Helicobacter pylori promotes hepatic fibrosis in the animal model

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Helicobacter pylori infection has been reported to be very common in patients with chronic liver diseases, including cirrhosis. To elucidate the pathological effect of *H. pylori* infection on the progression of hepatic fibrosis, C57BL/6 mice and Sprague–Dawley rats were orally inoculated with *H. pylori*, and hepatic fibrosis was induced with carbon tetrachloride (CCl₄) administration. We observed the histopathological changes and the presence of *H. pylori* genes by PCR in the liver. Significant increase in the fibrotic score as well as in serum alanine aminotransferase and aspartate aminotransferase levels was shown in the CCl₄ + *H. pylori* group compared with that in the CCl₄-treated group. Compared with the CCl₄-treated group, α -smooth muscle actin and transforming growth factor- β 1 were enhanced; however, senescence marker protein-30, a multifunctional protein protecting hepatocytes against oxidative stress and apoptosis, was suppressed in the CCl₄ + *H. pylori* group. The 16S rRNA (400 bp) was demonstrated by PCR for *H. pylori* genes from genomic DNA extracted from the liver, and *H. pylori*-infected mice showed 93.8% (15 of 16) seropositivity by contrast with seronegativity in all *H. pylori*-noninfected mice. In addition, immunohistochemical study against *H. pylori* showed positive antigen fragments in the liver of the infected groups. Consequently, our data suggest that *H. pylori* infection could be an important contributing infectious factor to the development of liver cirrhosis.

Laboratory Investigation (2009) 89, 1291–1303; doi:10.1038/labinvest.2009.90; published online 7 September 2009

KEYWORDS: *Helicobacter pylori*; hepatic fibrosis; liver; senescence marker protein 30; 16S rRNA

Helicobacter pylori is well known as an important pathogen that results in chronic gastritis associated with the development of peptic ulcer disease, atrophic gastritis and gastric cancer.¹ Recently, evidence showing a possible association between *H. pylori* infection in the human stomach and chronic liver diseases is emerging.^{2–4} Many serological studies have found that patients with hepatitis have an increased *H. pylori* infection rate.^{3,5} Moreover, the prevalence of *H. pylori* in patients with cirrhosis has been reported to be remarkably higher than in non-cirrhotic patients.^{6,7} *Helicobacter*-like DNA that showed a high similarity to *H. pylori* and enterohepatic *Helicobacter* species was detected in the liver by PCR.^{8,9} Taken together, these observations support the idea that *H. pylori* infection may have a critical role in the pathogenesis of various liver disorders. Hepatic cirrhosis is clinically very important as high risk conditions underline

the development of hepatocellular carcinoma (HCC), leading to a high rate of morbidity and mortality.¹⁰ Several factors such as toxins, viruses, oxidative stress, necrosis, apoptosis and growth factors are responsible for the activation of resting hepatic stellate cells (HSCs) which have an important role in the pathogenesis of hepatic cirrhosis. The transformation of quiescent HSC into myofibroblast-like cells with the expression of cytoskeletal protein α -smooth muscle actin (α -SMA) initiates the chronic process of hepatic fibrosis, finally leading to the fatal stage of liver cirrhosis.¹¹

A few papers have recently reported that *H. pylori* caused liver damages as an independent etiological factor, and that the gene belonging to it was demonstrated by PCR in mice.¹² After being infected with *H. pylori* for 2 years, the mice developed primary HCC, and bacteria were shown by immunohistopathological staining.¹³ Although some data

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Received 7 March 2009; revised 1 July 2009; accepted 2 July 2009

suggest that *H. pylori* caused the cytopathic effect in a liver and HCC cell line *in vitro*,^{14,15} the role of *H. pylori* infections in the development of chronic liver diseases including cirrhosis in humans is still not well characterized. Therefore, our study aimed at investigating the role of *H. pylori* infection in the pathogenesis of the damaged liver tissues by carbon tetrachloride (CCl₄) administration in the murine model.

MATERIALS AND METHODS

Bacterial Strains

H. pylori strain ATCC 43504 (American Type Culture Collection, a *cagA*⁺, *vacA* s1-m1 strain) and Sydney Strain (SS1, a *cagA*⁺, *vacA* s2-m2 strain)¹⁶ were used in this study. Each *H. pylori* was grown on Mueller Hinton (MH) agar supplemented with an antibiotic mixture and incubated for 48 h at 37 °C under microaerophilic conditions. The cells were harvested in phosphate-buffered saline (PBS), centrifuged at 2000 × *g* for 10 min, and resuspended in PBS at a final concentration of 10⁹ colony-forming units (CFU)/ml. In all experiments, cultures grown for 48 h on MH agar plates were used.

Animals and Experimental Design

Animals

SPF female Sprague–Dawley rats 14 weeks of age and 8-week-old SPF female C57BL/6 mice, absent from *Helicobacter bills* and *Helicobacter hepaticus*, were housed under clean conditions, fed a commercial diet and given water *ad libitum*. All animals were managed under the animal guideline of NIH, USA. Experimental liver fibrosis was produced by intraperitoneal administration of 10% CCl₄ in olive oil at 1.0 ml/kg thrice a week, and normal control animals received the same volume of vehicle (olive oil; 1.0 ml/kg b.w, i.p.) at the same time. After an overnight fast, each animal was intragastrically inoculated either with a suspension of *H. pylori* containing 10⁹ CFU/ml or sham infected with an equal volume of PBS using gastric intubation needles.

Experimental design-1

The rats (*n* = 24) were divided into the following three groups: (1) normal controls (*n* = 8), (2) only CCl₄-treated group (*n* = 8) and (3) CCl₄ + *H. pylori* group (*n* = 8). The only *H. pylori* group was not involved considering the low relationship between *H. pylori* and the liver in this preliminary study. CCl₄ was administered on three separate occasions over a 5-day period for 8 weeks to induce liver fibrosis.¹⁷ From week 1, when constructing the animal model of hepatic injury was begun, the *H. pylori* strain ATCC 43504, 1.0 ml of inoculum per rat, was inoculated orally in a daily alternative manner with CCl₄ treatment. Rats were killed at the end of the eighth week; from each rat, blood samples were collected from the inferior vena cava, and the liver and stomach were removed.

