedule is accepted for children in Poland by Polish Interferon Study Group[7]). The presence of HBeAg/antiHBe seroconversion 1 year after therapy discontinuation was considered to be the criterion of treatment efficacy. Informed consent was obtained from all parents of the patients. The study protocol was approved by the local ethical committee.

Table 1 Morphological evaluation of liver biopsies in children with chronic hepatitis B treated with IFN-α (n = 93)

Scoring system	Baseline biopsy	Final biopsy
Batts grade	1.516±0.619	1.312±0.51[a]
Batts stage	1.892±0.598	1.839±0.631
Ishak grade	4.054±1.683	3.344±1.514[b]
Ishak stage	2.462±1.006	2.398±1.034
METAVIR stage	1.667±0.712	1.645±0.702

[a]P<0.05 vs baseline biopsy; [b]P<0.01 vs baseline biopsy.

Table 2 Morphological evaluation of liver biopsies in children with chronic hepatitis B treated with IFN-α in the subgroups of responders (n = 35) and nonresponders (n = 58)

Scoring system	Subgroups	Baseline biopsy	Final biopsy
Batts grade	Nonresponders	1.431±0.596	1.379±0.524
	Responders	1.657±0.639	1.2±0.473[b]
Batts stage	Nonresponders	1.793±0.554	1.81±0.606
	Responders	2.057±0.639	1.886±0.676
Ishak grade	Nonresponders	3.81±1.503	3.43±1.403
	Responders	4.457±1.899	3.2±1.694[b]
Ishak stage	Nonresponders	2.293±0.878	2.431±0.901
	Responders	2.743±1.146	2.343±1.255
METAVIR stage	Nonresponders	1.62±0.644	1.638±0.667
	Responders	1.743±0.816	1.657±0.764

[b]P<0.01 vs baseline biopsy.

Table 3 Correlations between staging and grading according different histological scoring systems

Scoring system	Batts grade	Batts stage	Ishak grade	Ishak stage	METAVIR stage
Batts grade	-	r = 0.385	r = 0.825	r = 0.392	r = 0.405
		P = 0.00014	P = 0.0000001	P = 0.0001	P = 0.00005
Batts stage	r = 0.385	-	r = 0.522	r = 0.713	r = 0.713
	P = 0.00014		P = 0.0000001	P = 0.0000001	P = 0.0000001
Ishak grade	r = 0.825	r = 0.522	-	r = 0.409	r = 0.41
	P =0.0000001	P = 0.0000001		P = 0.000045	P = 0.000045
Ishak stage	r = 0.392	r = 0.713	r = 0.409	-	r = 0.758
	P = 0.0001	P = 0.0000001	P = 0.000045		P = 0.0000001
METAVIR stage	r = 0.405	r = 0.713	r = 0.41	r = 0.758	-
	P = 0.00005	P = 0.0000001	P = 0.000045	P = 0.0000001	

Histological analysis

The percutaneous liver biopsies were obtained immediately before the treatment and 12 mo after the end of the IFN-α treatment. The liver biopsies were fixed in 10% buffered formalin and embedded in paraffin. Four-micrometer histological sections were prepared and stained with hematoxylin and eosin, Masson's trichrome, Masson's-Goldner and reticulin stain. Fibrosis stage and inflammation grade were assessed in a blinded fashion by a single pathologist according to Batts and Ludwig[3] and Ishak et al[2]; METAVIR scoring system[5] was additionally used for assessing only fibrosis.

Statistical analysis

The results were expressed as mean±SD. Differences in the respective groups were analyzed using Wilcoxon's test for pairs. Spearman's correlation coefficient was used to define correlations between the examined scoring systems. Differences were considered statistically significant at P<0.05.

RESULTS

Table 1 shows the results of morphological evaluation of hepatic inflammation and fibrosis in children with chronic hepatitis B prior to the IFN-α treatment (baseline biopsy) and 12 mo after IFN-α therapy discontinuation (final biopsy).

There was a significant improvement of histological inflammation both in the system of Batts and Ludwig (P = 0.039) and Ishak et al (P = 0.0019). However, there were no significant differences in liver fibrosis of the three semiquantitative scoring systems used.

HBeAg/antiHBe seroconversion 12 mo after therapy termination was observed in 38% of treated children. Therefore, two subgroups were distinguished: responders (35 children) and nonresponders (58 children).

The results of morphological examinations of liver biopsy before the treatment in both subgroups showed more severe intensification (although statistically insignificant) of the inflammation grade and the stage of fibrosis in the subgroup of responders. There was significant improvement of histological inflammation only in the subgroup of responders (P = 0.0058 according to Batts and Ludwig; P = 0.0039 according to Ishak et al), but there were no significant changes in liver fibrosis in either subgroup according to the three respective scoring systems (Table 2).

Significantly positive correlations were found between fibrosis stage and inflammation grade in the respective systems (Table 3).

DISCUSSION

The improvement of liver morphology evaluated not earlier than 12 mo after treatment discontinuation in

comparison to the baseline biopsy is one of the criteria of IFN-α therapy efficacy.

The comparison of three histological scoring systems used to evaluate chronic hepatitis in children with HBV infection revealed a high coefficient of positive correlation between the respective scales. Thus, the systems seem comparable in the estimation of the inflammation grade and stage of fibrosis in children and therefore they can be used according to the preferences of a hepatological center. In adults, Rozario and Ramakrishna[8] were in agreement with our results; they concluded that concordance between Ishak and METAVIR scoring systems was good for necroinflammatory change and excellent for fibrotic change. We also showed a close correlation between liver fibrosis and inflammatory activity, which is in agreement with Lu *et al*[9]. Assy and Minuk[10] also confirmed good correlation between the previously and currently used histological assessment of chronic hepatitis, according to the International Group[11] and Desmet *et al*[4]. As no comparative analysis of the above systems in children have been known to the authors, the current study may inspire further analysis performed on a large group of patients in order to make up the standard morphological estimation of liver biopsy in childhood.

We found no significant changes in the stage of liver fibrosis in children with chronic hepatitis B, neither in responders nor in nonresponders to IFN-α, while a statistically significant decrease was noted in the intensification of necrotic-inflammatory process in the whole group and in the subgroup of responders.

The inhibition of HBV replication was most widely discussed in the previous reports on the treatment of HBV infections in children[12,13]. Considerably less attention was paid to the changes in the morphological picture of liver biopsy after antiviral therapy (changes in necrotic-inflammatory process intensification were mainly evaluated); single reports discuss the changes of liver fibrosis. So far, a statistically significant decrease has been reported in the inflammation grade[14-20], which is in agreement with our analysis. In literature, the reduction in inflammatory activity in IFN-α treated children is estimated at 80%[16,17].

Only a few studies in the world literature analyze the effect of IFN-α therapy on the stage of fibrosis in children with chronic hepatitis B. Most authors did not show improvement of the stage of liver fibrosis caused by antiviral treatment[14,20,21]. Gregorio *et al*[15] found significant improvement only in staging in the responders. But these trials included a relatively small number of liver biopsies. Further studies by Ruiz Moreno *et al*[19] were conducted on a representative group of 60 children with chronic hepatitis B treated with IFN-α, who underwent two liver biopsies (baseline and final). They found that when biopsies were obtained within 12 mo after HBeAg clearance, the degree of histological fibrosis was significantly higher than that seen in biopsies taken after 12 mo. These data seem to indicate that in order to achieve a reliable evaluation of final hepatic fibrosis, histological examination should be performed not earlier than 12 mo

after HBeAg/antiHBe seroconversion. In the present study, the liver biopsies were taken not earlier than after 12 mo of IFN-α therapy discontinuation, as this length of time seems sufficient to evaluate the therapy efficacy determined by histopathological analyses.

Studies conducted on the population of adults with chronic hepatitis B and C also do not provide a satisfactory answer to the question whether IFN-α treatment reduces the stage of liver fibrosis. Many authors have shown no significant changes of liver fibrosis due to this cytokine[22-24]; others, however, have proved a beneficial effect of IFN-α therapy on the reduction of the stage of fibrosis[25-29], particularly in responders[30-32]. Yet, they all underline the effect of IFN-α on the reduction of inflammation grade. The analysis of natural history of HBV infection shows that the inflammatory process in the liver is a triggering mechanism preceding fibrosis. It also seems that the IFN-α-induced reduction in fibrosis is preceded by the regression of inflammatory activity. Considerable divergence in the evaluation of the effect of IFN-α on fibrotic lesions in the liver of patients with chronic viral hepatitis seems to suggest that longer observation period (a few years) prior to the final biopsy is necessary to properly evaluate therapy-induced changes in fibrotic processes.

In conclusion, the treatment with IFN-α did not improve histological fibrosis but decreased inflammatory activity in children with chronic hepatitis B. The various semiquantitative histological scoring systems seem to be comparable in the estimation of the inflammation grade and fibrosis stage in this group of children.

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ELSEVIER

Effects of *Helicobacter pylori* infection on gastric epithelial cell kinetics in patients with chronic renal failure

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Abstract

AIM: To evaluate the effects of *Helicobacter pylori* infection on gastric epithelial cell kinetics in patients with chronic renal failure (CRF).

METHODS: Forty-four patients were enrolled in this study and divided into four groups with respect to their *Helicobacter pylori (H pylori)* and CRF status. Groups were labeled as follows: 1a: normal renal function, *H pylori* negative (n = 12), 1b: normal renal function, *H pylori* positive (n = 11), 2a: CRF, *H pylori* negative (n = 10), 2b: CRF, *H pylori* positive (n = 11). Upper gastrointestinal endoscopy was done in all the patients involved in the study. During endoscopical investigation, antral biopsy specimens were taken from each patient. In order to evaluate the cell apoptosis and proliferation in gastric epithelial cells, Bax and proliferating cell nuclear antigen (PCNA) labeling indexes (LI) were assessed with immunohistochemical staining method.

RESULTS: For groups 1a, 1b, 2a, and 2b, mean Bax LI was identified as 34.4±13.7, 44.1±16.5, 46.3±20.5, 60.7±13.8, respectively and mean PCNA LI was identified as 36.2±17.2, 53.6±25.6, 59.5±25.6, 67.2±22, respectively. When the one-way ANOVA test was applied, statistically significant differences were detected between the groups for both Bax LI (P = 0.004 <0.01) and PCNA LI (P = 0.009 <0.01). When groups were compared further in terms of Bax LI and PCNA LI with Tukey's HSD test for multiple pairwise comparisons, statistically significant difference was observed only between groups 1a and 2b (P = 0.006 <0.01).

CONCLUSION: In gastric epithelial cells, expression of both the pre-apoptotic protein Bax and the proliferation marker PCNA increase with *H pylori* infection. This increase is more evident in patients with uremia. These findings suggest that uremia accelerates apoptosis and proliferation in gastric epithelial cells.

INTRODUCTION

Helicobacter pylori (H pylori) is the most common chronic infection in human beings. *H pylori* infection has been related to peptic ulcer disease, gastric lymphoma and stomach cancers[1,2]. It is an important problem for public health as it can cause several diseases.

Gastrointestinal mucosa is characterized with rapid epithelial cell turnover and homeostasis that is mainly provided by apoptosis[3]. Therefore, it can be considered that the impaired apoptosis may have a role in the pathogenesis of many gastrointestinal system diseases.

In many studies, it has been shown that apoptotic cells are increased after colonization of *H pylori* in the stomach[4-7], and those increased apoptotic cell levels decrease to normal levels after *H pylori* eradication[5,8,9]. Increase in apoptotic cell rates causes compensatory hyperproliferative response in order to maintain gastric mucosal tissue mass[10-12].

The mechanisms by which *H pylori* induces apoptosis of gastric epithelial cells have remained unclear. It has been suggested that many bacterial products might be inducing apoptosis[5,13-15]. One of the factors that has been responsible for apoptosis of gastric epithelial cells is ammonia, which is produced via the degradation of urea by the urease enzyme of *H pylori*[5,16]. Because of high intragastric urea concentrations in patients with chronic renal failure (CRF), levels of ammonia produced by *H pylori* are significantly high[16]. Therefore, disorders associated with excessive ammonia production are more

prevalent[17,18].

Although the relationship of *H pylori* with apoptosis and proliferation in gastric epithelial cells has been mostly studied in cases with normal renal functions, gastric epithelial cell kinetics in patients with CRF still remains unknown. This study aims to investigate the effect of *H pylori* on gastric epithelial cell kinetics in patients with CRF.

MATERIALS AND METHODS

Subjects
The study included 44 patients, of whom 23 with normal renal function and 21 with CRF who were on hemodialysis treatment.

Those with gastric and/or duodenal ulcers as confirmed by upper gastrointestinal endoscopy were excluded from the study. None of the patients included had a history of gastric surgery. Patients who had been treated for *H pylori* or who had used any antibiotics, proton-pump inhibitor, H$_2$ receptor blocker or any compound that includes bismuth during the last one month were also excluded from the study.

The CRF patients were on a thrice-weekly hemodialysis program. The dialysis prescription included 4-5 h of bicarbonate hemodialysis with standard cuprophane membranes (hollow fiber 1-1.2 m2 surface area, Gambro, Sweden).

We divided the patients into four groups based on their *H pylori* and renal function status:
  Group 1a: normal renal function, *H pylori* negative (12 patients)
  Group 1b: normal renal function, *H pylori* positive (11 patients)
  Group 2a: CFR, *H pylori* negative (10 patients)
  Group 2b: CRF, *H pylori* positive (11 patients)

Endoscopy method
All patients were examined by using the upper gastrointestinal endoscopy (video-endoscope Olympus GIP Q240) after premedication with intravenous midazolam (2.5-7.5 mg), after an overnight fast.

During upper gastrointestinal endoscopy, multiple biopsy materials were taken from the antral region of the stomach. The biopsy specimens were transferred to the pathology laboratory in 10% buffered formalin. This study was conducted after getting permission from the local ethics committee of the Baskent University School of Medicine, Ankara, Turkey.

Identification of H pylori infection
H pylori was detected under microscopy on the histological sections stained with Giemsa staining method.

Laboratory methods
After a 12-h fasting period, venous blood samples were taken from the forearm-superficial veins of the patients in the morning. In the HD patients, the blood samples were taken before HD session. Serum levels of creatinine (normal <1.2 mg/dL) and blood urea nitrogen (BUN, normal <20 mg/dL) were measured in the central laboratory of our hospital by using routine automated
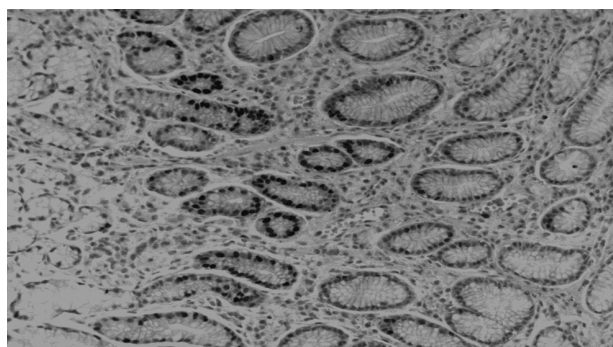

Figure 1 Proliferating cell nuclear antigen (PCNA) immunostaining of the gastric mucosa, original magnification ×250.
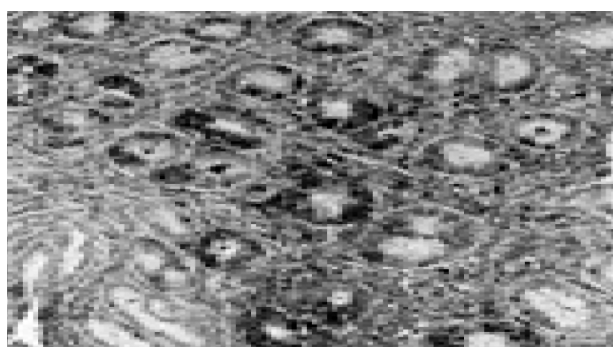

Figure 2 Bax immunostaining of the gastric mucosa, original magnification ×400.

techniques.

Immunohistochemical staining for proliferating cell nuclear antigen and Bax
All biopsies were fixed in formalin, embedded in paraffin and processed routinely. Briefly, 4-mm-thick sections were deparaffinized and mounted on poly-L-lysine-coated slides. The sections in a citrate buffer (0.01 mol/L, pH 6) were heated in a microwave oven for 15 min at a maximum power (700 W), and then cooled at room temperature for 20 min. A standard three-step immunoperoxidase avidin-biotin peroxidase complex (ABC) technique was used to identify the proliferating cell nuclear antige (PCNA) (PC10, Neomarkers, CA, USA) (Figure 1). A catalyzed signal amplification (CSA) system was used to detect the polyclonal antibody Bax (K1500, Dako, Denmark) (Figure 2). PCNA and Bax positive cells were counted in the glandular neck region, which corresponds to the area of cell proliferation[19,20]. The field to be counted was chosen under ×400 magnification from the well-labeled area. About 1 000 cells were counted in each case to determine the PCNA and Bax labeling indexes (LI). The percentage of positively stained cells over total cells counted was then calculated and used as a labeling index for PCNA and Bax expression[21,22]. All histological slides were reviewed by the same experienced pathologist. The pathologist who rated the PCNA and Bax LI was unaware of the *H pylori* and renal function status of the patients.

Statistical analysis

Statistical analyses were carried out using the program SPSS 9.0 for Windows. To compare the groups, we used Student's *t*-test, Mann-Whitney *U* test, one-way ANOVA and Tukey's HSD test when appropriate. *P* values <0.05 were considered statistically significant.