Experimental design-2

The mice (*n* = 32) were divided into the following four groups: (1) normal controls (*n* = 8), (2) only *H. pylori*-infected group (*n* = 8), (3) only CCl₄-treated group (*n* = 8) and (4) CCl₄ + *H. pylori* group (*n* = 8). The only *H. pylori* group was inserted to observe the independent effect of *H. pylori* on the liver. CCl₄ was administered for 15 weeks as a previously described regimen to induce liver fibrosis.^{18,19} In these animal models, CCl₄ treatment was conducted after the first week, when inoculation of the mice with *H. pylori* was initiated. Mice were infected with *H. pylori* strain SS1, 0.2 ml of inoculum per mouse, killed at the end of the sixteenth week and then their organs were collected as described above.

Histopathology

For histopathological analysis, samples of the liver and stomach were fixed in 10% neutralized buffered formalin, processed using the standard method and embedded in paraffin. Sections of 4-μm thickness were then stained with hematoxylin and eosin (H&E) or with Azan staining for collagen fiber. Liver samples were scored on histopathological changes including fibrosis using the scoring system proposed by Mendler *et al*,²⁰ and additional parameters related to hepatic injury were analyzed with Periodic Acid Schiff (PAS) stain and oil-red O stain.

Immunohistochemistry

Sectioned liver samples were assessed by the routine immunohistochemistry method, using the monoclonal mouse antibodies of α-SMA (Sigma, Saint Louis, MO, USA), polyclonal rabbit antibodies of transforming growth factor-β1 (TGF-β1) (Santa Cruz Biotechnology, CA, USA), polyclonal rat antibodies of SMP30 (obtained from Achito Ishigami), polyclonal rabbit antibodies of *H. pylori* (Dako, Glostrup, Denmark) and monoclonal mouse antibodies of proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology). Immunoreactive materials were visualized with avidin–biotin–peroxidase complex solution using an ABC Kit (Vector Laboratories, Burlingame, CA, USA) with 3,3-diaminobenzidine (Zymed Laboratories, San Francisco, CA, USA). Terminal deoxynucleotidyl transferase-mediated nick-end labeling staining was performed using a commercial apoptosis detection kit (Roche Diagnostics GmbH, Penzberg, Germany). The number of positively stained cells of a total of 1000 hepatocytes in the ×200 field was quantified by counting, and the results were reported as the mean percentage of positively stained cells compared with the total hepatocytes.

Immunoblotting

Electrophoresis of proteins was performed routinely on sodium dodecyl sulfate gel containing polyacrylamide (SDS-PAGE) gels using standard methods. For immunoblotting, 50–100 μg of purified protein per lane were run on an SDS-PAGE 10% gel and transferred into a PVDF membrane

(Schleicher & Schuell, Dassel, Germany). α -SMA (Sigma), TGF- β 1 (Santa Cruz Biotechnology) and SMP30 (obtained from Akihito Ishigami) were detected using a commercially enhanced chemiluminescence system (ECL, Amersham, Buckinghamshire, UK), and exposed to medical X-ray film (Kodak, Tokyo, Japan).

Biochemical Serum Transferase Assessment

The serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, total bilirubin and cholesterol levels were measured using an automated instrument (Konelab 60I Analyser, Konelab, Finland).

Hepatic Hydroxyprolin Content

Hepatic hydroxyprolin content was determined using the method described by Jeong *et al.*²¹ with some modifications. In brief, frozen liver tissue (50 mg for mouse and 200 mg for rat) was hydrolyzed in 4 ml of 6 N HCl at 120 °C for 30 min. The hydrolysate was filtered, and then 50 μ l of filtrate was evaporated under vacuum. Dried samples were dissolved in 125 μ l of isopropanol and incubated with 62.5 μ l chloramines-T/citrate buffer (pH 6.0) at 25 °C for 4 min. Freshly prepared 750 μ l of Ehrlich's reagent was added and then incubated at 60 °C for an additional 25 min. Absorbance at 560 nm was recorded using a spectrophotometer (Tecan, Salzburg, Austria). The absorbance value of each liver was taken to represent the hydroxyproline content of the tissue, and this was expressed in μ g hydroxyproline per g of tissue (wet weight).

Serum Anti-*H. Pylori* Antibody

The enzyme-linked immunosorbent assay (ELISA) method was applied to detect anti-*H. pylori* IgG antibodies. Flat bottom 96-well plates (Nunc, Denmark) were coated with 0.5 μ g of *H. pylori*-whole cell lysate antigen in 100 μ l of 20 mM carbonate buffer (pH 9.6) and then incubated overnight at 4 °C. The plates were washed three times with 0.05% Tween 20/Tris-buffered saline (TBS), and the nonspecific binding sites were blocked with 1% skim milk for 1 h at room temperature. Serum samples (diluted to 1:100) were added in duplicate, and the plates were incubated for 2 h at 37 °C. The plates were washed as described above and then incubated with horseradish peroxidase-conjugated anti-mouse IgG (Cell Signaling Technology, Beverly, MA, USA) for mouse samples and anti-rat IgG (Santa Cruz Biotechnology) for rat samples, respectively, at a dilution of 1:5000 in 0.05% Tween 20/TBS for 1 h at 37 °C. The plates were once again washed, then developed using a 1-Step Ultra TMB-ELISA Kit (Pierce, Rockford, IL, USA). Upon optimal color development, the reaction was stopped by adding 2 M sulfuric acid to the plates. Optical densities (450 nm) were measured using a plate reader (Tecan). The absorbance value was presented as mean $\pm$ s.d.