RESULTS

The number of patients, their mean ages and HD duration are shown in Table 1 for each group separately. There were no statistically significant differences between the groups with regard to mean ages, gender, and HD duration ($P>0.05$).

Creatinine levels were 0.7±02, 0.8±0.2, 7.3±1.1, 7.1±1.3 mg/dL, and BUN levels were 13.8±3.5, 15.4±4.1, 98.7±12.2, 96.1±11.5 mg/dL in groups 1a, 1b, 2a, 2b, respectively (Table 2). Considering the creatinine and BUN levels, there were no statistically significant differences between the groups 1a and 1b ($P>0.05$), and between the groups 2a and 2b ($P>0.05$) but levels were higher in groups 2a and 2b than 1a and 1b ($P<0.001$).

Table 1 Number, gender, mean age, and hemodialysis duration of patient groups

	Group 1a	Group 1b	Group 2a	Group 2b
Patients (*n*)	12	11	10	11
Sex (M/F)	7/5	6/5	5/5	6/5
Mean age (yr)	37±7	39±7	35±8	36±11
Hemodialysis duration (mo)	–	–	10.4±4.5	9.3±4.4

Group 1a: normal renal function, *H pylori* negative; group 1b: normal renal function, *H pylori* positive; group 2a: CRF, *H pylori* negative; group 2b: CRF, *H pylori* positive.

For groups 1a, 1b, 2a, 2b, mean Bax LI was identified as 34.4±13.7, 44.1±16.5, 46.3±20.5, 60.7±13.8, respectively, and mean PCNA LI was identified as 36.2±17.2, 53.6±25.6, 59.5±25.6, 67.2±22, respectively.

When the one-way ANOVA test was applied, statistically significant differences were detected between the groups for both Bax LI ($P = 0.004 <0.01$) and PCNA

Table 2 Creatinine, BUN, Bax, and PCNA LI in different patient groups

	Group 1a	Group 1b	Group 2a	Group 2b
Creatinine	0.7±0.2	0.8±0.2	7.3±1.1[a,b]	7.1±1.3[c,d]
BUN	13.8±3.5	15.4±4.1	98.7±12.2[a,b]	96.1±11.5[c,d]
Bax labeling index	34.4±13.7	44.1±16.5	46.3±20.5	60.7±13.8[e]
PCNA labeling index	36.2±17.2	53.6±25.6	59.5±25.6	67.2±22.1[e]

Group 1a: normal renal function, *H pylori* negative; group 1b: normal renal function, *H pylori* positive; group 2a: CRF, *H pylori* negative; group 2b: CRF, *H pylori* positive. [a]$P<0.001$ group 1a *vs* 2a; [b]$P<0.001$ group 1b *vs* 2a; [c]$P<0.001$ group 1a *vs* 2b; [d]$P<0.001$ group 1b *vs* 2b; [e]$P<0.01$ group 1a *vs* 2b.

LI ($P = 0.009 <0.01$). When groups were compared further in terms of Bax LI and PCNA LI with Tukey's HSD test for multiple pairwise comparisons, statistically significant difference was observed only between groups 1a and 2b ($P = 0.006 <0.01$).

In patients with *H pylori* infection and CRF, Bax LI and PCNA LI were found to be increased. However, the increase was more prominent in patients with CRF compared to patients with *H pylori* infection.

DISCUSSION

This is the first study where cell turnover of gastric epithelial cells is investigated in patients with CRF. We have shown that both *H pylori* infection and uremia cause increases in Bax and PCNA LI in gastric epithelial cells. Other studies in the literature that show *H pylori* increases the proliferation and apoptosis in gastric epithelial cells in cases with normal renal function support the results of our study. In addition, we also noted that both apoptosis and proliferation in gastric epithelial cells are more increased in patients with CRF.

Maintaining gastric mucosal integrity is a complex biological process[23]. This subject is provided with the balance between programmed cell death, which is also called apoptosis, and epithelial cell proliferation[23–26]. Apoptosis has been accepted as a physiological form of death and is a genetically programmed process where the cell commits suicide[27,28]. Unlike necrosis, dead cells do not cause inflammatory response in apoptosis[28]. Deranged apoptosis has been implicated in carcinogenesis, autoimmune diseases, and various infectious diseases including *H pylori* infection[5,23,28,29]. Regulation of apoptosis is a complex process. This process includes the activation of various apoptosis-related proteins such as the Bcl-2 family, p53, Fas and its ligand (FasL), and the interleukin-1b-related concerning enzyme (ICE) family[23,30].

There are several methods for evaluating cell division[31]. PCNA is a co-factor of DNA polymerase and mainly determined in late G_1 or S phase. There are several evidences that PCNA assessment is a useful tool to evaluate cell proliferation and previous studies have shown that PCNA index correlates with S phase fraction of tumor cells determined by DNA flow cytometry[32-35]. Furthermore, it has been found that PCNA immunostaining correlates with Ki67, the latter marking cells in G_1 and G_2 phases in addition to those in S phase and also with tymidine labeling index[36] and with bromodeoxyuridine uptake in cancer cell lines[20,37].

Many genes regulate the balance between apoptosis and cell proliferation. The pro-apoptotic protein, Bax, is a member of Bcl-2 gene family and is one of the genes that regulate apoptosis. Bax gene is a tumor suppressor gene and induces apoptosis via encoding Bax protein. It has been suggested that proliferation and apoptosis in the antral mucosa caused by *H pylori* is associated with the alterations in genes that regulate the process[8,22,38,39]. Konturek *et al*[23] showed that apoptosis induced by *H pylori* in gastric epithelial cells is associated with the upregulation

of proapoptotic Bax.

In many studies, it has been shown that the number of apoptotic cells in the stomach increase after *H pylori* colonization[4-6]. In addition, it has also been shown that the apoptotic cell number in stomach decreases to normal after *H pylori* eradication[5,8,9]. This increase in apoptotic rate causes compensatory hyperproliferative response in order to maintain gastric mucosa tissue mass[11].

In the literature, there are some data which suggest that apoptosis is associated with both bacterial and host factors[13-15,23]. However, the mechanism of apoptosis in *H pylori* infection is still not clear.

Some of the factors accused within the apoptosis are lipopolysaccharides, ammonia, and monochloramine (NH₂Cl), which is a highly toxic substance generated as a result of the reaction of ammonia with neutrophil-derived free radicals[13-15]. There are still debates on the role of cytotoxic agents such as *H pylori*-CagA and *H pylori*-VacA in apoptosis. In recent *in vivo* and *in vitro* studies, it has been found that *H pylori* strains can induce apoptosis without any dependency on Cag-A and Vac-A genotype[23]. In addition to the bacterial factors in infections of *H pylori*, characteristics of the host can also play important roles. Evidences are increasing about the inflammatory reactions that are induced by *H pylori* in gastric mucosa, especially cytokines such as nitric oxide and TNFα might assist to induce apoptosis[6].

One of the major factors that are responsible for *H pylori* derived apoptosis is ammonia. *H pylori* has a strong urease activity and the hydrolysis of urea causes production of high level of ammonia concentration in mucous gel layer[40]. *In vitro* studies have shown that *H pylori* derived ammonia causes lesions in insulated gastric epithelial cells. Ammonia concentrations, determined in *H pylori* infected subjects, cause gastric mucosal lesions[40-42], delay in mucosal improvement[43], and induce apoptosis in gastric epithelial cells[13,44].

Ammonia level, which is produced by *H pylori*, is controlled by the existence of urea in gastric juice. Patients with CRF have high intragastric urea concentrations and levels of ammonia produced by *H pylori* are significantly high[16]. Therefore, disorders associated with excessive ammonia production may be more prevalent in patients with uremia. In our cases, increased apoptosis and proliferation rate that are observed in uremia patients can be associated with excessive ammonia production by *H pylori* infection in uremia patients compared with cases that have normal renal functions.

In conclusion, in gastric epithelial cells, expression of both the pre-apoptotic protein Bax and the proliferation marker PCNA increase with *H pylori* infection. This increase is more evident in patients with uremia. These findings suggest that uremia accelerates apoptosis and proliferation in gastric epithelial cells.

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ELSEVIER

• *RAPID COMMUNICATION*•

Intrahepatic and peripheral T-cell responses in genotype 1b hepatitis C virus-infected patients with persistently normal and elevated aminotransferase levels

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Abstract

AIM: To evaluate whether the cytokine responses in liver and serum differ in chronic hepatitis C patients with normal and high alanine aminotransferase (ALT) levels.

METHODS: Thirty-three (16 with normal ALT level as group 1 and 17 with elevated ALT level as group 2) patients infected with genotype 1b hepatitis C virus (HCV) were examined. Liver infiltrating lymphomononuclear cells (LILMCs) were isolated from liver biopsy by collagenase type 1 and stimulated with phytohemagglutinin and interleukin 2 (IL-2). IL-10, IL-12, interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) were determined in serum and LILMCs by ELISA.

RESULTS: Serum cytokine levels were similar in both groups ($P>0.05$). Stimulated IFN-γ and TNF-α levels in LILMCs were increased in both groups. IL-12 and IL-10 levels stimulated with IL-2 were higher in group 1 than in group 2 ($P = 0.023$). Histological activity index (HAI) and stage had a negative correlation with TNF-α and IFN-γ levels in group 2.

CONCLUSION: Increased T-helper type 2 (Th2) cytokine response may regress inflammatory and biochemical activity. Progression of histological abnormalities in persons with elevated ALT probably depends on insufficient Th2 cytokine response, which does not balance Th1 cytokine response.

Key words: Liver cytokines; HCV; ALT

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INTRODUCTION

The liver is the primary site of hepatitis C virus (HCV) replication[1]. Clearance and control of HCV infection depend on immune responses. T lymphocytes and immunoregulatory cytokines play an important role in the host response to HCV infection[2]. Intrahepatic T-cell response to HCV may determine the hepatic injury of HCV infection[3,4]. T cells also play a role in HCV clearance[5]. Th1 cytokines such as IL-2 and IFN-γ are required for host antiviral immune response, while Th2 cytokine (IL-10) can inhibit the development of these responses[2]. TNF-α also triggers a partially overlapping set of antiviral defense mechanisms and serum level of TNF-α reflects the progression of inflammation[6,7].

The natural course of HCV infection is not clear. Also, the behavior of HCV infection cannot be estimated. Different immune responses in each patient may determine the clinical course. Approximately 30% of patients with chronic HCV infection show persistently normal ALT levels that are positive for HCV-RNA. Only a small number of these patients (0-20%) have normal histology. These people are referred as healthy HCV carriers[8]. Immunological differences between patients with normal and elevated ALT levels are not well known. Most patients with normal ALT levels have a certain degree of liver damage and show a slow progression[9].

This study aimed to evaluate whether the cytokine responses in liver and serum differ in chronic hepatitis C patients with normal and high ALT levels.

MATERIALS AND METHODS

Thirty-three patients infected with genotype 1b HCV were studied. Sixteen of them had persistently normal ALT levels (group 1), while 17 had high ALT levels (group 2) by at least four analyses in a year. Anti-HCV was determined by UBI EIA 4.0 (Organon Teknika, Holland). HCV-RNA was determined by polymerase chain reaction (COBAS Amplicor 2, Roche Diagnostics, Germany)

and HCV genotyping was carried out with INNO-LiPA HCV assay (Innogenetics NV, Belgium). Hepatitis B surface antigen (HBsAg) and anti-HIV were negative in all patients. HBsAg was detected by immunoenzymatic assay [Hepanostika HBsAg Uni-Form II (Organon Teknika, Holland)]. All patients were also negative for any other etiology of liver diseases. Blood was drawn from all patients and centrifuged at 2 500 r/min for 5 min and separated serum samples were stored at -85 ℃. Liver biopsy was performed in all the patients before the treatment. Histological changes (HAI and extent of fibrosis) were assessed according to the Knodell's scoring system[10].

LILMCs were isolated from liver biopsies as previously described by Bertoletti *et al*[11]. Biopsies were washed twice in RPMI 1640 medium to remove contaminated blood and digested with collagenase type-I (1 mg/mL CO130, Sigma Chemical Co., USA) and deoxyribonuclease I (25 mg/mL D4263, Sigma Chemical Co., USA) for 1 h at 37 ℃ on a shaking device. The liver specimen was then pipetted vigorously to disrupt the hepatic tissue and to release infiltrating lymphomononuclear cells. The mononuclear cell suspension was washed twice and the cells were recovered by centrifugation over a Ficoll-hypaque density gradient. Cells were then suspended in RPMI 1640 (GIBCO Lab) containing 10% heat inactivated fetal calf serum, 25 mmol/L HEPES, 2 mmol/L L-glutamine and 50 μg/mL gentamycin. Viability was >95% by the trypan blue dye exclusion test. Cells were cultured in duplicate (1.0×10^5 viable cells/100 μL) in flat-bottomed 96-well plates at 37 ℃ in an atmosphere containing 50 mL/L CO_2 with medium alone (unstimulated culture) and stimulated with 10 μg/mL phytohemagglutinin (1:100 PHA, Sigma Chemical Co., USA) and recombinant IL-12 (20 U/mL, R&D Systems, USA). Restimulation was performed every 10 d. Supernatants were collected at the end of the proliferation assay and kept frozen at -70 ℃ for later analyses. Culture supernatants and sera were thawed and the serum level of cytokines was measured by ELISA.

The local ethic committee approved this study. Informed consent was obtained from each patient included in this study. Data were presented as mean±SD. Data analysis was made by the χ^2, Fisher's exact, Mann-Whitney *U* and Pearson's correlation tests using SPSS for Windows (Chicago, IL, USA) when appropriate. *P*<0.05 was considered statistically significant.

RESULTS

Age, gender and viral load were similar in groups 1 and 2 (Table 1). The results of biochemical analyses except for aspartate aminotransferase (AST) and ALT were also similar in both the groups. In group 1, five patients underwent liver biopsy before being enrolled in the study. Though liver histology was similar in three patients, histological progression from stage 1 to stage 3 was observed in two of them.

Serum levels of IFN-γ (1.2±1.8; 1.1±0.9 pg/mL), IL-12 (0.2±0.4; 0.11±0.26 pg/mL), IL-10 (17.7±55.6; 18.2±47.8 pg/mL) and TNF-α (0.9±1.2; 0.4±0.25 pg/mL) were similar in groups 1 and 2 (*P*>0.05). Both stimulated IFN-γ and TNF-α levels in LILMCs were increased in comparison to their serum levels in both groups.

In ALT normal patients, there was a positive correlation between TNF-α and IFN-γ levels (both serum and liver) (*P*<0.05, *r* = 0.5). IL-12 and IL-10 levels stimulated by IL-2

Table 1 Demographic and biochemical features of patients (mean±SD)

	Normal ALT	Elevated ALT	*P*
n	16	17	
Age	51.4±10	47±10.7	>0.05
Female/male	10/6	8/9	>0.05
Duration of infection (mo)	33±32 (12-108)	21±22 (6-72)	>0.05
ALT (IU/L)	36±6	115±60	<0.05
AST (IU/L)	32±7.8	70±21	<0.05
ALP (IU/L)	187.9±95	219.8±104.5	>0.05
GGT (IU/L)	50.2±92.7	102.5±81.6	>0.05
Total bilirubin (mg/dL)	0.7±0.5	0.6±0.2	>0.05
Albumin (g/dL)	4.1±0.2	4±0.3	>0.05
Globulin (g/dL)	1.2±0.2	1.4±0.4	>0.05
Histology			
Minimal abnormalities	3	0	
Stage (1/2/3/4)	9/1/3/0	010/5/0/2	
HAI	4.4±2.8	6.1±3.5	>0.05
HCV-RNA (copies/mL)	456 532±277 761	461 710±324 860	>0.05

ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; GGT: gamma-glutamyl transpeptidase, HAI: histological activity index.

Table 2 Cytokine levels stimulated by IL-2 and phytohemagglutinin in LILMCs (mean±SD)

pg/mL	Normal ALT	Elevated ALT	P
IFN-γ			
IL-2	1.8±2.3	2.2±3.4	>0.05
PHA	2.1±2.7	2.8±3.7	>0.05
IL-12			
IL-2	0.6±0.5	0.24±0.5	0.027
PHA	0.9±0.6	0.7±0.7	>0.05
IL-10			
IL-2	12.2±23.8	4.1±5.3	0.023
PHA	15.9±30.6	9.2±7.1	>0.05
TNF-α			
IL-2	2.5±1.06	2.5±2.9	>0.05
PHA	3.3±1.4	3.8±3.2	>0.05

ALT: alanine aminotransferase; PHA: phytohemagglutinin; LILMCs: liver infiltrating lymphomononuclear cells.

were higher in group 1 than in group 2 ($P<0.05$, Table 2).