DNA Extraction

In all, 60–80 mg of ground frozen liver tissues under liquid nitrogen were transferred into a sterile 1.5-ml Eppendorf tube. The samples were incubated overnight at 50 °C in 500 μ l lysis buffer (50 mM Tris pH 8.0, 100 mM NaCl, 100 mM EDTA, pH 8.0, 0.1% SDS) containing 0.5 mg/ml proteinase K (Sigma). Proteins were extracted with equal volume of phenol–chloroform–isoamyl alcohol in a ratio of 25:24:1 (Sigma) and centrifuged at 1410 $\times$ g for 5 min at 10 °C, followed by transferring the supernatants into a new tube. Subsequently, an equal volume of chloroform–isoamyl alcohol in a ratio of 24:1 (Sigma) was added, centrifuged at 1410 $\times$ g for 5 min at 10 °C and the supernatants were transferred into a new tube. The genomic DNA in the supernatant was recovered by precipitation with ethanol, followed by centrifuging at 20 800 $\times$ g at 4 °C for 5 min. The precipitated DNA was washed in 70% ethanol and dried overnight. Finally, DNA was resuspended in 500 μ l ultra pure water and measured in Qubit Fluorometer (Invitrogen, The Netherlands). In all, 40 ng of DNA extract was used in each PCR reaction.

PCR Amplification

DNA extracted from the liver tissue samples were amplified by *Helicobacter* genus-specific 16S rRNA primers described by Fox *et al.*⁴ sense primer: 5'-GCT ATG ACG GGT ATC C-3' (C97F); antisense primer: 5'-GAT TTT ACC CCT ACA CCA-3' (C98R). The forward and reverse primers amplified a product of $\sim$ 400 bp.⁴ A volume of 2 μ l of 40 ng DNA and 1 μ l of 20 pmol from each primer were added to the PCR PreMix Kit (Bioneer, Daejeon, Korea) containing 250 μ M of each dNTPs, 40 mM KCl, 1.5 mM MgCl₂, 1 U of Taq polymerase and 10 mM Tris-HCl (pH 9.0). The final mixture was amplified after a PCR cycle program: initial activation of the Taq-polymerase at 94 °C for 10 min, denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s (35 cycles) and a final extension step at 72 °C for 5 min. The 40 ng amplified products were loaded onto 1.5% (w/v) agarose (SeaKem LE Agarose, Cambrex, Bio Science Rockland, Rockland, ME, USA) gels containing ethidium bromide, and the PCR product sizes were estimated by comparison with a 100-bp DNA size marker (MBI Fermentas, Vilnius, Lithuania). *H. pylori* strain SS1 was used as a positive control and ultra pure water was used as the negative control at each amplification event. Bands were visualized under UV light.

Statistical Analysis

When experiments included only two groups, the two-tailed *t*-test was used. When the experimental design included more than two groups, statistical differences were determined by analysis of variance. Comparisons between particular groups were then made by the Bonferroni test, keeping an overall probability of error lesser than 0.05.

Results

Hepatic Fibrosis in the CCl₄ + Hp Livers

Histological analysis using H&E stain showed marked microvesicular and macrovesicular fatty changes with thin fibrotic septa in the CCl₄-treated livers. Bile duct epithelial proliferation in thick fibrotic septa and a more severe mononuclear inflammatory cell infiltration were observed in the CCl₄ + Hp livers (Figure 1a). Liver collagen content was determined by morphometry using Azan staining (Figure 1b), and histological findings were assessed and then scored with regard to the fibrotic stage (Table 1). CCl₄ induced long collagenous septa connecting the central veins across the liver parenchyma at 8 weeks in rats and at 16 weeks in mice. Interestingly, *H. pylori* infection during CCl₄ treatment remarkably increased the development of liver fibrosis as compared with CCl₄ alone in both rats and mice. The CCl₄ + Hp livers of experimental animals showed a marked increase in the collagen band intersecting the liver parenchyma to form bridging fibrotic septa, which progressed into pseudolobules (Figure 1b). To confirm these results, total hydroxyprolin concentration was evaluated in both species of animals (Table 1). The hydroxyprolin level was

significantly higher in the CCl₄ + Hp livers than in the only CCl₄-treated ($P < 0.05$) livers. This result corresponded to the severity of fibrosis in both sets of animal models.

Effects of *H. Pylori* on Biochemical Factors in the CCl₄-Treated Livers

As shown in Table 1, *H. pylori* infection significantly contributed to the CCl₄-induced increase in AST and ALT levels in both mouse and rat models. In rats, plasma alkaline phosphatase and total bilirubin activities in the CCl₄ + Hp groups (151.4 ± 85.1 IU/l and 0.48 ± 0.44 mg/dl, respectively, $P < 0.05$) were markedly elevated than in the CCl₄ groups (78.3 ± 31.1 IU/l and 0.22 ± 0.13 mg/dl, respectively), whereas plasma total cholesterol was significantly reduced (79.8 ± 36.0 mg/dl in CCl₄ groups and 56.2 ± 13.5 mg/dl in CCl₄ + Hp groups, $P < 0.05$) (data not shown), indicating a more severe hepatic injury.

Expression of Chronic Liver Damage Markers in the CCl₄ + Hp Livers

We performed immunohistochemical staining and immunoblotting using chronic liver injury markers (Figure 2).