In elevated ALT patients, there was a negative correlation between serum IL-12 and IFN-γ levels ($P = 0.01$, $r = –0.6$). HAI and stage had a negative correlation with TNF-α ($P<0.05$, $r = –0.8$; $P<0.05$, $r = –0.6$, respectively) and IFN-γ ($P<0.05$, $r = –0.5$; $P<0.05$, $r = –0.4$, respectively) levels (Figure 1). IL-10 stimulated by IL-2 had a positive correlation with HAI ($P<0.05$, $r = 0.49$) and stage ($P<0.05$, $r = 0.59$) (Figure 2). TNF-α and IFN-γ levels in LILMCs were positively correlated ($P<0.05$, $r = 0.8$). Cytokine levels in serum and LILMCs were not correlated with serum HCV-RNA loads in both groups.
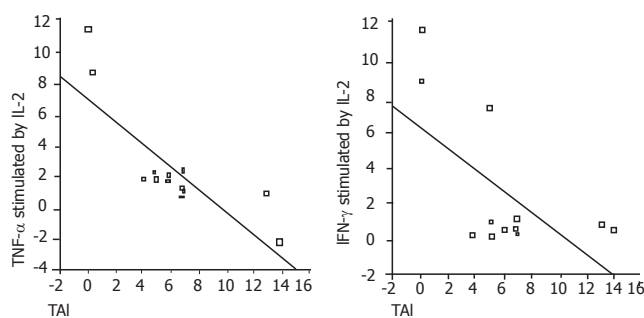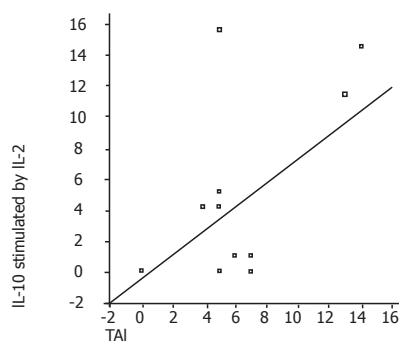
DISCUSSION

Factors involved in the progression to end-stage liver disease in HCV-infected patients are not well characterized. It is thought that cytotoxic T lymphocyte (CTL) response early in infection may be important for viral clearance, while continuous low-level anti-HCV CTL-dependent immune response may be responsible for accumulated liver damage[12]. Prezzi *et al*[13] showed that LILMCs have phenotypic and functional characteristics distinct from peripheral blood lymphocytes. All these immunological processes define natural progress of HCV infection. Patients with normal and elevated ALT levels show different clinical patterns[14]. Generally, HCV carriers with normal ALT have mild and stable diseases with a favorable prognosis[8]. Liver histology was normal in 20% of our cases with persistently normal ALT. Progression of liver fibrosis was observed in two of five patients (40%) who had a second liver biopsy in a period of 41.5±22.1 months.

Immunological studies concerning HCV infection generally focus on T lymphocytes. However, the serum cytokine levels have been found to be different in chronic hepatitis C patients[2,15,16]. Rico *et al*[1] showed that HCV specific CD4+ T-cell proliferation responses do not parallel in LILMCs and peripheral blood mononuclear

cells. The magnitude of T-cell response is higher in the liver than in peripheral blood. In our study, serum levels of cytokines were similar in patients with persistently normal and elevated ALT, suggesting that liver cytokine levels are more important than serum levels in mediating T-cell responses.

IL-10 and IL-12 levels stimulated with IL-2 were higher in patients with normal ALT than in those with elevated ALT ($P<0.05$). While IL-10 showed Th2 response, IL-12 promoted Th1 cell induction and cell-mediated immunity. Interestingly both of them were high in patients with normal ALT, suggesting that strong Th2 response may be the cause of the mild biochemical and histological activity in patients with normal ALT.

Sobue *et al*[4] revealed that disease activity and progression correlate with dominant Th1 response in chronic hepatitis C patients. On the other hand, Tsai *et al*[17] showed that predominant Th1 response is stronger in patients with resolved infection than in those with chronic diseases. In our study, while histological stage and HAI were increased, TNF-α and IFN-γ levels were decreased in patients with elevated ALT (Figure 1). TNF-α and IFN-γ trigger antiviral defense mechanisms and have a principal effect on inflammation[6,7]. This means that the magnitude of antiviral immune response is decreased, while the histological activity and stage are increased. Though Th1 and Th2 responses were both strong in patients with normal ALT, Th1 response was not as high as that

Figure 1 Negative correlations between histological activity index (HAI) and TNF-α and IFN-γ levels in LILMCs of patients with elevated ALT.

Figure 2 Positive correlation between IL-10 stimulated by IL-2 and HAI in patients with elevated ALT.

in patients with elevated ALT, suggesting that strong antiviral defense against HCV infection can normalize liver enzymes. On the other hand, histological abnormalities might be impaired by the increased Th2 response.

In conclusion, both Th1 and Th2 responses in liver are elevated in patients with normal ALT. Increased Th2 response may regress inflammatory activity. In patients with normal ALT, progression of histological findings may probably depend on insufficient Th2 response, which does not balance Th1 response. A differentiation between virus-specific and non-specific T-cell populations is the greatest challenge in future studies.

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• *RAPID COMMUNICATION* •

Diagnostic accuracy of serum biochemical fibrosis markers in children with chronic hepatitis B evaluated by receiver operating characteristics analysis

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Abstract

AIM: To investigate the diagnostic accuracy of potent serum biochemical fibrosis markers in children with chronic hepatitis B evaluated by receiver operating characteristics (ROC) analysis.

METHODS: We determined the serum level of apolipoprotein A-I (APO A-I), haptoglobin (HPT) and a-2 macroglobulin (A2M) with an automatic nephelometer in 63 children (age range 4-17 years, mean 10 years) with biopsy-verified chronic HBeAg-positive hepatitis B. Fibrosis stage and inflammation grade were assessed in a blinded fashion according to Batts and Ludwig. We defined mild liver fibrosis as a score ⩽2 and advanced fibrosis as a score equal to 3. ROC analysis was used to calculate the power of the assays to detect advanced liver fibrosis (AccuROC, Canada).

RESULTS: Serum concentrations of APO A-I, HPT and A2M were not significantly different in patients with chronic hepatitis B compared to controls. However, APO A-I level of 1.19 ng/L had a sensitivity of 85.7% and a specificity of 60.7% (AUC = 0.7117, P = 0.035) to predict advanced fibrosis. All other serum biochemical markers and their combination did not allow a useful prediction. None of these markers was a good predictor of histologic inflammation.

CONCLUSION: Apolipoprotein A-I may be a suitable serum marker to predict advanced liver fibrosis in children with chronic hepatitis B.

Key words: Chronic hepatitis B; Liver fibrosis; Children; Apolipoprotein A-I; Haptoglobin; a-2 macroglobulin

INTRODUCTION

Liver biopsy is regarded as the gold standard for the determination of the stage of liver fibrosis. However, in clinical practice, the use of invasive liver biopsy has several limitations such as complications, sampling variability, low reproducibility and it only provides static information about the fibrotic process[1-3]. There is a clinical need for noninvasive measurement of liver fibrosis both to diagnose significant hepatic fibrosis and to monitor the effects of antiviral or antifibrotic therapy.

Several matrix-derived surrogate markers of extracellular matrix (ECM) turnover have been studied in adults so far and some were found to be correlated with matrix deposition, while their role as predictors of ECM turnover remains unclear[4-6]. This applies particularly for children whose serum levels of ECM-derived parameters are usually influenced by body growth[7,8]. Therefore, there is an urgent need for noninvasive parameters that better define those children who should undergo liver biopsy in order to assess fibrosis stage, to decide whether they should receive antiviral therapy or not, to predict fibrosis progression or to monitor potential antifibrotic treatment. Serum levels of type IV collagen[9,10], hyaluronic acid[10-13], laminin[14], collagen VI[15], transforming growth factor beta 1 (TGF beta 1)[16] and metalloproteinases (MMPs) or tissue inhibitors of metalloproteinases (TIMPs)[10,17] have recently been studied in children, but almost exclusively in patients with secondary biliary fibrosis due to biliary atresia and cystic fibrosis.

To our knowledge, serum fibrosis markers predicting liver fibrosis have not been assessed before in children with chronic hepatitis B (HBV) except our previous studies[18-20]. From a broad panel of matrix-derived serum markers (collagen IV, collagen VI, PIIINP, laminin-2, hyaluronan, MMP-2, TIMP-1, MMP-9/TIMP1 complex, tenascin-C), the combination of serum hyaluronan and laminin-2 can accurately predict significant liver fibrosis[18].

In our previous studies, we have reported that serum TGF beta 1 and cystatin C level did not predict advanced liver fibrosis in children with chronic hepatitis B[19,20].

In the present study, the serum levels of apolipoprotein A-I (APO A-I), haptoglobin (HPT) and a-2 macroglobulin (A2M) were measured in children with chronic hepatitis B and compared to liver histological features to determine whether the measurement of these biochemical tests have any clinical usefulness as markers of liver fibrosis. Receiver operating characteristics (ROC) analysis was used to determine the sensitivity and specificity of the assays in detecting advanced liver fibrosis.

MATERIALS AND METHODS

Patients
The study was carried out in 63 children, including 41 boys and 22 girls (range 4-17 years, mean age 10 years) with biopsy-verified chronic HBeAg-positive hepatitis B prior to antiviral therapy. Other causes of chronic liver diseases, such as HCV coinfection, autoimmune hepatitis and metabolic liver disorders, were excluded. Children with diagnosed liver cirrhosis and evidence of other acute or chronic infections were also excluded from this study. Informed consent was obtained from all the parents of patients and the protocol used was approved by the Ethics Committee of the Medical University of Bialystok, Poland. As a control group, 16 children (mean age 10 years) were included without anamnestical, clinical or laboratory signs of liver or other systemic diseases.

Measurement of serum level of biochemical markers
APO A-I, HPT and A2M were measured in serum samples (obtained after an overnight fast) with an automatic nephelometer (BNII, Dade Behring; Marburg, Germany).

Histological analysis
All children underwent liver biopsy on the day after serum sampling. Liver specimens were fixed in buffered formalin and embedded in paraffin. Histological sections were stained using hematoxylin-eosin, Masson's-Goldner, Masson's trichrome and reticulin. Fibrosis stage and inflammation grade were assessed in a blinded fashion by a single pathologist who was without the knowledge of the patients' laboratory or clinical data. In order to determine specificity and sensitivity of the assay, we arbitrarily defined mild liver fibrosis or inflammation as a score ⩽ 2 and advanced liver disease as fibrosis or inflammation score equal to 3 according to Batts and Ludwig[21].

Statistical analysis
Serum concentrations of biochemical tests were expressed as mean±SD. Statistical analysis was performed using the Mann-Whitney two-sample test for nonparametric data. The relationship between the enzymes and liver histology scores was analyzed by the Spearman's rank-correlation test for nonparametric data. Tests were considered statistically significant at $P<0.05$. Receiver operating characteristics (ROC) analysis (AccuROC, Montreal, Canada) was used

to calculate the power of the assays to detect advanced liver fibrosis. The best cut-off points for the diagnosis of advanced fibrosis are those which maximize the sum of sensitivity and specificity. Sensitivity of the assays was plotted against the false positivity (1-specificity). Comparison of the area under the curve (AUC) was performed using a *P*-test, which compares the AUC to the diagonal line of no information (AUC 0.5)[22].

RESULTS

Characteristics of the patients
Selected biochemical and histological data are presented in Table 1.

Table 1 Initial characteristics of children with chronic hepatitis B

Parameters	Mean	SD	Minimum	Maximum
Age (yr)	10	3.41	4	17
ALT (IU/L)	84	57	12	312
AST (IU/L)	68	37	27	264
GGT (IU/L)	15	9	3	69
Bilirubin (μmol/L)	10.26	4.45	2.74	32.83
APO A-I (g/L)	1.14	0.3	0.5	1.77
HPT (g/L)	0.58	0.36	0.31	1.69
A2M (g/L)	2.27	0.57	1.0	3.59
Staging	1.9	0.56	1	3
Grading	1.49	0.56	1	3

Serum concentration of APO, HPT and A2M
Serum concentrations of APO A-I, HPT and A2M were not significantly different in patients with chronic hepatitis B as compared with the controls (1.14±0.3 *vs* 1.13±0.2; 0.58±0.36 *vs* 0.71±0.62, 2.27±0.57 *vs* 2.53±0.3, respectively). There were no significant correlations of examined markers with liver fibrosis and inflammation according to Batts and Ludwig[21].
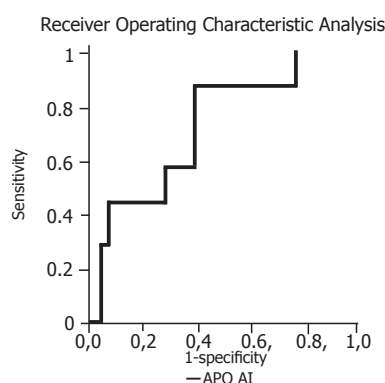
Diagnostic value of biochemical markers for identification of patients with advanced liver fibrosis and inflammation
Seven children (11.1%) had advanced liver fibrosis. The ability of examined biochemical markers to differentiate the children with advanced liver fibrosis from those with mild fibrosis was not significant except APO A-I (Table 2 and Figure 1). Using the APO A-I and the Batts and Ludwig score, 34 and 6 out of 63 children could be correctly allocated either to the group with mild or advanced fibrosis, respectively, potentially avoiding biopsy in 40 (63.5%) of the examined children. HPT and A2M did not reach the predictive power of APO A-I (Table 2). None of the combination of examined markers resulted in a significant increase in sensitivity and specificity for the identification of patients with advanced liver fibrosis. None of these markers was a good predictor of histologic inflammation (Table 3).

Table 2 Diagnostic value of serum markers for predicting advanced fibrosis

Markers	Cut-off (g/L)	Se (%)	Sp (%)	PPV (%)	NPV (%)	AUC	P values
APO A-I	1.19	85.7	60.7	21.4	97.1	0.7117	0.035
HPT	1.01	14.3	87.5	12.5	89.1	0.3878	NS
A2M	1.79	100.0	23.2	14.0	100.0	0.5268	NS

Se: sensitivity; Sp: specificity; PPV: positive predictive value; NPV: negative predictive value; AUC: area under curve; NS: no statistical significance.

Figure 1 ROC curve of ability of serum APO A-I to detect advanced liver fibrosis according to Batts and Ludwig in children with chronic hepatitis B.

Table 3 Diagnostic values of serum markers for predicting advanced inflammation

Markers	Cut-off (g/L)	Se (%)	Sp (%)	PPV (%)	NPV (%)	AUC	P values
APO A-I	0.87	89.7	20.6	49.1	70.0	0.3803	NS
HPT	0.59	34.5	70.6	50.0	55.8	0.4701	NS
A2M	1.6	96.6	14.7	49.1	83.3	0.3813	NS

Se: sensitivity; Sp: specificity; PPV: positive predictive value; NPV: negative predictive value; AUC: area under curve; NS: no statistical significance.

DISCUSSION

Infection with the hepatitis B virus remains one of the most important epidemiological problems all over the world. It was estimated that chronic HBV infection, which is the single most common cause of cirrhosis and hepatocellular carcinoma (HCC), affects more than 400 million people worldwide[23,24].

During the early phase of chronic hepatitis B, the underlying disease is usually subclinical and can be quite mild, particularly in children[25]. This is referred to as the immune-tolerant phase of infection[26]. While the natural history of chronic hepatitis B in children is usually marked by an indolent course, advanced disease with significant fibrosis can be found in up to one-third of the children, and decompensated cirrhosis and even HCC have been reported[27,28]. For this reason, noninvasive diagnosis and monitoring of liver fibrosis in children are urgently needed.

There are several features required for an ideal serum fibrosis marker. It should be liver-specific, independent of metabolic alterations, minimally influenced by impaired urinary and biliary excretion, easy to perform and it should measure either the dynamic processes of fibrogenesis or fibrolysis and reflect the degree of fibrosis. Unfortunately, no current assay fulfills enough of these criteria[5].

Recently, algorithms of non-ECM-derived serum markers have been proposed as the predictors of fibrosis stage in adult patients with chronic hepatitis C[29-31]. APO A-I, HPT and A2M are the components of FibroTest[32] and APO A-I and A2M are the components of PGAA index[33,34]; they have also been validated as single serum fibrosis markers[6,35-38].

In the present study, we did not find significantly different levels of examined serum fibrosis markers in children with chronic hepatitis B as compared with the controls. There were also no significant correlation of APO A-I, HPT, and A2M levels with histologically assessed stage of liver fibrosis and inflammation grade. Because liver biopsy is not necessarily a gold standard for assessing liver histology, noninvasive markers will not have complete concordance with histological staging. Only 11.1% children had advanced fibrosis according to Batts and Ludwig[21] and probably for this reason, we also did not observe statistically significant correlation between the examined markers and fibrosis stage.

These findings confirmed previous observation in children by Selimoglu et al[35,37] who also demonstrated that mean APO A-I level of the patients with chronic hepatitis B was not different from controls and did not correlate with fibrosis and inflammation scores. To our knowledge, A2M and HPT as potent serum fibrosis markers have not been assessed in children yet.

In adults with chronic hepatitis B, Oberti et al[6] and Huang and Gong[36] observed that APO A-I and A2M were significantly correlated with the staging of liver fibrosis. Lu et al[34] confirmed that A2M but not APO A-I was significantly correlated with fibrosis stage. However, HPT was negatively associated with fibrosis[38]. These opposing correlation of A2M and HPT with fibrosis staging could be explained by the different roles of hepatocyte growth factor (HGF) and TGF beta 1 in fibrogenesis and acute phase response[39-41]. As seen in experimental fibrosis, transduction with HGF gene suppresses increase of TGF beta 1[39] and the factor stimulates synthesis of A2M[40] and reduces synthesis of haptoglobin[40,41].

Recently, ROC analysis has been recommended to calculate the power of the assays to detect advanced liver fibrosis[42-46]. In this study, the ability of examined biochemical markers to differentiate children with advanced liver fibrosis from those with mild fibrosis was not significant except APO A-I. The high negative predictive value of this marker could potentially be useful in the selection of patients without significant fibrosis and in avoiding invasive liver biopsy in this group of children. Our study is not consistent with the findings of Selimoglu et al[35], because they did not find diagnostic performance of APO A-I for discriminating patients with advanced liver

disease. Usefulness of APO A-I as well as HPT and A2M as potent serum fibrosis markers in patients with chronic liver disease needs to be evaluated in large controlled studies.