Figure 1 Histopathological changes in the livers. (a) The livers were stained by H&E staining and the pathological scores were represented as mean $\pm$ s.d. $*P < 0.05$. In CCl₄-treated groups, hepatocytes with cytoplasmic microvesicular and macrovesicular fatty changes were accompanied with fine collagenous fibers surrounding degenerative hepatocytes. In CCl₄ + Hp groups, perivenular zones showed extensive collagen deposition with a prominent increase in activated hepatic stellate cells (HSCs) and small lymphocytes. Bile duct (arrow) proliferation in the fibrotic septa was also observed. In a *H. pylori*-infected group, the hepatocytes contained various sized cytoplasmic vacuoles. Scale bar, 50 μ m. (b) The liver was stained by Azan staining and scoring on fibrosis was represented as mean $\pm$ s.d. $*P < 0.05$. The marked increase in hepatic fibrosis showing micronodular formation by interconnective fibrotic septa in the CCl₄ + Hp group, in comparison with activity in the CCl₄ group is noted. Scale bar, 200 μ m.

Table 1 Effects of infection of *H. pylori* on fibrosis grade, serum biochemical tests and tissue HYP levels in experimental animals

Animals and groups	Fibrosis grade	AST (IU/l)	ALT (IU/l)	HYP (μ g/g)
<i>Rats</i>				
Con	0	112.9 $\pm$ 23.0	32.4 $\pm$ 7.9	223.6 $\pm$ 8.3
CCl ₄	3 $\pm$ 1.2 ^{††}	569.6 $\pm$ 291.5 ^{††}	440.4 $\pm$ 24.3 ^{††}	271.4 $\pm$ 43.7 [†]
CCl ₄ +Hp	7.4 $\pm$ 1.7*	2408 $\pm$ 2003.8*	1454.5 $\pm$ 814.0*	326.8 $\pm$ 62.0*
<i>Mice</i>				
Con	0	61.6 $\pm$ 5.4	21.4 $\pm$ 3.20	190.8 $\pm$ 53.0
CCl ₄	5.1 $\pm$ 0.9 ^{††}	1364.8 $\pm$ 724.1 ^{††}	2340 $\pm$ 1027.5 ^{††}	269.3 $\pm$ 40.3 ^{††}
CCl ₄ +Hp	8.1 $\pm$ 0.7**	3085 $\pm$ 674.4*	5038 $\pm$ 1006.2*	321.9 $\pm$ 46.7*
Hp	0	79.5 $\pm$ 7.8	20 $\pm$ 1.4	204.9 $\pm$ 57.5

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HYP, hydroxyproline.

Results are expressed as the mean $\pm$ s.d.

* P < 0.05; ** P < 0.01 vs CCl₄ group.

[†] P < 0.05; ^{††} P < 0.01 vs control group.

The expression of α -SMA is a characteristic feature of activated HSCs and this is considered as a marker for exacerbation of hepatic fibrosis and related cellular events. In the control livers, α -SMA-immunoreactive cells were detected mainly in the portal area as elements of vascular walls. Focal accumulated α -SMA-positive cells around the central vein branches and the portal area were detected in only the CCl₄-treated livers. In the livers of the CCl₄ + Hp group, more intensely stained α -SMA-positive HSCs associated with vascular walls were observed in the liver parenchyma (Figure 2a). α -SMA was hardly detected in the normal liver by immunoblotting. In contrast, α -SMA expression was dramatically increased in the CCl₄ + Hp livers when compared with that in the CCl₄-only treated animals that exhibited slightly increased levels when compared with control animals (Figures 2b and c). This result completely correlated with our immunohistochemical observation in both species of the murine model.

Immunohistochemistry for triggering hepatic fibrosis was performed in the mouse and rat livers using anti-TGF- β 1 antibodies (Figure 3a). Macrophages expressing TGF- β 1 were infiltrated at the chronic injured area constituted with fibrotic septa around the central vein in the CCl₄-treated livers. *H. pylori* infection markedly increased the recruit of macrophages showing a positive reaction with TGF- β 1 in the CCl₄-treated livers. The weak activity of TGF- β 1 was detected by immunoblotting assay in controls and in the *H. pylori*-infected livers in a similar level, although it was difficult to detect by immunohistochemistry. Immunoblotting data showed that TGF- β 1 was expressed significantly higher in the

CCl₄ + Hp livers than in the only CCl₄-treated livers (Figures 3b and c). Overall, the expression of α -SMA and TGF- β 1 in the liver sections was modulated by orogastric *H. pylori* infection in the hepatic fibrosis-induced murine model.

Expression of SMP30 as an Anti-Oxidative Stress Marker in the CCl₄ + Hp Livers

Jung *et al* suggested that increased oxidative condition result in the downregulation of the SMP30 gene expression and in the subsequent decrease in SMP30 protein.¹⁶ *H. pylori* alone decreased the expression of SMP30, mainly located around the central vein, in the livers compared with that in the control livers on immunohistochemical staining (Figure 4a). The reduction in SMP30 in the CCl₄-treated livers was remarkably enhanced by *H. pylori* infection, suggesting an increased magnitude of oxidative stress (Figure 4a). Correlative trends were shown on immunoblotting (Figures 4b and c). *H. pylori* infection significantly decreased SMP30 expression in hepatocytes when compared with the control livers as shown through the immunohistochemical study. CCl₄ more severely reduced SMP30 levels in the livers than did *H. pylori*. Significant downregulation of SMP30 in the CCl₄-treated livers with concomitance of *H. pylori* infection in immunoblotting precisely agreed with the findings of immunohistochemical study. This was particularly true for mice infected with *H. pylori* that showed significantly decreased SMP30 expression in the CCl₄-treated livers.

Immunohistochemical Identification of *H. Pylori* Infection

Gram-negative curved bacterium was found in colonies obtained from incubation of the gastric mucosa (Figure 5b). Similar organisms in the gastric pits of infected animals were confirmed to be *H. pylori*-antigen positive by immunohistochemical stain using anti-*H. pylori* antibodies (Figure 5a). Immunohistochemical stain for *H. pylori* showed positively reacted particles within the *H. pylori*-infected liver tissue, especially in Kupffer cells existing along the adjacent sinusoid, and occasionally in hepatocytes (Figures 5c and d). Immunopositivity for *H. pylori* was 75% (6 of 8) in the *H. pylori*-infected mouse livers without CCl₄ administration, and 62.5% (5 of 8) in the *H. pylori*-infected mouse livers with CCl₄ administration (data not shown). Consequently, these immunohistochemical data support the results of the PCR reaction, indicating the presence of the *H. pylori* gene in the infected liver tissue.

Determination of *H. Pylori* Infection

Overall, 75% of the inoculated mice (12 of 16) had a typical *H. pylori* colony from plated homogenized stomach on MH agar plate with antibiotics, and Gram-negative spiral bacterium was found in these colonies by gram staining. Mice showing *H. pylori* colonies had organisms colonizing the gastric pit of the stomach on histopathological analysis (Figures 5a and b). There was no evidence of cross-infection

Figure 2 α -SMA expression of the livers. (a) The livers were stained for α -SMA and the number of positive cells was represented as mean $\pm$ s.d. $*P < 0.0001$. Normal expression of myofibroblasts (MFBs) was identified by α -SMA-positive staining and was restricted to the vascular walls in the portal area and the central vein. Plentiful activated hepatic stellate cells (HSCs) (arrowhead), strongly positive, in the liver parenchyma showed increased continuity with each other in CCl₄ + Hp compared with those in CCl₄ groups. CV, central vein; PV, portal vein; Scale bar, 100 μ m. (b and c) α -SMA expression in liver tissues was assessed by immunoblotting. Histogramic representation of relative expression of α -SMA in the liver tissues was measured and expressed as mean $\pm$ s.d. $*P < 0.005$ compared with only CCl₄-treated group. Upregulation of α -SMA activities in the liver infected by *H. pylori* accompanied with the administration of CCl₄ was observed in comparison with only CCl₄-treated livers.

as *H. pylori* was not present in any of the 16 sham-infected mice. To examine whether *H. pylori* could be detected in the liver tissue, DNA extracted from the liver tissue samples in mice were tested by PCR amplification using primers for the *Helicobacter* genus-specific 16S rRNA (Figure 6). The size of all PCR products corresponded to the expected size of 400 bp. The number of positive samples in the only *H. pylori*-infected group was 87.5% (7 of 8) and in the CCl₄ + Hp group, 100% (8 of 8) of samples were positive (data not shown).

Detection of Serum IgG Anti-*H. Pylori*

The level of serum anti-*H. pylori* total IgG in the *H. pylori*-infected groups is shown in Table 2. Sera were collected at week 8 in rats and at week 16 in mice, and the antibody response was determined by ELISA. We detected specific *H. pylori*-reactive IgG antibodies in 50% of the

infected rats (4 of 8) and in 87.5% of the infected mice (14 of 16). In mice, increased levels of the total IgG antibody titers against *H. pylori* were observed in the Hp group ($P = 0.03$) and in the CCl₄ + Hp group ($P = 0.005$) in comparison with the control group. To investigate the relationship between CCl₄ administration and rate of *H. pylori* infection, levels of IgG antibodies against *H. pylori* were compared as well as the sera of the only *H. pylori*-infected subjects and different groups accompanied with CCl₄ administration. Interestingly, *H. pylori* infection in the CCl₄-treated group resulted in significantly increased specific serum IgG ($P = 0.02$), in comparison with the only *H. pylori*-infected group. Taken together, it was confirmed that *H. pylori*-infected groups showed seropositivity in contrast to seronegativity in all *H. pylori*-noninfected groups in both rats and mice.

Figure 3 TGF- β 1 expression in the livers. (a) The livers were stained for TGF- β 1 and the number of positive cells was represented as mean $\pm$ s.d. * P < 0.0001. No expressed cell was identified by TGF- β 1-positive staining in the control and Hp groups. In CCl₄ groups, TGF- β 1-positive macrophages (arrowhead) can be seen in the fibrotic septa emerging from the central vein (CV). Remarkably increased macrophages (arrowhead), strongly positive, along the increased thick fibrotic septa are observed in CCl₄ + Hp groups. CV, central vein; scale bar, 100 μ m. (b and c) TGF- β 1 expression in the liver tissues was assessed by immunoblotting. Histogramic representation of relative expression of TGF- β 1 in the liver tissues was measured and expressed as mean $\pm$ s.d. * P < 0.005 compared with only CCl₄-treated group. Upregulation of TGF- β 1 activities in the liver infected by *H. pylori* accompanied by the administration of CCl₄ was observed compared with only CCl₄-treated liver.

Histopathological Assessment of the *H. Pylori*-Infected Livers

As shown in Figure 7, the *H. pylori*-infected livers showed extensively degenerative changes such as cytoplasmic vacuolation and increased binucleation, although the foci of hepatocytic death were rarely noted. This vacuolation showed mild lipid droplets by oil-red O staining (Figure 8). In addition, PAS stain showed reduction in glycogen storage in the *H. pylori*-infected livers (Figure 8). In the *H. pylori*-infected mice, the percentage of binucleated hepatocytes was significantly greater than in the respective control mice (Figure 7). To determine changes in hepatocyte proliferation and apoptosis, the expression of PCNA was assessed and the TUNEL method conducted, respectively, in the liver (Figure 7). Differences in PCNA levels among the groups were also statistically significant (P < 0.05). PCNA-positive cells, including binucleated hepatocytes, markedly increased

in the *H. pylori*-infected livers, in comparison with the control livers, whereas apoptosis assessed by TUNEL-positive count showed no difference between the two groups (Figure 7).