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• *RAPID COMMUNICATION* •

Effects of L-carnitine in patients with hepatic encephalopathy

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Abstract

AIM: To evaluate the influence of L-carnitine on mental conditions and ammonia effects on patients with hepatic encephalopathy (HE).

METHODS: One hundred and fifty patients (10 patients with alcoholism, 41 patients with hepatitis virus B infection, 78 patients with hepatitis C virus infection, 21 patients with cryptogenetic cirrhosis) meeting the inclusion criteria were randomized into group A receiving a 90-d treatment with L-carnitine (2 g twice a day) or into group B receiving placebo in double blind.

RESULTS: At the end of the study period, a significant decrease in NH4 fasting serum levels was found in patients with hepatic encephalopathy ($P<0.05$) after the treatment with levocarnitine (LC). Significant differences were also found between symbol digit modalities test and block design in patients with hepatic encephalopathy ($P<0.05$).

CONCLUSION: Results of our study suggest an important protective effect of L-carnitine against ammonia-precipitated encephalopathy in cirrhotic patients.

INTRODUCTION

Hepatic encephalopathy (HE) commonly occurs in patients with liver cirrhosis and is characterized by impaired mental function, neuromuscular disorders and altered states of consciousness. The pathogenesis of HE is controversial although ammonia has been found to induce alterations of cerebral neurotransmitter balance especially at the astrocyte-neuron interface.

Hyperammonemia induces abnormalities such as brain edema and intracranial hypertension in cirrhotic patients. In cirrhotic patients, the relation between blood levels of ammonia and brain events is also influenced by the changes in the permeability-surface area of the blood-brain barrier[1].

In hepatic encephalopathy, the Alzheimer type II astrocytes are the main neuropathological alteration, characterized by swollen cellular nuclei, with their chromatin displaced to the periphery.

The accumulation of glutamine, a product of ammonia detoxification generated within astrocytes expressing activity of glutamine synthetase, is a major factor for the swelling of astrocytes[2].

Previous studies have reported a significant protective effect of L-carnitine in mice, rats, and human beings. In fact L-carnitine treatment is associated with a significant reduction of blood and brain ammonia concentration[3-6]. Carnitine is a natural substance involved in regulating substrate flux and energy balance across cell membranes.

The pharmacological activity of L-carnitine presents various aspects. The carnitine acts by shuttling acetyl-CoA into mitochondria, enhances the metabolic flux in the tricarboxylic acid cycle by sparing free CoA and activating the transport of adenine nucleotides across the inner mitochondrial membrane and prevents adenylate translocase inhibition of the activity of pyruvate dehydrogenase by decreasing the acetyl-CoA/CoA ratio, thus enhancing the oxidative utilization of glucose.

Increased extra-intestinal ammonia production and reduced ammonia detoxification capacity in patients with cirrhosis[7-9] and HE, have not been studied[10].

L-carnitine, inducing ureagenesis, may decrease blood and brain ammonia levels[4,5]. In order to assess the clinical efficacy of L-carnitine in the treatment of HE, a randomized, double-blind, placebo-controlled study with oral administration in cirrhotic patients with hyperammonemia was carried out.

MATERIALS AND METHODS

Patients

One hundred and fifty patients (10 with alcoholism, 41 with hepatitis B virus infection, 78 with hepatitis C virus infection, 21 with cryptogenetic cirrhosis) meeting

the following inclusion criteria were enrolled in the study: chronic hepatitis with spontaneous manifest HE (mental state grade 1 or 2 according to the West Haven criteria) minimal HE (MHE) (mental status grade 0) and a number connection test performance time >30 s[11-13]; hyperammonemia (venous ammonia concentration >50 mmol/L); cooperative, hospitalized, adult patients with liver cirrhosis diagnosed by clinical, histological, and ultrasonographic findings.

Exclusion criteria included: major complications of portal hypertension, such as gastrointestinal blood loss, hepatorenal syndrome or bacterial peritonitis; acute superimposed liver injury; patients with other neurological disease and metabolic disorders, diabetes mellitus, unbalanced heart failure and/or respiratory failure or end-stage renal disease; severe HE (mental state grade 3-4); administration of anti-HE medications such as neomycin, lactulose, lactitol, branched-chain aminoacids; any additional precipitating factors such as high protein intake (additional high-protein meals), constipation or intake of psychostimulants, sedatives, antidepressants, benzodiazepines, or benzodiazepines-antagonists (flumazenil); patients with fever, sepsis or shock were also excluded to avoid variations caused by body temperature.

Study design

Patients meeting the inclusion criteria were randomized either into group receiving a 90-d treatment with L-carnitine (2 g twice daily) or into group receiving placebo in double-blind. Concomitant medications throughout the study included diuretics and beta-blockers.

Patients were visited weekly throughout the treatment period for the assessment of adherence to the study protocol, blood pressure and cognitive functions, as well as recording of adverse events. Throughout the trial, levo-carnitine was supplied as 2 g vials for oral use.

All administered drugs were identical in appearance, and neither investigators nor patients were informed of the selected agents at the end of the study. Administration instructions were provided with each patient pack. All patients were instructed to take the trial medication as prescribed. Patients were considered compliant, if the number of returned vials was between 80% and 120% of the planned treatment regimen. For the duration of the trial, concomitant drugs were administered at the lowest possible therapeutic dosage, and unchanged as far as possible.

Study series

A total of 150 cirrhotic hepatopathic patients met the criteria for inclusion in this study performed between January 2000 and December 2001. They were randomly assigned to receive placebo (75 patients) or L-carnitine (75 patients). The two groups had similar demographic characteristics, etiology, course of disease and Child-Pugh grade. All study series were subdivided into groups of MHE or HE 1 or HE 2 according to the initial HE grade (West-Haven criteria). Group A consisted of patients with MHE (21 patients receiving LC, 19 patients receiving

placebo). Group B consisted of patients with initial HE 1 (30 patients receiving LC, 30 patients receiving placebo). Group C consisted of patients with initial HE 2 (25 patients receiving LC, 25 patients receiving placebo). The effectiveness of therapy was compared and evaluated separately in the different subgroups.

The groups were homogeneous with regard to anamnestic and diagnostic criteria. Differences in composition of the two groups with respect to precipitant factors might be minimized, because the patient population was well defined by inclusion and exclusion criteria.

Diagnosis of minimal hepatic encephalopathy

Psychometric tests and automated EEG analysis were performed for all the patients.

Minimal hepatic encephalopathy was defined as the presence of at least one of the abnormal psychometric tests.

Trail making test: The trail making test was used to evaluate abstract reasoning, tactile performance, tactile-visual and spatial memory, rhythm perception and memory, speech-sound perception, primary motor speed, intelligence, psychomotor speed, sequencing abilities, language function, sensory function, grip strength, and personality functioning.

Time was recorded in seconds. This test included part A and B.

In part A, patients were asked to serially connect digits that were scattered on a page as quickly as possible. In part B, patients were asked to sequentially alternate numbers and letters (i.e. 1-A-2-B-3-C) as quickly as possible. A decrease in the time indicated an improvement in neuropsycological function.

Wechsler adult intelligence scale-revised: Block design test and symbol digit modalities test. The Wechsler adult intelligence scale-revised (WAIS-R) could provide information on global intellectual functioning.

Cognitive functions assessed in the block design test (BDT) were construction, praxis, spatial reasoning motor function, and processing speed. The symbol digit modalities test (SDMT) was used to assess attention, concentration executive function, motor function, and processing speed.

An increase in the number of points in the BDT and SDMT indicated an improvement in neurological function.

Diagnosis of hepatic encephalopathy

Hepatic encephalopathy grade was diagnosed on the basis of the evaluation of consciousness, intellectual functions, behavior and neuromuscular functions according to West Haven criteria introduced by Conn and Liebertahl[11].

Neurophysiological assessment

The EEG was recorded using standardized techniques. Five electrodes were attached to the skin at the positions T3, T4, O1, O2, and Cz according to the international "10-20 system". Electrode impedance was kept lower than 5 KQ. After applying the usual handpass filters (0.53-35 Hz), 2 runs of 100 s each were recorded and

compared for reproducibility. Artefact-free recordings were selected and fed into a computer after digital conversion (sample frequency 102.4 Hz). Ten epochs of 10 s each were analyzed by applying fast Fourier transformation, and the mean power spectrum was calculated.

Patients were graded into different studies of hepatic encephalopathy according to their mean dominant frequency (MDF) and the relative powers of delta and theta activity[14].

Liver function assessment

The Child-Pugh[15] score was determined to assess the severity of cirrhosis, including three biochemical variables (serum albumin, bilirubin, and prothrombin time) and two clinical characteristics (presence or absence of ascites and clinical hepatic encephalopathy).

A patient had a Child-Pugh score A cirrhosis if the score was ≤6 points, a Child-Pugh B cirrhosis if the score was 7-9 points and a Child-Pugh C cirrhosis if the score was >9 points. Patients without signs of ascites were scored as two points for ascites[15].

Venous ammonia concentration

The ammonia was determined according to the enzymatic determination of ammonia with glutamate dehydrogenase in a rapid and interference-free photometric determination (340 nm) of NH_4^+ in native blood plasma as previously described[16].

Due to reasons of safety, blood was immediately sent to the laboratory for determination of NH_4^+.

Safety parameters

Safety parameters included blood tests (hemoglobin, hematocritus, white blood cell count, and thrombocytes) and liver function tests (alanine amino transferase, aspartate amino transferase, gamma glutamyl-transpeptidase, cholinesterase activity, serum bilirubin concentrations, prothrombin time and partial thromboplastin time) on d 0, 30, 60 and 90.

Statistical analysis

Descriptive statistics was proposed from the study sample and results were expressed as mean±SD.

Statistical analyses were performed by two-way analysis of variance (ANOVA), as well as by controlling for multiple correction of Bonferroni.
All *P* values were two-sided, using α = 0.05 as the reference standard for determining the significance of the principal outcomes.

Statistical Analysis System (Cari, NC, USA) software version 6.11 was used for all analyses.

The primary population for statistical analysis was to intent to treat population of all randomized patients (I.T.T.).

RESULTS
Baseline values

The two groups had similar general characteristics. Serum NH_4^+ fasting concentrations were not significantly different before the treatment.

Comparison with baseline

In MHE, ammonia serum levels were significantly decreased to 13.10 μmol/L (CI-24.3 to-1.8; *P*<0.05), to 19.10 μmol/L (CI-30.2 to-8.0; *P*<0.001), to 28.1 μmol/L (CI-38.5 to 17.6; *P*<0.001) after 30, 60, and 90 d of treatment, respectively.

The trial making test-A and trial making test-B were significantly decreased to 13.80 s (CI-19.87 to-7.73; *P*<0.05), to 25.00 s (CI-29.09 to −20.91; *P*<0.05), to 18.00 s (CI-23.50 to-12.50; *P*<0.05) and to 26.00 s (CI-29.61 to-22.39; *P*<0.05), to 21.50 s (CI-26.62 to-16.38; *P*<0.05), and to 28.9 (CI-32 to-25; *P*<0.05) respectively, after 30, 60 and 90 d of treatment.

The symbol digit modalities test was significantly increased from 4.00 points (CI 3.51 to 4.49; *P*<0.05), after 30 d of treatment, to 8.00 points (CI 7.56 to 8.44; *P*<0.05), and 8.00 points (CI 7.53 to 8.47; *P*<0.05) after 60 and 90 d of treatment, respectively.

The block design in MHE were significantly increased from 4.10 points (CI 3.59 to 4.61; *P*<0.05), to 7.90 points (CI 7.35 to 8.45; *P*<0.05), and to 12.90 points (CI 12.53 to 13.27; *P*<0.05) respectively after 30, 60, and 90 d of treatment.

In HE1, ammonia serum levels were significantly decreased to 12.0 μmol/L (CI-22.57 to-3.65; *P*<0.05), to 23.90 μmol/L (CI-33.23 to-14.57; *P*<0.05) and to 41.00 (CI -50.23 to 31.77; *P*<0.05) respectively after 30, 60, 90 d of treatment.

The TMT A and B were significantly decreased to 11.60 s (CI-18.08 to-5.12; *P*<0.05) and to 9.20 s (CI-12.62 to-5.78; *P*<0.05), to 21.60 s (-27.73 to-15.47; *P*<0.05) and to 19.10 s (CI-22.70 to-15.50; *P*<0.05), to 28.80 s (CI-34.87 to-22.73; *P*<0.05) and to 23.80 s (CI-27.06 to 20.54; *P*<0.05) respectively after 30, 60, 90 d of treatment.

The SDMT were significantly increased from 5.10 (CI 4.45 to 5.75; *P*<0.05) to 8.00 (CI 7.30 to 8.70; *P*<0.05) and to 14.00 (CI 13.39 to 14.10; *P*<0.05) respectively after 30, 60, 90 d of treatment.

The BDT were significantly increased from 3.00 points (CI 2.40 to 3.60; *P*<0.05) from 6.10 points (CI 5.47 to 6.73; *P*<0.05) and from 12.10 points (CI 11.62 to 12.58; *P*<0.05) respectively after 30, 60 and 90 d of treatment.

In HE2, ammonia serum levels were significantly decreased to 15.10 μmol/L (CI-23.44 to-6.76; *P*<0.05) and to 36.00 μmol/L (CI-44.84 to-27.16; *P*<0.05) respectively after 60 and 90 d of treatment.

The TMT A and B were significantly decreased to 10.50 s (CI-16.97 to-4.03; *P*<0.05) and to 9.50 s (CI-13.31 to-5.60; *P*<0.05), to 26.20 s (CI 32.97 to-22.39; *P*<0.05) and 21.79 s (CI-25.70 to 17.88; *P*<0.05), to 30.20 s (CI-36.48 to 23.92; *P*<0.05) and 35.00 s (CI 39.13 to 30.87; *P*<0.05) respectively after 30, 60, and 90 d of treatment.

The SDMT were significantly increased to 3.10 points (CI 2.56 to 3.64; *P*<0.05), to 7.00 points (CI 4.15 to 9.85; *P*<0.05) and to 12.90 points (CI 12.27 to 13.53; *P*<0.05) respectively after 30, 60, and 90 d of treatment.

The BDT were significantly increased from 4.10 points (CI 3.5 to 4.7; $P<0.05$) to 8.10 points (CI 7.60 to 8.60; $P<0.05$) and to 13.00 points (CI 12.40 to 13.60; $P<0.05$) respectively after 30, 60, and 90 d of treatment.

No significant differences were observed in the patients treated with placebo compared to baseline (Table1).

Table 1 Baseline data of patients

Parameter	Carnitine group, $n = 75$	Placebo group, $n = 75$
Male/female	50/25	45/30
Age (yr)	51.7 ± 9.6	53.2 ± 9.2
Cirrhosis etiology		
Alcohol	7	5
Post-hepatitis B	20	22
Post-hepatitis C	38	38
Cryptogenetic	10	10
Child–Pugh class		
A	30	31
B	34	34
C	11	10
Prothrombin time (%)	62.8 ± 6.9	63.1 ± 6.8
Serum albumin level (g/dL)	2.9 ± 0.7	2.8 ± 0.9
Serum bilirubin level (mg/dL)	3.1 ± 1.2	3.2 ± 1.4
Serum alanine aminotransferase level (IU/L)	119 ± 74	116 ± 77
Blood urea nitrogen (mg/dL)	40 ± 9	39 ± 11
Serum creatinine level (mg/dL)	0.88 ± 0.21	0.82 ± 0.30
Natriemia (mEq/L)	136 ± 3.4	138 ± 4.7
Kaliemia (mEq/L)	4.1 ± 1.2	4.2 ± 0.9

Comparison between treatments

At the end of the study period, fasting serum levels of NH4, TMT-A, TMT-B were significantly decreased in patients with hepatic encephalopathy compared to controls ($P<0.05$) after treatment with LC and placebo respectively. MHE-28.10 *vs* -2.00, $P < 0.05$; HE1-41.00 *vs* -1.50, $P<0.05$; HE2-36.00 *vs* 3.90, $P < 0.05$; TMT-A MHE -21.50 *vs* -7.30, $P < 0.05$, HE1 -28.8 *vs* -7.30, $P<0.05$; HE2 -30.20 *vs* -2.90, $P < 0.05$; TMT-B MHE-28.9 *vs* 1.0, $P < 0.05$; HE1-23.8 *vs* -4.00, $P < 0.05$; HE2-12.29 *vs* 0.90, $P < 0.05$.

Significant differences were also found between symbol digit modalities test and block design.

The SDMT was increased to 4.00 *vs* 0.20, 2.90 *vs* 0.80, 3.90 *vs* 0.20, respectively in MHE, HE1, and HE2 ($P<0.05$). The block design was increased to 3.80 *vs* 0.40, 3.10 *vs* 0.40, 4.00 *vs* 1.00, respectively in MHE, HE1, and HE2 ($P<0.05$) (Table 2).

DISCUSSION

The pathogenetic mechanisms underlying the development of hepatic encephalopathy are complex.

Symptoms of hepatic encephalopathy are generally reversible, suggesting a metabolic cause. Among the possible neurotoxins implicated in hepatic encephalopathy, ammonia is considered as a leading candidate[17,18].

The majority of blood ammonia results from muscle protein catabolism at the intestinal level. The remnant is produced by the action of colic bacteria on the nitrium present in digested foods. Ammonia is vehicled to the liver throughout the portal flux and normally eliminated as urea. The liver damage or the presence of porto-systemic shunts increases its serum levels[19-21]. The excess ammonia is eliminated from the blood by transforming glutamate into glutamine in skeletal muscle and central nervous system. Neurotoxicity of ammonia is probably due to a direct action on neurons, because the reduction of glutamate and the increase of glutamine may induce swelling of the astrocytes[22,23].

O'Connor *et al*[19] observed that mice treated with L-carnitine experience a continuous rise in blood urea N until a plateau is reached 1 h after injection, whereas mice not treated with L-carnitine experience a rise in blood urea N, but one died within 15 min after ammonium acetate administration.

Intravenous L-carnitine significantly can lower plasma ammonia N levels in ewes after an oral urea challenge even though the concentration of ruminal free, non-ionized ammonia N is similar to those in ewes treated only with the urea solution, suggesting that L-carnitine administration may prevent hyperammonemia in ruminants[24].

Carnitine has been shown to be efficacious in valproic acid hyperammonemia[25]. Preventive supplementations with L-carnitine might afford some benefits. Ohtani *et al*[26] have showed that oral administration of carnitine for 4 wk corrects hyperammonemia in patients treated with valproic acid. Studies in valproate-treated rats indicate that L-carnitine supplementation can correct hyperammonemia, hypocarnitinemia and protect rat liver against mitochondrial swelling[27].

Results of our study showed a protective effect of L-carnitine against ammonia-precipitated encephalopathy in cirrhotic patients[6].

In patients with MHE, HE 1, or 2, we observed a significant reduction of ammonia serum levels after 30 d of treatment. After 60 and 90 d of treatment, we observed a persistent trend of decreased ammonia serum levels.

A significant therapeutic effect of carnitine was also observed in the NCT-A, which is an accepted and reliable psychometric test for the assessment of mental function in cirrhotic patients with HE[12,28]. L-carnitine crosses the hemato-encephalic barrier slowly (brain uptake index 5.5%) but its amount in brain is relatively large[29]. The protective effect of L-carnitine is accompanied with a significant attenuation of the increased cerebrospinal fluid and brain alanine as well as cerebrospinal fluid lactate content, caused by ammonium acetate administration[30], suggesting that mitochondrial respiration is at least partially restored in L-carnitine-treated animals. The possible beneficial effect of carnitine may be related to an improved pyruvate oxidation, Krebs cycle and flux through

Table 2 Comparison between evaluated parameters of the two groups (mean+SD)

		Carnitine group (A)				Placebo group (B)			
Duration of treatment (days)		0	30	60	90	0	30	60	90
NH₄ fasting	MHE	68.2±38.2	55.1±31.4	49.1±30.1	40.1±25.1	69.1±39.2	68.2±38.2	68.2±33.6	67.1±34.1
	HE1	82.1±29.1	69.2±28.2	58.2±28.7	41.4±28.1	80.1±30.1	78.7±36.2	77.2±33.4	76.2±31.8
	HE2	89.2±34.2	80.1±28.1	74.1±12.9	53.2±18.2	88.2±34.1	87.1±35.2	86.1±38.1	85.4±39.2
Trail making test-A	MHE	48.9±18.7	35.1±18.9	30.9±15.2	274±12.4	51.4±19.2	50.2±18.9	52.1±20.1	50.4±20.2
	HE1	62.4±21.2	50.8±18.9	40.8±16.5	33.6±16.1	60.9±19.9	59.9±19.8	58.4±20.1	53.6±19.8
	HE2	69.3±21.8	58.8±18.1	43.1±20.1	39.1±16.8	64.3±22.4	65.6±20.4	64.9±21.9	61.4±22.9
Trail making test-B	MHE	66.1±12.1	41.1±13.2	40.1±10.2	37.2±12.1	65.2±13.1	61.1±14.1	60.1±13.2	59±14.1
	HE1	74.2±10.1	65±11.1	55.1±12.1	50.4±10.1	75.8±11.9	74.2±12.1	70.11±12.8	71.4±13.2
	HE2	96.9±12.4	87.4±11.2	75.11±11.8	61.9±13.2	91.4±12.1	90.6±10.8	91.5±10.6	93.4±11.8
Symbol digit modalities test	MHE	36±1.2	40±1.8	44±1.5	44±1.7	35±1.9	36±2.1	36.2±11.8	36.4±22.2
	HE1	30±1.9	35.1±2.1	38±2.4	44±1.9	31.2±1.8	32.4±1.9	33.2±1.8	34.1±1.9
	HE2	25.1±1.4	28.2±1.9	32.1±12.4	38±2.4	26.1±2.1	27.2±2.4	28.1±1.9	27.9±1.8
Block design test	MHE	31.1±1.2	35.2±1.9	39±2.1	44±1.1	30.1±2.8	32±2.6	32.4±2.9	32.7±1.8
	HE1	27±1.8	30±1.9	33.1±2.1	39.1±1.1	26±2.9	28±1.9	28.4±2.1	27.1±2.2
	HE2	22±1.9	26.1±1.8	30.1±1.1	35±1.8	22.6±21	23.2±1.4	24.2±1.9	24.8±2.1

glutamate dehydrogenase. The latter could then explain the lowering of blood ammonia levels that follows L-carnitine administration .

The best-known function on L-carnitine is the facilitation of b-oxidation by transforming activated long-chain fatty acids into mitochondria. High concentration of fatty acids has numerous deleterious effects upon mitochondrial metabolism.

Acyl-CoA derivates competitively inhibit the activation of the gluconeogenetic enzyme pyruvate carboxylase with acetyl-CoA[31]. Thus when mitochondrial b-oxidation is inhibited, not only is acetyl-CoA, but also the activating effect of acetyl-CoA is further inhibited by non-esterified acyl-CoA esters, an effect that further decreases gluconeogenesis.

High levels of acyl-CoA derivates also may inhibit ureagenesis (resulting in hyperammonemia and the tricarboxylic acid cycle)[32].

Treatment with levocarnitine decreases the serum levels of ammonia and improves the mental functions in cirrhotic patients, and has been proven effective with small adverse events.

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ELSEVIER

• *RAPID COMMUNICATION* •

Synergistic action of famotidine and chlorpheniramine on acetic acid-induced chronic gastric ulcer in rats

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Abstract

AIM: To assess the synergistic action of famotidine (FMD) and chlorpheniramine (CPA) on acetic acid-induced chronic gastric ulcer in rats.

METHODS: Chronic gastric lesions were induced in male Sprague-Dawley (SD) rats by serosal application of the acetic acid. Forty SD rats were randomly divided into blank group ($n = 8$), control group ($n = 8$), FMD group ($n = 8$), CPA group ($n = 8$), and FMD+CPA group ($n = 8$). Each group was given intraperitoneally (i.p.) 0.5 mL/100 g distilled water, 9 g/L NaCl saline, 4 mg/kg FMD, 10 mg/kg CPA, 4 mg/kg FMD+10 mg/kg CPA, respectively, daily for 10 d. On d 10, ulcer area was determined by planimetry. The level of myeloperoxidase (MPO) in the liver homogenation was determined by biochemical methods and the plasma levels of 6-ketoprostaglandin F1 alpha (6-keto-PGF$_{1a}$) and IL-8 were determined by radioimmunoassay.

RESULTS: The synergistic effects of FMD+CPA group on the lesion, IL-8, 6-keto-PGF$_{1a}$ and MPO were confirmed. The effect of FMD+CPA group was significantly different as compared to the control and FMD groups. The lesion (mm^2) was reduced from 40.18±2.6 in control group to 6.83±2.97 in PMD+CPA group, $P<0.01$, and from 32.9 ±3.27 in FMD group to 6.83±2.97 in PMD+CPA group, $P<0.01$. The plasma levels of IL-8 decreased from 0.69± 0.11 ng/L in control group to 0.4±0.04 ng/L in PMD+CPA group, $P<0.01$, and from 0.51±0.08 ng/L in FMD group to 0.4±0.04 ng/L in PMD+CPA group, $P<0.05$. The level of 6-keto-PGF$_{1a}$ increased from 7.55±1.65 ng/L in control group to 16.62±0.97 ng/L in PMD+CPA group, $P<0.01$, and from 13.15±1.48 ng/L in FMD group to 16.62±0.97 ng/L in PMD+CPA group, $P<0.05$. The levels of MPO in the liver homogenate decreased from 9.12±2.05 u/L

in control group to 4.33±0.95 u/L in PMD+CPA group, $P<0.01$, and from 8.3±1.29 u/L in FMD group to 4.33± 0.95 u/L, $P<0.01$.

CONCLUSION: The synergistic action of FMD and CPA on acetic acid-induced chronic gastric ulcer in rats decreases the incidence of ulcer and also enhances the healing of ulcer.

INTRODUCTION

Peptic ulcer is a common disorder of the gastrointestinal system and the pathogenesis of peptic ulcer disease is multifactorial, including *Helicobacter pylori*, gastric acid, pepsin, gastroduodenal motility, smoking, use of nicotine, and complex interaction between so-called aggressive and protective factors[1]. Mast cells are initiators and regulators of inflammation. After mast cell degranulation, histamine causes the secretion of gastric acid by triggering H$_2$-receptors, marked infiltration of inflammatory cells into the gastric mucosa, and expression of cytokines by triggering the H$_1$-receptors. Consequently, mast cells are considered as important effector cells in the pathogenesis of gastritis, especially in *H pylori*-associated peptic ulcer[2]. Histamine H$_2$-receptor antagonists that possess a potent antisecretory activity can greatly enhance the healing rate of peptic ulcers. However, after H$_2$-receptor antagonist therapy peptic ulcer recurs rapidly and frequently. The possible reasons why the recurrence rate is high after H$_2$-antagonist therapy are acid rebound after the cessation of treatment, deficiency in gastric defensive factors such as gastric prostaglandin levels, and low maturity of regenerated mucosa. Recently, H$_1$-receptor antagonists are potent anti-inflammatory compounds. Erwin[3] reported that H$_1$-receptor antagonists have anti-inflammatory activity, including their effects on eicosanoid production

and cytokine release and their influence on the release rate of proinflammatory mediators. Cetirizine reduces the attraction of inflammatory cells to the inflammatory focus after the antigen challenge and inhibits the expression of the intercellular adhesion molecule 1 (ICAM-1) on the surface of epithelial cells[4]. It also might be able to block the antigen-induced production of leukotrienes (LTC4)[5]. Loratadine and its metabolite decarboethoxyloratadine inhibit the release of tryptase and amacroglobulin[6], interleukins (IL) 6 and 8[7], leukotrienes and prostaglandin D2 (PGD2)[8], and suppress the expression of ICAM-1 and of HLA-II antigens on the surface of epithelial cells[9]. Therefore, H_1 and H_2 blockers have not only antisecretory activity but also anti-inflammatory activity when they are used in the treatment of gastric ulcer. The aim of the present study was to assess the synergistic action of famotidine (FMD) and chlorpheniramine (CPA) on acetic acid-induced chronic gastric ulcer in rats.

MATERIALS AND METHODS

Materials
IL-8 and 6-ketoprostaglandin F1 alpha (6-keto-PGF$_{1a}$) kits were obtained from Beijing North Institute of Biological Technology. Myeloperoxidase (MPO) kit was obtained from Nanjing Jiancheng Bioengineering Institute.

Animals
Male Sprague-Dawley (SD) rats (180-220 g) were provided by the Medical Experimental Animal Center, Tongji Medical College, Huazhong University of Science and Technology (No: TJLA-2004-159). Animals were fed with standard laboratory chow and tap water, and kept in a room with constant humidity and temperature (25 °C) in a 12-h light-dark cycle for 1 wk before the experiments.

Induction of gastric ulcer
Gastric ulcers were induced using the method described by Takagi et al[10] with some modifications. Gastric ulcers were produced by the application of round filter paper (diameter: 5 mm) immersed in a 100% acetic acid on the serosal surface of anterior wall of the stomach approximately at the center of the corpus for 30 s and the process was repeated twice. This produced an immediate necrosis of the entire mucosa and submucosa (but not serosa) within the area (20 mm^2), where the acetic acid was applied. The excess of acetic acid was then removed and the serosa was gently washed with saline. Control animals received no surgery. The abdomen was then sutured, and the animals were allowed to recover and returned to their cages with free access to food and water.

Experimental groups
Two days after the ulcer induction, 40 male SD rats, weighing 180-220 g, were randomly divided into blank group (n = 8), control group (n = 8), FMD group (n = 8), CPA group (n = 8), FMD+CPA group (n = 8). Rats were given intraperitoneally (i.p.) 0.5 mL/100 g distilled water, 0.5 mL/100 g, 9 g/L NaCl saline, 4 mg/kg FMD, 10 mg/kg CPA, 4 mg/kg FMD+10 mg/kg CPA for 10 d. On d 8, the rats were deprived of food for 48 h prior to the experiment but allowed free access to water. After the last dose, each animal was anesthetized with 10 g/L sodium thiopental. Five milliliters of blood was collected by intracardiac puncture. Each sample contained 0.22 mL 6-keto EDTA, and was centrifuged for 15 min at 5 500 r/min. The plasma from each sample was stored at -80 °C for the determination of the level of interleukin IL-8 and 6-keto-PGF1a by RIA using IL-8 and 6-keto PGF1a kits, respectively. Subsequently, the abdomen was opened and the stomach was exposed after the esophagus and pyloric were ligated, and then 5 mL 40 g/L neutral buffered formalin was instilled into the stomach via the incision for pathological examination.

Ulcerated area (mm^2) determination
After being fixed in formalin overnight, the stomach was opened along the greater curvature and spread out with pins on a cork board, and then photographed. The ulcerated area (mm^2)[11] was computed using the following equation: $S = \pi(d_1/2) \times (d_2/2)$, where S represents the ulcerated area (mm^2), d_1 and d_2 represent the longest longitudinal and transverse diameters of the ulcer respectively.

Light microscopy
After fixation, the stomach was divided into two parts and each part was subdivided into four tissue strips by cutting along the whole width of the half stomach. The blocks were embedded in paraffin wax. Five micrometer thick sections were cut in a standard fashion and stained with hematoxylin and eosin.

Radioimmunoassay (RIA) for IL-8 and 6-keto-PGF1a
All the blood samples for IL-8 and 6-keto-PGF$_{1a}$ determination were stored in the tubes. Serum level of IL-8 and 6-keto-PGF$_{1a}$ was determined by RIA with the IL-8 and 6-keto-PGF$_{1a}$ RIA kits according to their manufacturer's instructions.

MPO activity
The level of MPO in the liver homogenate was determined by biochemical methods with the MPO kits according to its manufacturer's instructions.

Statistical analysis
All the values were expressed as mean±SD. One-way ANOVA was used to analyze the differences among them. Student's t-test was applied to comparisons between the two groups. $P < 0.05$ was considered statistically significant. Software SPSS10.0 was used in all statistical analyses.
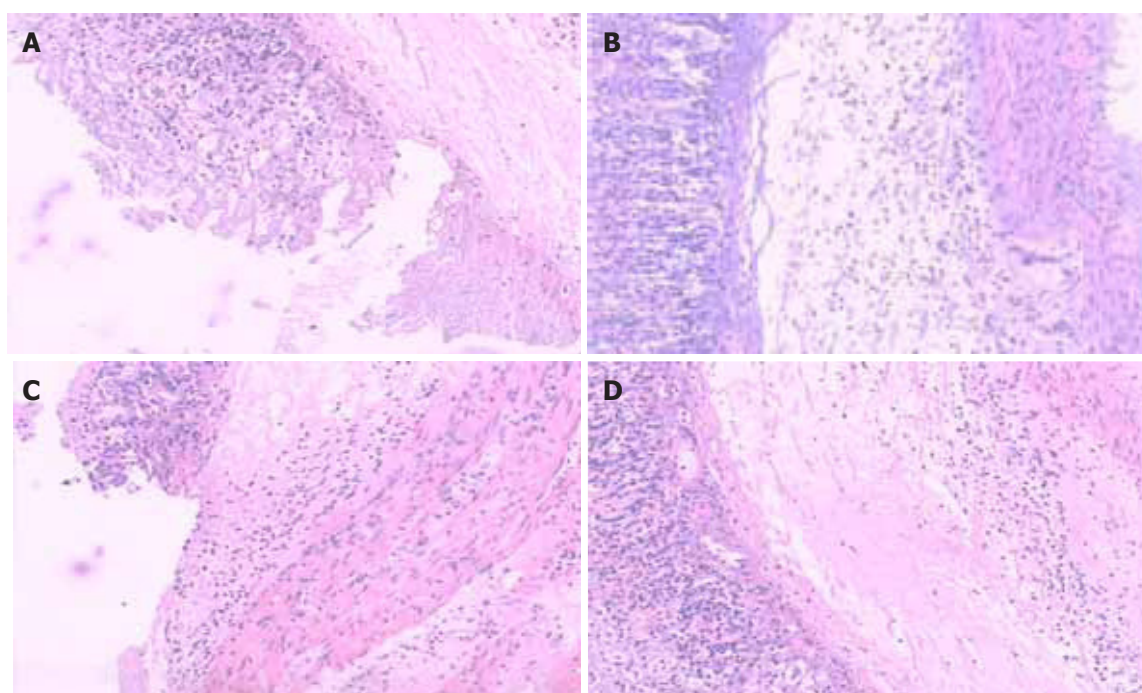
RESULTS

Ulcerated area (mm^2)
The lesion (mm^2) decreased from 40.18±2.6 in control

Table 1 Effects of combined famotidine (FMD) and chlorpheniramine (CPA) on the ulcerated area in different groups (mean±SD)

Group	Dose (mg/kg)	Area of ulcer (mm^2)	6-keto-PGF$_{1a}$ (ng/L)	IL-8 (ng/L)	MPO (μ/L)
Blank		0	22.74±5.03	0.3±0.09	3.13±0.39
Control		40.18±2.6[b]	7.55±1.65[b]	0.69±0.11[b]	9.12±2.05[b]
FMD	4	32.9±3.27[d]	13.15±1.48[d]	0.51±0.08[d]	8.3±1.29
CPA	10	35.15±3.0[d]	10.79±1.48[c]	0.41±0.07[d]	5.7±1.00[d]
FMD+CPA	4+10	6.83±2.97[d,f]	16.62±0.97[d,e]	0.4±0.04 [d,e]	4.33±0.95[d,f]

[a]$P<0.05$; [b]$P<0.01$ *vs* blank; [c]$P<0.05$; [d]$P<0.01$ *vs* control; [e]$P<0.05$; [f]$P<0.01$ *vs* FMD.