Discussion

We previously reported the development of primary biliary cirrhosis in a C57BL/6 mouse infected with *H. pylori* over 1 year²² and another author's experimental study on healthy C57BL/6 mice showed that *H. pylori* infection for 8 months led to hepatic inflammation characterized by the infiltration of lymphocytes.¹² In this study, we established a hepatic fibrosis model with repeated administration of CCl₄. To demonstrate the role of *H. pylori* in the progression of hepatic fibrosis, we constructed two separate experimental designs using two different rodent models to observe a potentially different effect between pre-infection and post

Figure 4 SMP30 expression in the livers. (a) Livers were stained for SMP30 and the number of positive cells was represented as mean $\pm$ s.d. * $P < 0.0001$. Strongly expressed normal hepatocytes within the cytoplasm and nucleus were identified around the hepatic central vein in the control group. Positively reacted cells in the CCl₄ + Hp group remarkably decreased in comparison with those in CCl₄ group. The area of positive immunoreaction around the central vein was significantly reduced in the Hp group compared with the normal control group. Scale bar, 400 μ m. (b and c) SMP30 expression in the liver tissues was assessed by immunoblotting. Histogrammic representation of relative expression of SMP30 in the liver tissues was measured and expressed as mean $\pm$ s.d. * $P < 0.005$ compared with only CCl₄-treated group. Downregulation of SMP30 activities in the liver infected by *H. pylori* accompanied by the administration of CCl₄ was observed in comparison with only CCl₄-treated liver.

infection of *H. pylori* after exposure to CCl₄ administration. In the preliminary study with rats, we did not include the only *H. pylori* group as we speculated whether *H. pylori* influenced liver injury induced by CCl₄. First, ATCC 43504 *H. pylori* strains which were not recognized as strong antigens was inoculated after the induction of liver injury to rats, from which the results suggested a possible role of *H. pylori* on liver fibrosis. Using the results obtained from the rat model, we experimented with the mouse model to inspect the independent role of *H. pylori* in the liver. To investigate whether pre-infection causes the same phenomenon, the SS1 *H. pylori* strain, much used as the standard mouse-adapted strain for experimental infection, was used for mice. Mice and rats were killed when known as inducing fibrotic stage by CCl₄ administration.^{17–19}

H. pylori infection for 4 months caused functional and morphological degenerative changes in hepatocytes, such as

the depletion of glycogen particles, hydropic degeneration, binucleation and accumulation of numerous small fat droplets in our present research (Figures 7 and 8). These histological findings regarding hepatocytes suggest that *H. pylori* causes a disarrangement of hepatic architecture with some showing slight focal necrosis and inflammatory changes in spite of there being no severe hepatitis present in the liver samples used in this study (Figure 7). The hepatocyte in those degenerated liver samples is more vulnerable to external harmful stimuli.²³ Consequently, another concomitant liver disorder may provide a synergistic combination of liver injury, such as steatosis, oxidative damages that aggravate liver injury.²⁴ Therefore, microvesicular fatty changes in the *H. pylori*-infected liver could be susceptible to the oxidative stress, and then deteriorate hepatic damage on the basis of liver fibrosis induced by CCl₄ administration. In addition, an increase in binucleated hepatocytes (Figure 7) indicates

Figure 5 Demonstration of *H. pylori* in the stomach and liver tissues by immunohistochemistry (IHC) using antibodies for *H. pylori* and Gram stain. Colonization of gastric tissue by spiral or curved *H. pylori* was observed in a gastric pit by IHC for *H. pylori* (a). The small curved *H. pylori* with Gram negativity was observed in the colonies plated from gastric mucosa of *H. pylori*-infected mice (b). IHC using antibodies for *H. pylori* in the liver tissues that were only *H. pylori* infected (c) and *H. pylori* infected with CCl_4 treatment (d). Positive immunoreactivity was observed along the hepatic sinusoid (arrowhead) and the intracytoplasm of hepatocyte (large arrow). Kupffer cells (small arrow) lining the sinusoid represent positive-reacted particles, may be phagocytosed (c). Immunopositive particles were observed in the cytoplasm of degenerated hepatocyte (d). Scale bar, 20 μm .

Figure 6 A schematic representation of the PCR products of the *H. pylori* DNA from *H. pylori*-infected mouse liver samples. Gel image showing amplification products of 16S rRNA PCR with *Helicobacter* genus-specific 16S rRNA primers at 400 bp. 'M' represents 100-bp molecular weight marker, and lanes 'N' and 'P' represent reaction negative control and positive control, respectively. Lanes 1 and 2 represent *H. pylori*-infected liver DNA samples without CCl_4 treatment, whereas lane 3 represents the control liver. Lane 4 represents only CCl_4 -treated liver without *H. pylori*-infected DNA sample. Lanes 5 and 6 represent the CCl_4 -treated liver with *H. pylori*-infected DNA samples.

pathological effects of *H. pylori* infection, as it suggests that under oxidative stress, the increase in binucleated hepatocytes can be caused either by a fusion of two hepatocytes with damaged cell membranes or by a regenerative process against hepatic damage.²⁵ Immunohistochemistry for PCNA showed that increased binucleated cells were correlated with a positive reaction in hepatocytes of the *H. pylori*-infected livers, suggesting that hepatocytes proliferated against liver damage (Figure 7).

One study suggested that *H. pylori* infection may be related to increasing endotoxins (LPS) found in Gram-negative bacteria in patients with cirrhosis.²⁶ Previous studies have described glycogen depletion in various infectious and pathological conditions, although the mechanism of depletion of liver glycogen stores in endotoxemia is still unclear.²⁷ Histological findings in this study suggest a possible derangement of carbohydrate metabolism in rodents infected with *H. pylori*. Moreover, reductions in hepatic glycogen suggested increased glucose utilization and were consistent

with the expected depletion of hepatic ATP after mitochondrial impairment, assuming that there was a consequent increase in energy production from glycolysis. Damaged mitochondria due to endotoxin result in hydropic degeneration in hepatocytes.²⁸ Therefore, functional changes such as the depletion of hepatic glycogen could point to impending liver damage during *H. pylori* infection.