Figure 1 Pathological feature of gastric mucosa in the control group (**A**), FMD group (**B**), CPA group (**C**), and FMD+CPA group (**D**) under light microscope, HE ×200.

group to 0 in the blank group. The lesion (mm^2) was reduced from 40.18±2.6 in the control group to 6.83±2.97 in the PMD+CPA group ($P<0.01$), and from 32.9±3.27 in FMD group to 6.83±2.97 in PMD+CPA group ($P<0.01$) (Table 1).

Light microscopy
Microscopic findings on the gastric mucosal scar showed that the mucosal architecture of the scar healed by FMD+CPA group (Figure 1D) was better restored than that of the scar in the control group or in the case of treatment with FMD alone which exhibited much lymphocyte infiltration, and surface epithelial lesions (Figures 1A and B).

Plasma level of 6-keto-PGF$_{1a}$
The level of 6-keto-PGF$_{1a}$ in the FMD+CPA group was significantly different from that in the control and the FMD groups, which increased from 7.55±1.65 ng/L in the control group to 16.62±0.97 ng/L in the PMD+CPA group ($P<0.01$) ng/L, and from 13.15±1.48 ng/L in

the FMD group to 16.62±0.97 ng/L in the PMD+CPA group, $P<0.05$ (Table 1).

MPO level in liver homogenate
In the liver homogenate, the level of MPO activity decreased from 9.12±2.05 μ/L in the control group to 4.33±0.95 μ/L in the PMD+CPA group, $P<0.01$, and from 8.3±1.29 μ/L in the FMD group to 4.33±0.95 μ/L ($P<0.01$) (Table 1).

The plasma levels of IL-8
The plasma level of IL-8 decreased from 0.69±0.11 ng/L in the control group to 0.4±0.04 ng/L, $P<0.01$, and from 0.51±0.08 ng/L in the FMD group to 0.4±0.04 ng/L in the PMD+CPA group ($P<0.05$) (Table 1).

DISCUSSION

The acetic acid injection-induced ulcer model is a mature classical model. The drawback of the model is that the dose of injection is not rigorous. We used a round filter paper (diameter: 5 mm) immersed in a 100% acetic acid

on the serosal surface to establish the model, which is characterized by high success rate, small coefficient of variation, low rate of perforation.

Prostaglandins (PGs) are well-known mucosal defense factors, protecting the gastric mucosa against injury caused by a variety of toxic stimuli[12,13]. PGE2 stimulates the secretion of gastric mucus and bicarbonate, increases mucosal blood flow, inhibits acid secretion, and reduces gastric motility. In addition, downregulation of proinflammatory cytokine expression by PGs is also likely to be important for mucosal protection against *H pylori* infection. 6-Keto PGF$_{1a}$ is a PGI2 metabolite, and PGI2 plays an important role in gastric cytoprotection. This study also approved that the ulcerated area decreased with the increase in the level of 6-Keto PGF$_{1a}$ and this supports that prostaglandins possess gastric cytoprotection function.

Gastric mucosal integrity is maintained by an interplay of some aggressive and defensive factors controlling cell apoptosis and proliferation. Disturbing this balance leads to ulcer. Proinflammatory cytokines play an important role. IL-8 is an important cytokine in the host inflammatory response to *H pylori*[14-16], which correlates with its induction in gastric epithelial cells co-cultured with *H pylori in vitro*[17-19]. Upregulation of IL-8 by *H pylori* may lead to free radical generation and the release of proteolytic enzymes from activated neutrophils, affecting mucosal integrity[20]. Eradication of *H pylori* in ulcer patients results in a reduction in antral IL-8 mRNA expression, neutrophil infiltration, and surface epithelial lesions[16], suggesting that inflammatory cytokines may play an important role in mucosal damage due to *H pylori* infection. Leukocyte infiltration in gastric mucosa is assessed by determining tissue activity of MPO[21], an enzyme used as a marker for leukocyte infiltration in a variety of tissues including the rat gastric mucosa. Recently, experimental data on H$_1$ receptor antagonists demonstrate potentially anti-inflammatory effects in addition to their anti-allergic effects on histamine production, the histamine-induced response. Cook et al[22] reported that human conjunctival mast cells can be purified (>95%) from cadaveric tissues and that olopatadine inhibits anti-IgE antibody-mediated release of TNF-α from human conjunctival mast cells. Bakker[23] showed that the histamine H$_1$ receptor, which is also an important player in allergic and inflammatory conditions, activates NF-κB in a constitutive- and agonist-dependent manner and that the observed constitutive NF-κB activation is inhibited by various H$_1$-receptor antagonists, suggesting that inverse agonism may account, at least in part, for their ascribed antiallergic properties. Miki et al[24] showed that histamine concentration-dependently enhances the TNF-α-induced expression of E-selectin and intercellular adhesion molecule-1 (ICAM-1) in vascular endothelial cells. Arnold[25] suggested that cetirizine reduced the release of IL-8 from A549 cells stimulated with PMA and TNF-α, by lowering IL-8 gene expression. Modlin et al[26] reported that histamine may also act as an autocrine growth factor for ECL hyperplasia via H$_1$ receptors. Therefore, histamine may be an important regulator of all gastric endocrine cells. It was reported that

a high dose FMD administration significantly increases serum gastrin concentration and proliferates G and ECL cells[27] indicating that the synergistic action of histamine H$_1$ receptor antagonists and histamine H$_2$ receptor antagonists may reduce the incidence rate of carcinoids.

The present experimental study demonstrated that FMD, a H$_2$ receptor antagonist, afforded against gastric ulcers. Chlorpheniramine, a H$_1$ receptor antagonist could give partial protection. When both H$_1$ and H$_2$ blockers were given, they decreased the incidence of ulcer and also enhanced the healing of ulcers. At the same time, this study showed that H$_1$-antagonists reduced the release of proinflammatory mediators (IL-8 and MPO) from mast cells in comparison with the controls. The synergistic effect indicates that H$_1$ receptors play a role in gastric ulceration as the H$_2$ receptors.

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• *RAPID COMMUNICATION* •

Effect of liniment levamisole on cellular immune functions of patients with chronic hepatitis B

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Abstract

AIM: To explore the effects of liniment levamisole on cellular immune functions of patients with chronic hepatitis B.

METHODS: The levels of T lymphocyte subsets and mIL-2R in peripheral blood mononuclear cells (PBMCs) were measured by biotin-streptavidin (BSA) technique in patients with chronic hepatitis B before and after the treatment with liniment levamisole.

RESULTS: After one course of treatment with liniment levamisole, the levels of $CD3^+$, $CD4^+$, and the ratio of $CD4^+/CD8^+$ increased as compared to those before the treatment but the level of $CD8^+$ decreased. The total expression level of mIL-2R in PBMCs increased before and after the treatment with liniment levamisole.

CONCLUSION: Liniment levamisole may reinforce cellular immune functions of patients with chronic hepatitis B.

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INTRODUCTION

It has been reported that cellular immune functions are abnormal in patients with hepatitis B[1]. To explore the effects of liniment levamisole on cellular immune functions of patients with chronic hepatitis B, we measured the levels of $CD3^+$, $CD4^+$, $CD8^+$ and the ratio of $CD4^+/CD8^+$ cells as well as the expression level of mIL-2R in PBMC in patients with chronic hepatitis B before and after the treatment with liniment levamisole. The results support that levamisole can improve cellular immune functions of patients with chronic hepatitis B.

MATERIALS AND METHODS

Patients

According to the diagnostic criteria modified during the 10th National Conference on Viral Hepatitis and Hepatopathy 2 000, 76 patients with chronic hepatitis B (45 males and 21 females) aged 25-64 years (average 48.25 years) were enrolled in this study and randomly divided into treatment group ($n = 38$, 27 males and 11 females, aged 32-64 years and averaged 51.8 years) and control group ($n = 38$, 18 males and 10 females, aged 25-58 years and averaged 44.7 years).

Treatment methods

Vitamins B and C, silymarin compound and other liver-protecting drugs were used in the 76 patients. The treatment group was primed with liniment levamisole at a dose of 500 mg twice a week for 3 mo (a course of treatment). Five milliliters of liniment levamisole was smeared on the surface of skin inside the four limbs near the body. The area of the skin should be as large as possible and the smeared skin was washed within 24 h to assure absorption of the drug. No antiviral agent or immunomodulator was used in the two groups.

Samples

Five milliliters of peripheral vein blood was collected at 8:00 a.m. from each patient with hepatitis B and 2.5 mL was put into a sterile test tube and 2.5 mL into an anticoagulant test tube with heparin.

Separation of PBMC and detection of T cell subsets, mIL-2R

After the heparinized blood was mixed with an equal

volume of Hanks' liquid without Ca^{2+} and Mg^{2+}, PBMCs were harvested from heparinized whole blood by centrifugation on Ficoll-Hypaque sedimentation gradient and diluted to $(1-3)\times10^6$/L cell suspension with RPMI 1 640 culture liquid. Ten microliter suspension of PBMCs was smeared on sheet glass pores so that the cells with $CD3^+$, $CD4^+$, $CD8^+$ and the rest phrase of mIL-2R could be detected. Of the PBMC suspension, 0.5 mL was mixed with RPMI 1640 culture liquid. The cells were grown in continuous culture (37 ℃, 50 mL/L CO_2 in atmosphere) for 72 h and mIL-2R could be measured by antibodies against the membrane of T cells[1].

Immunocytochemical BSA technique

Different monoclonal antibodies (mAb) against $CD3^+$, $CD4^+$, $CD8^+$ and Tac with biotin and SA-HRP were smeared on different sheet glasses. The smears were left to dry naturally and fixed with acetone for 15-20 min. The cells were incubated in continuous culture (37 ℃, 50 mL/L CO_2 in atmosphere) for 30 min. The immune sheet glass pores were measured after being stained with color-developing agents and washed with Tris buffer solution (TBS). The total number of PBMCs was counted and the positive cells were analyzed with the help of high power lens.

Reagents and instruments

Antibodies against T-lymphocyte subsets were provided by Shanghai Jing'an Medical Institute; Ficoll-Hypaque sedimentation gradients were offered by Shanghai Second Reagent Factory. Carbon dioxide incubator (MDF-135) was from Japan.

Statistical analysis

Statistical analysis was performed using t test to determine the difference between the two groups.

RESULTS

Before the treatment, there was no significant difference in the levels of $CD3^+$, $CD4^+$, $CD8^+$, mIL-2R and the ratio of $CD4^+$/$CD8^+$ between the two groups. After one course of treatment, the levels of $CD3^+$, $CD4^+$ and the ratio of $CD4^+$/$CD8^+$ increased, while the level of $CD8^+$ decreased. The total expression level of mIL-2R in PBMCs increased before and after the treatment with liniment levamisole.

DISCUSSION

This study demonstrated that levamisole could exert its immunopotentiating activity in patients with chronic hepatitis B. As an immune stimulant, levamisole could be connected with receptors of thymopentin on the surfaces of immunologic cells and induces inhibited T lymphocytes into immunologilly competent cells, which take part in cell-mediated immune, induce interferon production and prime the lymphocytes and macrophages[2-11]. According to clinical experiments, levamisole has therapeutic

effectiveness and boosts immunity in a variety of infectious diseases and some cancers[11-24]. Krastev *et al*[25] treated 25 viremic patients with chronic HBV infection with LMS and found that LMS may benefit some patients with chronic ongoing viral replication including patients who are contraindicated for TNF-α. It was reported that LMS acts by resetting the immune balance toward a type 1 response via the induction of IL-18[26].

We have previously shown that decreased $CD3^+$ and $CD4^+$ levels and $CD4^+$/$CD8^+$ ratio as well as increased $CD8^+$ level occur in patients with chronic hepatitis B[1]. We have also shown that the level of mIL-2R in PBMCs is lower than that in normal controls[1]. In the present study, all these parameters including the levels of $CD3^+$, $CD4^+$, $CD8^+$, mIL-2R and the $CD4^+$/$CD8^+$ ratio became normal in the levamisole-treated patients. This reversion of aberrant cellular immunity may improve symptoms of chronic hepatitis B patients.

Liniment levamisole is made from levamisole, which is applied on the surface of skin and can penetrate into the liver, spleen, lungs, kidneys and other internal organs through the skin. We suggest that liniment levamisole should be used in the treatment of patients with chronic hepatitis B.

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• *RAPID COMMUNICATION* •

Correlation between the expressions of gastrin, somatostatin and cyclin and cyclin-depend kinase in colorectal cancer

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Abstract

AIM: To explore the correlation between the expressions of gastrin (GAS), somatostatin (SS) and cyclin, cyclin-dependent kinase (CDK) in colorectal cancer, and to detect the specific regulatory sites where gastrointestinal hormone regulates cell proliferation.

METHODS: Seventy-nine resected large intestine carcinomatous specimens were randomly selected. Immunohistochemical staining for GAS, SS, cyclin D1, cyclin E, cyclin A, cyclin B1, CDK2 and CDK4 was performed according to the standard streptavidin-biotin-peroxidase (S-P) method. According to the semi-quantitative integral evaluation, SS and GAS were divided into high, middle and low groups. Cyclin D1, cyclin E, cyclin A, cyclin B1, CDK2, CDK4 expressions in the three GAS and SS groups were assessed.

RESULTS: The positive expression rate of cyclin D1 was significantly higher in high (78.6%, 11/14) and middle GAS groups (73.9%, 17/23) than in low GAS group (45.2%, 19/42) ($P<0.05$, $\chi^2_{high\ vs\ low}$ = 4.691; $P<0.05$, $\chi^2_{middle\ vs\ low}$ = 4.945). The positive expression rate of cyclin A was significantly higher in high (100%, 14/14) and middle GAS groups (82.6%, 19/23) than in low GAS group (54.8%, 23/42) ($P<0.01$, $\chi^2_{high\ vs\ low}$ = 9.586; $P<0.05$, $\chi^2_{middle\ vs\ low}$ = 5.040). The positive expression rate of CDK2 was significantly higher in high (92.9%, 13/14) and middle GAS groups (87.0%, 20/23) than in low GAS group (50.0%, 21/42) ($P<0.01$, $\chi^2_{high\ vs\ low}$ = 8.086; $P<0.01$, $\chi^2_{middle\ vs\ low}$ = 8.715). The positive expression rate of CDK4 was significantly higher in high (78.6%, 11/14)

and middle GAS groups (78.3%, 18/23) than in low GAS group (42.9%, 18/42) ($P<0.05$, $\chi^2_{high\ vs\ low}$ = 5.364; $P<0.01$, $\chi^2_{middle\ vs\ low}$ = 7.539). The positive expression rate of cyclin E was prominently higher in low SS group (53.3%, 24/45) than in high (9.1%, 1/11) and middle (21.7%, 5/23) SS groups ($P<0.05$, $\chi^2_{high\ vs\ low}$ = 5.325; $P<0.05$, $\chi^2_{middle\ vs\ low}$ = 6.212). The positive expression rate of CDK2 was significantly higher in low SS group (77.8%, 35/45) than in high SS group (27.3%, 3/11) ($P<0.01$, $\chi^2_{high\ vs\ low}$ = 8.151). There was a significant positive correlation between the integral ratio of GAS to SS and the semi-quantitative integral of cyclin D1, cyclin E, cyclin A, CDK2, CDK4 ($P<0.05$, $^{D1}r_s$ = 0.252; $P<0.01$, $^{E}r_s$ = 0.387; $P<0.01$, $^{A}r_s$ = 0.466; $P<0.01$, $^{K2}r_s$ = 0.519; $P<0.01$, $^{K4}r_s$ = 0.434).

CONCLUSION: The regulation and control of gastrin, SS in colorectal cancer cell growth may be directly related to the abnormal expressions of cyclins D1, A, E, and CDK2, CDK4. The regulatory site of GAS in the cell cycle of colorectal carcinoma may be at the G_1, S and G_2 phases. The regulatory site of SS may be at the entrance of S phase.