Remarkably, our findings indicate that *H. pylori* exacerbate the progression of hepatic injury from fibrosis to cirrhotic stage in the CCl₄-treated liver. The degree of hepatocellular

injury was shown by a dramatic increase in serum levels of AST, ALT and hepatic hydroxyproline content, and the substantial extension of fibrous areas was confirmed by Azan staining. Biochemical markers on liver injury were significantly increased; morphological architecture was more severely destructed; and the fibrotic area was remarkably extended in CCl₄ + Hp rather than in only CCl₄-administered livers. Furthermore, the expression of fibrosis-related cytokine was significantly upregulated in the CCl₄ + Hp livers, compared with that in the CCl₄ livers. Immunoblot analysis, which shows a significant correlation with immunohistochemical data, further confirmed this finding. TGF- β has been shown to be a key profibrogenic cytokine, as it has a prominent role in the production of extracellular matrix by activated HSC.²⁹ In particular, TGF- β 1, which is a well-studied mediator in hepatic fibrogenesis, promotes HSC transition into the myofibroblast²⁹ and is involved in the formation of α -SMA fiber formation in the myofibroblast.³⁰

The major cellular mechanisms of CCl₄ are suggested to produce free radicals, which damage the hepatocyte by causing lipid peroxidation and binding cell structures.³¹ Many studies have indicated that oxidative stress may represent a common link between chronic liver damage and hepatic fibrosis.³² Damaged hepatocytes, their membrane components, metabolites of toxic agents and infiltrating inflammatory cells activated Kupffer cells, and then these activated Kupffer cells released several important cytokines to act on the HSCs, which transitioned to myofibroblast-like cells with a remarkably increased capacity for collagen synthesis.²⁹

Table 2 *H. pylori*-specific IgG antibody titers in sera

Animals and groups (n = 8)	<i>H. pylori</i> strain 43504 specific IgG antibody titer	Animals and groups (n = 8)	<i>H. pylori</i> strain SS1 specific IgG antibody titer
	OD 450 nm (mean $\pm$ s.d.)		OD 450 nm (mean $\pm$ s.d.)
<i>Rats</i>		<i>Mice</i>	
Con	0.1085 $\pm$ 0.0063	Con	0.0283 $\pm$ 0.0248
CCl ₄	0.121 $\pm$ 0.0086	Hp	0.1957 $\pm$ 0.2050*
CCl ₄ +Hp	0.271 $\pm$ 0.2589	CCl ₄	0.0414 $\pm$ 0.0285
		CCl ₄ +Hp	0.775 $\pm$ 0.8198* [†]

OD, optical density.

Results are expressed as mean $\pm$ s.d.

* $P < 0.05$ compared with the noninfected control group, [†] $P = 0.02$ compared with the Hp group.

Figure 7 Histopathological findings of the *H. pylori*-infected livers. (A) Histopathological changes by *H. pylori* infection in the mouse liver tissues stained by hematoxylin–eosin staining. Normal mouse hepatocytes with eosinophilic cytoplasm were separated from the sinusoid (a). The *H. pylori*-infected mouse liver tissue showed compressed sinusoid by swollen hepatocytes with faint cytoplasm with vacuole (b). The *H. pylori*-infected mouse liver showed diffuse hepatocellular degeneration characterized by pale swollen hepatocytes with cytoplasmic rarefaction, anisokaryosis and binucleation (arrow) at high magnification (c). Scale bar: (a and b) 100 μ m; (c) 20 μ m. The number of binucleated hepatocytes significantly increased in *H. pylori*-infected mice compared with that in the controls. * $P < 0.001$ (d). (B) The expression of PCNA and TUNEL in normal and *H. pylori*-infected mouse liver. The normal liver showed nearly no activity for PCNA immunostain. Scale bar, 100 μ m (a). The *H. pylori*-infected liver showed increased immunopositive cells, especially binucleated hepatocytes, for PCNA antibody. Scale bar, 100 μ m (b). The number of TUNEL- and PCNA-positive cells was compared between the control and *H. pylori*-infected groups (c). TUNEL indicates a nonsignificant difference between groups on the basis of Student's *t*-test ($P > 0.05$). The increase in PCNA-positive hepatocytes caused by *H. pylori* infection ($P < 0.05$) is noted. PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase-mediated nick-end labeling.

Figure 8 Histopathological changes by *H. pylori* infection in the mouse liver tissues stained by PAS and oil-red O staining. To observe the location of hepatic glycogen granules and the glycogen density of hepatocytes, PAS stain was used. The noninfected liver stained with PAS visualized the densely deposited glycogen as purple granules in hepatocyte cytoplasm. Scale bar, 50 μ m. In the *H. pylori*-infected liver, glycogen storage was remarkably reduced compared with the noninfected liver. To differentiate intracellular vacuoles and lipid accumulation, a frozen section of the liver was stained with oil-red O. The noninfected liver stained with oil-red O showed several hepatocytes containing positively few lipid droplets. The *H. pylori*-infected liver showed increased number of small lipid droplets (red) in hepatocytes. Scale bar, 20 μ m.

Therefore, oxidative stress has been reported to modulate collagen gene expression.³³ Furthermore, hepatic tissue damage concurrent with pathogenic infection in the liver may increase the synergistic effect of oxidative stress in the pathogenesis of hepatic injury.

SMP30 has been known as a multifunctional protein, highly expressed in hepatocytes, preventing oxidative stress and cellular apoptosis.^{16,34} Jung *et al* proposed that increased oxidative status by LPS-treatment, well known to increase oxidative stress, causes downregulation of the SMP30 gene expression.⁴⁰ Decrease in SMP30 expression in this study provides the possibility that LPS originating from *H. pylori* could reach the liver and result in oxidative stress. Ishigami *et al*³⁵ suggested that susceptibility to harmful stimuli markedly increases, when SMP30 expression in the liver decreases due to continuous oxidative stress.