INTRODUCTION

Colorectal cancer is one of the most common human malignant tumors in the world, with a high incidence rate in North America, Western Europe, Australia, New Zealand and France, and is the second leading cause of gastrointestinal cancer-related mortality worldwide[1–3]. Although great progress has been made in understanding the molecular aspects of colorectal cancer and several therapeutic agents have been developed, it still poses a serious threat to public health and remains as the major killer among the Chinese. The general survival rate of colorectal cancer patients does not exceed

40%[4,5]. Studies demonstrate that the occurrence of colorectal cancer is directly related to the abnormal expression of gastrointestinal hormones such as gastrin, somatostatin, etc[6]. At the same time, some studies found that somatostatin is able to induce apoptosis of large intestinal cancer cells and inhibit cell proliferation, but the role of GAS (gastrin) is opposite[7-9]. However, the detailed molecule mechanism by which gastrin and somatostatin regulate and control the growth of large intestinal carcinoma is not fully known. We have used immunohistochemical staining standard streptavidin-biotin-peroxidase (S-P) method to detect the expressions of GAS, somatostatin (SS), cyclin D1, cyclin E, cyclin A, cyclin B1, CDK2, CDK4 proteins in large intestinal cancer tissue. The aim of our study was to explore the correlation between the expressions of SS, GAS and cyclins, cyclin-dependent kinase (CDKs) and to further confirm whether GAS, SS could regulate and control large intestinal cancer cell growth by affecting the expression of cyclins and CDKs.

MATERIALS AND METHODS

Seventy-nine cancer tissue samples were randomly selected from patients with large intestinal carcinoma hospitalized in the Department of Pathology of the First Affiliated Yijishan Hospital of Wannan Medical College from June 2001 to June 2003. All patients were confirmed to have colorectal carcinoma by clinical pathology. Among them, 37 were cases of rectum cancer, 42 were cases of colorectal carcinoma. Thirty-five were females and 44 were males. The median age was 52.9 ± 14.3 years, with a range of 27-78 years. Ulcerative type was found in 44 patients, protruded type in 33, infiltrating type in 2, papillary adenocarcinoma in 7, glandular adenocarcinoma in 40, mucoid carcinoma in 14, signet-ring cell carcinoma in 11, and undifferentiated carcinoma in 7. The clinical stage was determined according to the Dukes' stage. Dukes' stages A and B were found in 38 patients, Dukes' stages C and D in 41 patients.

The polyclonal rabbit antibodies against human SS and GAS, monoclonal mouse antibodies against human cyclins D1, E, A, B1, and CDK2, CDK4, and immunohistochemical staining kits were all purchased from Beijing Zhongshan Biological Technology Co., Ltd.

Specimens obtained during surgery were routinely fixed in 10% neutral formalin and embedded in paraffin. Serial 4-μm-thick sections were cut. Immunohistochemical staining for cyclins D1, E, A, B1, and CDK2, CDK4, GAS, SS was performed according to the S-P method. The detailed manipulation was conducted according to the introduction for users. Positive pancreatic tissue, stomach antrum mucous membrane, healthy amygdalae tissue, breast cancer tissue, reactive lymph node tissue, healthy skin tissue were used as a positive control for GAS, SS, cyclins A, B1, D1, E, and CDK2, CDK4, respectively. PBS (0.01mol/L) as a negative control replaced the primary antibody.

Positive SS and GAS were stained brown-yellow mainly in cell plasma, partly in cell membranes. When SS and GAS protein expression were scored, both the extent and intensity of immunopositivity were considered. The intensity of staining was scored as follows: 0 as no staining, 1 as weak-yellow, 2 as brown-yellow, and 3 as brown-black. The extent of positive cells was scored as follows (100 cells were counted by two independent observers, who did not know the clinicopathological features of these large intestinal cancers): 1 = positively stained cells <5%, 2 = positively stained cells being 5-10%, 3 = positively stained cells being 10-20%, 4 = positively stained cells >20%. The final score was determined by multiplying the intensity and extent of positivity scores, yielding a range from 1 to 12. According to the semi-quantitative integral evaluation, SS and GAS were divided into three groups as follows: scores 1-3 were defined as the low group, 4-6 as the middle group, and 7-12 as the high group.

Positive cyclin B1 was stained brown-yellow mainly in the cell plasma. Positive cyclins D1, E, A, and CDK2, CDK4 were stained brown-yellow mainly in karyons. The degree of their staining was estimated by semiquantitative evaluation and categorized by the extent and intensity of staining as follows[10]. The intensity of staining was scored as follows: 0 as negative, 1 as weak-yellow, 2 as brown-yellow, and 3 as brown-black. The extent of positively stained cells was scored as follows: 0=positively stained cells being 0-5%, 1 = positively stained cells being 6-25%, 2 = positively stained cells being 26-50%, 3 = positively stained cells being 51-75%, 4 = positively stained cells>75%. Combined staining score was used to evaluate the staining of cyclins D1, E, A, B1, and CDK2, CDK4. The final score was determined by adding the intensity and extent of staining scores, yielding a range from 0 to 7. Scores1-2 were defined as negative staining (-), 3 as weak staining (+), 4 as moderate staining (++), ≥5 as strong staining (+++).

Statistical analysis was performed using chi-square test to differentiate the positive rates of different groups and using Spearman's test to analyze the correlation between the ratio of GAS to SS and the integral of cyclins D1, E, A, B1, and CDK2, CDK4. All data were analyzed with SPSS version 10.0. $P<0.05$ was considered statistically significant.
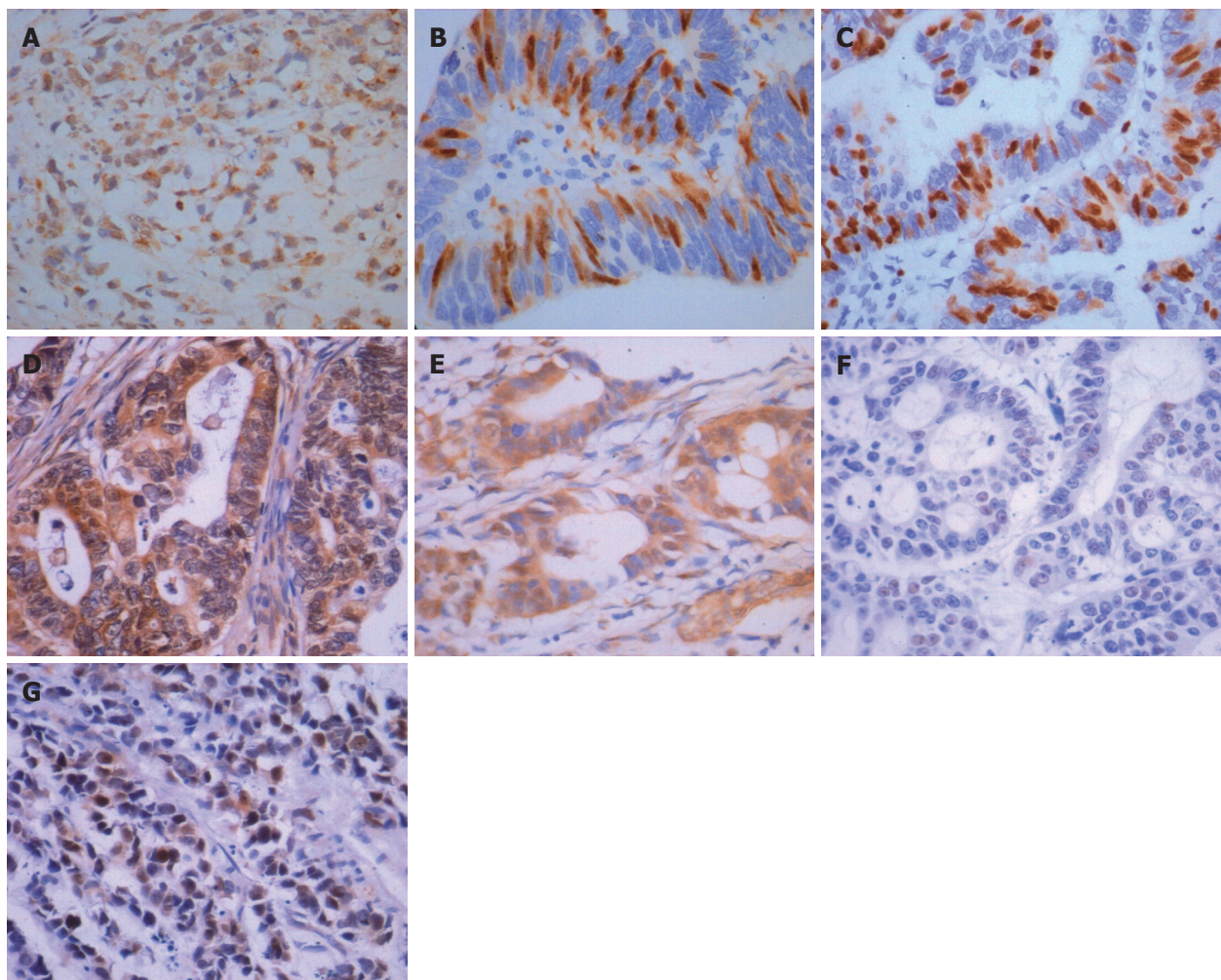
RESULTS

The positive expression rate of cyclin D1 was significantly higher in high (78.6%, 11/14) and middle GAS groups (73.9%, 17/23) than in low GAS group (45.2%, 19/42) ($P<0.05$, $\chi^2_{\text{high } rs \text{ low}} = 4.691$; $P<0.05$, $\chi^2_{\text{middle } rs \text{ low}} = 4.945$). The positive expression rate of cyclin A was significantly higher in high (100%, 14/14) and middle GAS groups (82.6%, 19/23) than in low GAS group (54.8%, 23/42) ($P<0.01$, $\chi^2_{\text{high } rs \text{ low}} = 9.586$; $P<0.05$, $\chi^2_{\text{middle } rs \text{ low}} = 5.040$). The positive expression rate of CDK2 was significantly higher in high (92.9%, 13/14) and middle GAS groups (87.0%, 20/23) than in low group (50.0%, 21/42) ($P<0.01$, $\chi^2_{\text{high } rs \text{ low}} = 8.086$; $P<0.01$, $\chi^2_{\text{middle } rs \text{ low}} = 8.715$). The positive

Table 1 CDK2, CDK4, cyclins D1, E, A, and B1 expressions in high, middle, and low SS and GAS groups of colorectal carcinoma

Groups	n	Cyclin D1		Cyclin E		Cyclin A		CyclinB1		CDK2		CDK4	
		-	+ (%)	-	+ (%)	-	+ (%)	-	+(%)	-	+ (%)	-	+ (%)
GAS													
High	14	3	11 (78.6)[a]	6	8	0	14 (100)[b]	1	13(92.9)	1	13 (92.9)[b]	3	11 (78.6)[a]
Middle	23	6	17 (73.9)[a]	12	11	4	19 (82.6)[a]	6	17	3	20 (87.0)[b]	5	18 (78.3)[b]
Low	42	23	19	31	11	19	23	14	28	21	21	24	18
SS													
High	11	6	5	10	1 (9.1)[c]	3	8	2	9	8	3 (27.3)[d]	7	4
Middle	23	9	14	18	5 (21.7)[c]	7	16	6	17	7	16	9	14
Low	45	17	28	21	24	13	32	13	32	10	35	16	29

[a]$P<0.05$, [b]$P<0.01$ *vs* low GAS group; [c]$P<0.05$, [d]$P<0.01$ *vs* low SS group.

Figure 1 Strong expressions of GAS (**A**), cyclin D1 (**B**), cyclin A (**C**), CDK4 (**D**), SS (**E**), cyclin E (**F**) and CDK2 (**G**) in colorectal carcinoma tissue, S-P×400.

expression rate of CDK4 was significantly higher in high (78.6%, 11/14) and middle GAS groups (78.3%, 18/23) than in low group (42.9%, 18/42) ($P<0.05$, $\chi^2_{\text{high vs. low}} = 5.364$; $P<0.01$, $\chi^2_{\text{middle vs. low}} = 7.539$). However, the positive expression rate of cyclins E and B1 was significantly higher in high (57.1%, 8/14; 92.9%, 13/14) and middle GAS groups (47.8%, 11/23; 73.9%, 17/23) than in low GAS group (26.2%, 11/42; 66.9%, 28/42), but there was no statistically significant difference among the three

groups when compared to three groups to each other ($P>0.05$, $\chi^2 = 5.608$; $P>0.05$, $\chi^2 = 4.417$) (Table 1, Figure 1 A-D).

The positive expression rate of cyclin E was prominently higher in low SS group (53.3%, 24/45) than in high (9.1%, 1/11) and middle (21.7%, 5/23) SS groups ($P<0.05$, $\chi^2_{\text{high vs low}} = 5.325$; $P<0.05$, $\chi^2_{\text{middle vs low}} = 6.212$). The positive expression rate of CDK2 was significantly higher in low SS group (77.8%, 35/45) than in high SS

group (27.3%, 3/11) ($P<0.01$, $\chi^2_{high\ vs\ low}$ = 8.151). However, the positive expression rate of CDK4, cyclin D1 was significantly higher in low SS group (64.4%, 29/45; 62.2%, 28/45) than in high SS group (36.4%, 4/11; 45.5%, 5/11), it was not statistically significant ($P>0.05$, χ^2 = 2.868; $P>0.05$, χ^2 = 1.038). There was no statistically significant difference in the positive expression rate of cyclins A and B1 in high (72.2%, 8/11; 81.8%, 9/11), middle (69.6%, 16/23; 73.9%, 17/23) and low SS groups (71.0%, 32/45; 71.1%, 32/45) when compared to each other ($P>0.05$, χ^2 = 0.039; $P>0.05$, χ^2 = 0.554) (Table 1, Figure 1 E-G).

There was a significant positive correlation between the integral ratio of GAS to SS and the semiquantitative integral of cyclins D1, E, A, and CDK2, CDK4 ($P<0.05$, $^{D1}r_s$ = 0.252; $P<0.01$, $^{E}r_s$ = 0.387; $P<0.01$, $^{A}r_s$ = 0.466; $P<0.01$, $^{K2}r_s$ = 0.519; $P<0.01$, $^{K4}r_s$ = 0.434). But there was no correlation between the integral ratio of GAS to SS and cyclin B1 integral ($P>0.05$, r = -0.108).

DISCUSSION

Cancer arises mainly from mutations in the somatic cells. However, conversion of normal cells to cancer cells is not the result of a single mutation; it is achieved through a multi-step process that is closely associated with the accumulation of multiple gene changes including both oncogenes and tumor suppressor genes[11-13]. Uncontrolled cell proliferation is one of the main hallmarks of cancer, and tumor cells have acquired damage to genes that are directly involved in regulating the cell cycle. The cell cycling process is carefully regulated. The switch or transition between phases is a hallmark of the cell cycle, with an extremely accurate timing and order of molecular events. However, if something goes wrong, the cells have several systems for interrupting the cell cycle[12,14,15].

In order to ensure the cell cycling process, CDK timing-activity is a critical step in the regulation and control mechanism of cell cycle. At least nine different CDKs are known today. However, only some of them seem to be involved in cell cycle regulation. CDKs that are required for cell cycle regulation contain an active kinase subunit in complex with a regulatory subunit, or activator, commonly called cyclin[16,17]. Cyclins are important mediators of CDKs activity, and their level fluctuates throughout the cell cycle, some being more abundant in specific cell phases than others[18]. These cyclins have been divided into three classes: G_1-S cyclin, S cyclin, and M cyclin. Cyclins response to mitogenic signals and unscheduled expression, leading to uncontrolled proliferation, has been implicated in different human cancers[19], such as colon cancer[20] and breast cancer[21]. The CDK/cyclin complex is subjected to several kinds of regulation factors, both positive and negative.

Cell cycle progression is positively regulated by multiple cyclins and CDKs and cyclin/CDK complexes are negatively regulated by a number of CDK inhibitors including p27[22,23]. P27 is a CDK inhibitor and plays an important role in the negative regulation of the cell cycle during G_0 and G_1 phases[12,13,24,25]. Proliferating cells pass through several cell cycle checkpoints, mainly the G_1 to S and G_2 to M transitions. The former checkpoint is considered as the most important one in the replication of DNA and mitosis. The G_1-S transition is a highly regulated and important transition in the cell cycle. At this stage, the cell cycle passes a point between G_1 and S phases (restriction point) with an irreversible commitment to a new cycle. The underlying molecular mechanisms are the induced expression of CDKs and cyclins required for the cells to progress from early G_1 phase into late G_1 phase of the cell cycle, reaching the restriction point. This is a critical point in the late G_1 phase where the mammalian cells become committed to entering the S phase and to complete the cell cycle, even in the absence of growth factors[23,26,27]. The main CDKs involved in the progression from mid- to late G_1 are CDK4 and CDK6, driven by three G_1-specific cyclins, D1, D2 and D3[15]. Cycle progression from G_1 to S phase is usually accompanied with Rb phosphorylation induced by cyclin D_1-CDK_4 and cyclin E-CDK_2 complexes in the late G_1 phase[28]. Cyclin-dependent kinase 2 (CDK2) activity is critical for S phase entry. CDK2 activation is apparently cyclin E-dependent. Late G_1 phase CDK2/cyclin E activity depends on early G_1 phase CDK2/cyclin E function[29,30].

Previous studies have shown that some tissue growth is regulated by hormones, and these tissues that have turned into tumors are still controlled by hormones[31]. Gastrointestinal hormones such as gastrin and somatostatin regulate the secretion, motility, absorption, blood flow and cell nutrition of the digestive tract. Abnormality of their secretion often affects the normal functions of the digestive tract, even causes clinical symptoms or syndromes[32,33]. Some studies demonstrated that there is a high correlation between the abnormal expressions of GAS, SS and the occurrence and development of colorectal cancer[34-36]. Recently, great progress has been made in understanding the cell cycle mechanisms of GAS and SS. Some studies showed that the abnormal expressions of GAS and SS are closely related to cell apoptosis and proliferation of colorectal cancer, and that the expression of gastrin protein and the proliferation index are higher in colorectal cancer tissue, while the action of somatostatin is opposite in colorectal carcinoma[9,37,38]. Though there is abundant evidence that gastrin plays an important role in promoting tumor growth in the stomach, as well as malignancies in the GI tract, the precise mechanisms governing the gastrin-induced and somatostatin-restrained proliferation are still largely unknown. To elucidate the mechanisms of gastrin and somatostatin in regulating mitogenesis, we have analyzed their effects on the expression of cyclins and CDKs in human colorectal cancer tissue.

Gastrin is a gastrointestinal (GI) peptide that possesses potent trophic effects on most normal and neoplastic mucosae of the GI tract. Gastrin is mainly secreted from gastrin secreting cells (G cells) in the antrum mucosa or upper small intestine, large intestine. Medulla oblongata and dorsal nuclei of vagus nerves in central nervous system also secrete gastrin[39,40]. Studies indicate that

chronic hypergastrinemia increases the risk of colorectal cancer and cancer growth, and that interruption of the effects of gastrin may be a potential target in the treatment of colorectal cancer[41]. Shen *et al*[42] showed that gastrin is able to promote DNA and protein synthesis in colorectal cancer tissue. However, gastrin-released peptide receptor antagonist proglumide could block these effects of gastrin, and restrain colorectal cancer cells from G_0/G_1 phase into S and G_2, M phase transitions. It was recently reported that gastrin (G-17) is able to induce a significant increase in G_1-specific marker cyclin D1 transcripts, protein, and promoter activity via the activation of beta-catenin and CRE-binding protein pathways in gastric adenocarcinoma cells, which promote transition of tumor cells from G_1 phase into S phase, and lead to uncontrolled proliferation of tumor cells[43]. Lefranc *et al*[44] studies found that gastrin is able to significantly modify the growth of a number of experimental gliomas. This effect seems to occur via a cytostatic effect, that is, an accumulation of tumor astrocytes occurs in the G_1 cell cycle phase. The cytostatic effect relates to a gastrin-induced decrease in the level of cyclin D3-CDK4 complex. In this study, we have found that the level of gastrin protein expression was higher and the positive expression rate of cyclins D1, A, and CDK2, CDK4 was higher in large intestinal carcinoma tissue, indicating that mechanism of gastrin in promoting colorectal cancer cell proliferation is via inducing the overexpression of cyclins D1, A, and CDK2, CDK4, thus leading to the rise of the level of cyclin D1-CDK4 and cyclin A-CDK2 complexes, which influence cell cycle progress and promote cell proliferation.

Somatostatin is a widely distributed inhibitory hormone that plays an important role in several biological processes including neurotransmission, inhibition of exocrine and endocrine secretions, and cell proliferation. Somatostatin acts as an inhibitory peptide of various secretory and proliferative responses. Somatostatin is secreted from somatostatin secreting cells (D cells). D cells are distributed mainly in intestinal nerve plexus, stomach and pancreas. The diverse biological effects of somatostatin are mediated by a family of five somatostatin receptors (sst1-sst5) that belong to the family of G-protein-coupled receptors and regulate diverse signal transduction pathways including adenylate cyclase, phospholipase C-β phospholipase A_2, guanylate cyclase, ionic conductance channels, and tyrosine phosphatase[45-48]. The mechanisms underlying the inhibition are the combined interaction of somtostatin and its analogs to SST1-5R in tumor tissues, either directly inhibiting division and proliferation of tumor cells or the activities of growth factors such as vascular endothelial growth factor, insulin-like growth factor, etc[49-51], thus counteracting tumorigenesis and tumor cell proliferation[52]. The ability of somatostatin and its stable analogs to inhibit normal and tumor cell growth has been demonstrated in various cell types including mammary, prostatic, gastric, pancreatic, colorectal, and small cell lung cancer cells. However, the mechanisms of somatostatin underlying cell growth arrest are still poorly understood. Pages *et al*[53] showed that activation of sst2 promotes

cell growth arrest through the ability of somatostatin to maintain high levels of p27^{Kip1} and inactivates cyclin E-CDK2 complexes, leading to hypophosphorylation of pRb, restraining transition of tumor cells from G_1 phase into S phase. Charland *et al*[54] reported that somatostatin is able to inhibit cyclin E expression in pancreatic cells and cyclin E-associated CDK2 activity, as well as pRb phosphorylation, and to restrain transition of cells from G_1 phase into S phase, thus inhibiting cell proliferation. Zhao *et al*[55] demonstrated that somatostatin analog, octreotide, inhibits the proliferation of cholangiocarcinoma cells through G_0/G_1 cell cycle arrest rather than through the process of apoptosis. These effects are partially mediated by enhancing the expression of p27kipl, and decreasing the level of cyclin E-CDK2 complex. In this study, the higher the integral of SS, the lower the positive expression rate of cyclin E and CDK2. Our data indicate that the mechanism of somatostatin in inhibiting colorectal cancer cell proliferation is via restraining the expression of cyclin E and CDK2, and decreasing the level of cyclin E-CDK2 complex, which inhibits transition of cells from G_1 phase into S phase and induces cell cycle arrest, thus restraining cell proliferation.

In the present study, we have found that the ratio of GAS to SS had an effect on the biological characteristics of large intestinal cancer[56]. The increased ratio of GAS to SS is an event of significance in large intestinal cancer occurrence and development[31]. Our results indicate that there is a positive correlation between the ratio of GAS to SS and the semi-quantitative integral expression of cyclins D1, A, E, and CDK2, CDK4. Furthermore, the expression of GAS and SS has a direct relation with the expression of cyclins D1, A, E, and CDK2, CDK4 in colorectal cancer.

In conclusion, the regulation and control of gastrin, somatostatin in colorectal cancer cell growth may be directly related to the abnormal expressions of cyclins D1, A, E, and CDK2, CDK4. The regulatory site of GAS in the cell cycle of colorectal carcinoma may be at the G_1, S and G_2 phases. The regulatory site of SS may be at the entrance of S phase.

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• *CASE REPORTS* •

Delayed development of hepatocellular carcinoma during long-term follow-up after eradication of hepatitis C virus by interferon therapy

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Abstract

A 42-year-old Japanese man with liver cirrhosis by hepatitis C virus (HCV) had successful interferon therapy in May 1991. Since then, serum HCV-RNA and liver function tests had been negative. He had continued to drink more than 100 g/d of alcohol as before. In June 2003, a 5-cm tumor was found in the posterior segment of the liver. The tumor was curatively resected and the surgical specimen showed a well-differentiated hepatocellular carcinoma (HCC). Non-cancerous lesions of the liver revealed fibrosis at stage F3 with minimal to mild inflammation of grade A1. Heavy drinking may retard the dissolution of fibrosis and accelerate HCC development in patients with sustained virological response.

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Key words: Hepatocellular carcinoma; Interferon; HCV; Eradication

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of death in Japan. Hepatitis C virus (HCV) infection is apparently a major cause of HCC[1-6]. Patients with chronic HCV infection seem destined for progression from milder forms of hepatitis to cirrhosis and, eventually, to HCC. The more advanced is the fibrosis of the liver, the higher is the risk of HCC development. This risk is surprisingly high in cirrhotic patients, the incident rate being about 7% per year[7]. Previously, those patients had no choice but to pass uneasy days in fear of HCC development. It was believed that liver fibrosis is an irreversible change and liver cirrhosis is an incurable disease. Recently, however, it has been shown that the risk of HCC is reduced in HCV-infected patients by interferon therapy, even in cirrhotic patients[8-13]. Interferon therapy can not only improve inflammation, but also ameliorate fibrosis and reduce the risk of HCC. More recently, it has been considered that HCC rarely develops long after HCV eradication[14,15].

Although interferon therapy has been employed for more than a decade, it is not clear how long the risk of HCC lasts among patients who have achieved sustained virological response (SVR). Recently, we had a case with HCC developing 12 years after HCV eradication. This case seems worthy of noting the details, and the data might be helpful for managing patients after HCV eradication.

CASE REPORT

A 42-year-old Japanese man had abnormal liver function tests at a health check-up in 1987. Serum ALT and AST were moderately elevated, 80 and 79 IU/L, respectively. He had no history of blood transfusion or drug abuse, but had been consuming more than 100 g/d of alcohol for 20 years. The liver biopsy specimen showed liver cirrhosis, diagnosed later as A1F4 according to the Classification by Desmet[17]. Alcohol hyaline was not found in the specimen (Figure 1).

In May 1991, he underwent successful interferon therapy after a diagnosis of HCV infection using a total dose of 390 MU of natural interferon-alpha (Sumiferon®, Sumitomo Pharmaceuticals Co., Ltd., Osaka, Japan). Serum HCV-RNA had been negative ever since, and liver function tests, including ALT and AST, had also been normalized. He had continued to drink alcohol heavily as before.

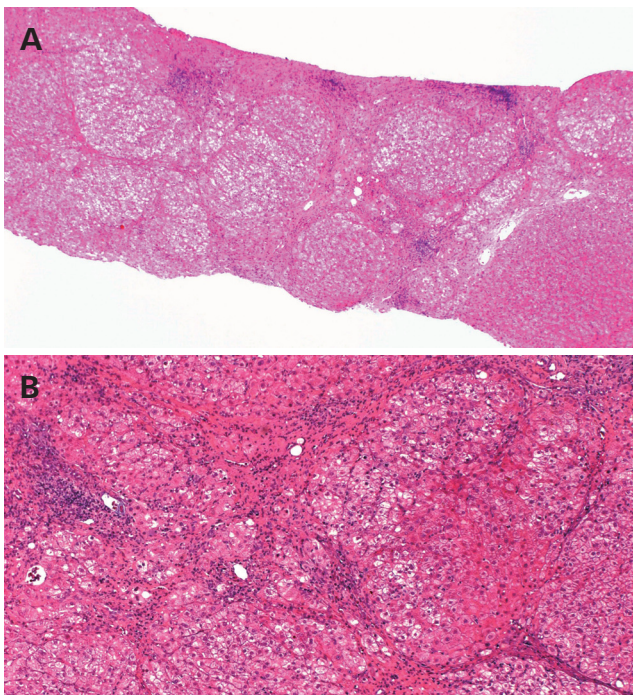His follow-up had included blood tests and ultrasound examinations every 4-6 mo. In June 2003, ultrasonography

Figure 1 (**A** and **B**) Liver biopsy specimen before interferon therapy revealed liver cirrhosis with mild inflammation. It was graded as A1F4 according to the Classification by Desmet. Alcohol hyaline was not found (H&E stain, original magnifications A: ×4, B: ×200).

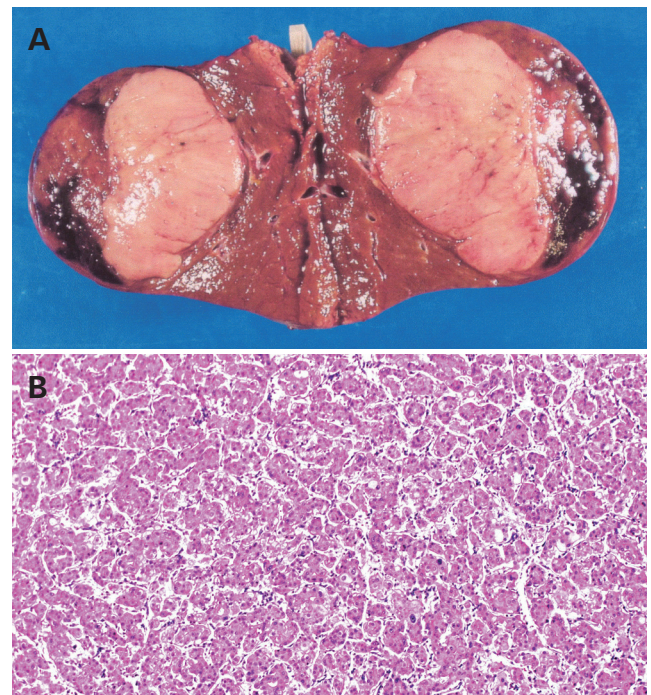Figure 3 (**A**) Surgical specimen showing an 8-cm tumor. (**B**) Histological examination revealing a well-differentiated hepatocellular carcinoma (H&E stain, original magnification ×200).

Figure 2 Contrast medium-enhanced CT image of the liver tumor of the right inferior segment (a white arrow).

and CT scan demonstrated a tumor of 5 cm in diameter in the posterior segment of the liver (Figure 2). Blood tests were normal except for elevated desgamma carboxy prothrombin (DCP, 44 mAu/mL, normal <40 mAu/mL). Angiography confirmed a hypervascular nodule, suggesting HCC.

The tumor was curatively resected and the surgical specimen showed a well-differentiated HCC (Figure 3). Non-cancerous lesions revealed minimal to mild inflammation and severe fibrosis of A1F3 (Figure 4).

The patient had recurrence of multiple HCCs, 12 mo after the surgery. Chemoembolization was performed repeatedly, and he was in remission up to September 2004.
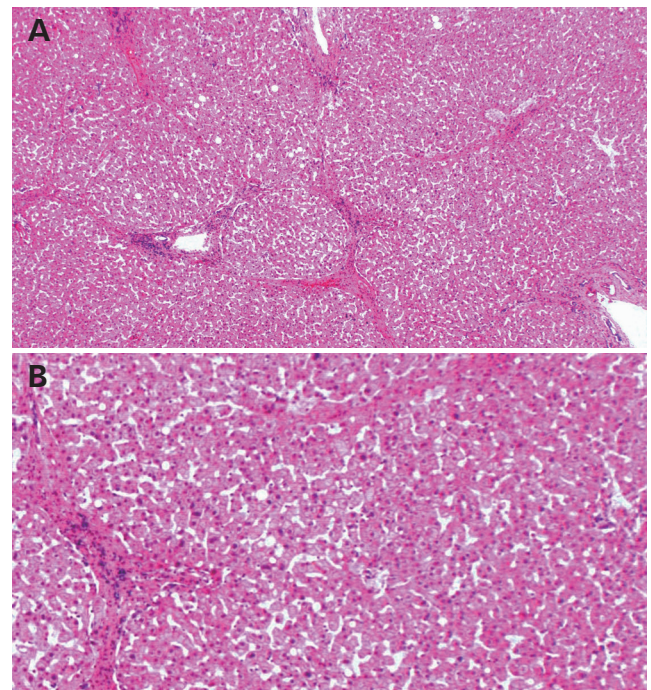

Figure 4 (**A** and **B**) Surgical specimen of non-cancerous lesion showing improvement of fibrosis and inflammation. However, great amounts of septa were observed, although fibrous bundles apparently decreased. Fibrosis resolved, revealing liver cirrhosis with mild inflammation. It was graded as A1F3 (H&E stain, original magnifications A: ×4, B: ×200).

DISCUSSION

Interferon therapy has been shown to reduce the risk of HCC development among the patients with chronic HCV infection. Their risk is decreased to one-third and their

survival is also significantly improved when they obtain SVR[17,18].

In patients achieving SVR, fibrosis is gradually dissolved and the risk for HCC development is reduced. Our concern is how long their risk of HCC lasts. There have been few documented cases of HCC development for more than 10 years after the interferon therapy, with more than 10 years having passed since its introduction. At that point, the possibility of HCC development is also believed to be notably decreased.

The present case developed HCC, 12 years after HCV eradication, perhaps the latest developed HCC case on record. It may be helpful for the management of post-eradication patients to review the details of this case in terms of the potential of HCC development.

The present patient had been showing normal aminotransferase values, including ALT and AST, and negative results for serum HVC RNA for 12 years after receiving interferon treatment. Although the histological findings of the liver were improved, great amounts of septa were still observed in the surgical specimen, suggesting F3. In patients with this status, the HCC risk is appraised at 5% per year. The dissolution of fibrosis may have been retarded in the present case. Shiratori et al[19] reported that the F values regress at a rate of -0.282/year among patients with SVR that is based on the method of Poynard[20]. The regression rate in the present case was from F4 to F3 for over the course of 12 years, a meager -0.083/year compared to the reported rate.

The patient had been consuming alcohol since the successful interferon therapy, perhaps accounting for the fact that the hepatic fibrosis had not been dissolved as expected. Alternatively, a long time after eradication, the regression rate in patients may not be as high as in those previously reported. As little data is available regarding regression or HCC development in patients with more than 10 years of follow-up, further studies will be needed.

In addition, alcohol itself may accelerate HCC development. Alcohol intake of 41-80 g/d and over 80 g/d is reported to increase the risk of HCC in HCV-infected patients by two- and four-fold, respectively[21]. Alcohol may also facilitate the growth of HCC, as the doubling time of tumor volume was reported as 148 d in abstemious patients and 78 d in imbibing patients[22].

The degree of liver fibrosis, male gender, higher age, and alcohol intake are well-known risk factors for HCC in chronic HCV-infected patients. The present patient met all these conditions, although little is known as to whether they serve as risk factors in patients with SVR. However, alcohol may have played a role in the development of HCC in this case. If he had stopped consuming alcohol, his liver fibrosis would likely have been improved to a greater extent, and he might have escaped from HCC entirely.

In conclusion, heavy drinking may retard the dissolution of fibrosis and accelerate HCC development in patients with SVR. A larger population of similar cases needs to be studied.

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