This is the first *in vivo* study to investigate the association between the presence of *H. pylori* DNA in the liver and hepatic fibrosis. Our study also successfully detected *Helicobacter* genomic materials in the mouse liver as well as showed that most *H. pylori*-infected animals had specific

antibodies in both experimental species (Table 2). One study demonstrated that immunostaining for *H. pylori* and electron microscopy showed morphologically intact bacteria in one patient with primary sclerosing cholangitis,³⁶ and Wang *et al*¹³ demonstrated the presence of *H. pylori* bacteria inside the HCC tissue by immunohistochemical stain for *H. pylori*. In addition, the study by Francavilla *et al*,³⁷ which stated that vacuolating toxin A (VacA), one of the bacterial virulence factors, was detected in hepatocytes of patients with isolated hypertransaminasemia supports the report that transaminases in humans infected by cytotoxic strains of *H. pylori* increase mildly.³⁸ In this study, we ascertained bacterial fragments by immunostaining for *H. pylori* in *H. pylori*-infected liver tissues. These data strongly support those of previous studies that *H. pylori* DNA and/or protein could reach the liver.

Finally, chronic *H. pylori* infection could have a crucial role as a co-risk factor for exacerbation of liver fibrosis under exogenous oxidative stimuli as well as being an independent troublemaker causing dysfunction in the livers of rodent animals. In addition, our findings indicate that influence of

H. pylori on hepatopathy is not only restricted to cytotoxic strain, ATCC 43504 (VacA, s1m1) but also to the SS1 (VacA, s2m2) strain which shows no vacuolating cytotoxic activity.³⁹ A possible explanation for this finding may be that another pathological factor from *H. pylori* causing oxidative stress besides cytotoxic VacA may participate in the pathogenesis of liver fibrosis.

Nonetheless, this study has a limitation to directly relate to the pathophysiological situation in humans. In humans, *H. pylori* infection is usually acquired in early childhood and results in the majority of subjects having chronic persistent gastritis with the establishment of equilibrium between the host and germ. Liver damage (alcohol or virus induced) occurs decades later, when a 'balanced gastritis' is present in subjects. Therefore, we speculate that it is difficult to totally relate to human condition, as the duration of *H. pylori* infection that lasts for several weeks in the hepatic fibrosis-induced animal models of this study is short to link to the pathophysiological situation of the humans. Further *in vitro* and *in vivo* studies are still necessary to delineate the precise mechanisms underlying the pathogenesis of *H. pylori*-related liver fibrosis in humans.

ACKNOWLEDGEMENT

This research was supported by a grant (code: CBM 31-B3003-01-01-00) from the Center for Biological Modulators of the 21st Century Frontier R&D Program, the Ministry of Science and Technology, Korea.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

1. Crowe SE. *Helicobacter* infection, chronic inflammation, and the development of malignancy. *Curr Opin Gastroenterol* 2005;21:32–38.
2. Stalke P, Zoltowska A, Orlowski M, *et al.* Correlation between liver damage and degree of gastric mucosae colonisation by *Helicobacter pylori* in subjects with parenchymatous liver damage. *Med Sci Monit* 2001;7(Suppl):271–276.
3. Pellicano R, Leone N, Berrutti M, *et al.* *Helicobacter pylori* seroprevalence in hepatitis C virus positive patients with cirrhosis. *J Hepatol* 2000;33:648–650.
4. Fox JG, Drolet R, Higgins R, *et al.* *Helicobacter canis* isolated from a dog liver with multifocal necrotizing hepatitis. *J Clin Microbiol* 1996;34:2479–2482.
5. Ponzetto A, Pellicano R, Redaelli A, *et al.* *Helicobacter pylori* infection in patients with hepatitis C virus positive chronic liver diseases. *New Microbiol* 2003;26:321–328.
6. Ponzetto A, Pellicano R, Leone N, *et al.* *Helicobacter pylori* seroprevalence in cirrhotic patients with hepatitis B virus infection. *Neth J Med* 2000;56:206–210.
7. Calvet X, Navarro M, Gil M, *et al.* Seroprevalence and epidemiology of *Helicobacter pylori* infections in patients with cirrhosis. *J Hepatol* 1997;26:1249–1254.
8. Nilsson H-O, Taneera J, Castedal M, *et al.* Identification of *Helicobacter pylori* and other *Helicobacter* species by PCR, hybridization, and partial DNA sequencing in human liver samples from patients with primary sclerosing cholangitis or primary biliary cirrhosis. *Clin Microbiol* 2000;38:1072–1076.
9. Avenaud P, Marais A, Monteiro L, *et al.* Detection of *Helicobacter* species in the liver of patients with and without primary liver carcinoma. *Cancer* 2000;89:1431–1439.
10. Desmet VJ, Roskams T. Cirrhosis reversal: a duel between dogma and myth. *J Hepatol* 2004;40:860–867.
11. George J, Tsutsumi M, Takase S. Expression of hyaluronic acid in *N*-nitrosodimethylamine induced hepatic fibrosis in rats. *Int J Biochem Cell Biol* 2004;36:307–319.
12. Tian XF, Fan XG, Fu CY, *et al.* Experimental study on the pathological effect of *Helicobacter pylori* on liver tissues. *Zhonghua Gan Zang Bing Za Zhi* 2005;13:780–783.
13. Wang X, Willen R, Svensson M, *et al.* Two-year follow-up of *Helicobacter pylori* infection in C57BL/6 and Balb/cA mice. *APMIS* 2003;111:514–522.
14. Taylor NS, Fox JG, Yan L. *In-vitro* hepatotoxic factor in *Helicobacter hepaticus*, *H. pylori* and other *Helicobacter* species. *J Med Microbiol* 1995;42:48–52.
15. Chen R, Fan XG, Huang Y, *et al.* *In vitro* cytotoxicity of *Helicobacter pylori* on hepatocarcinoma HepG2 cells. *Ai Zheng* 2004;23:44–49.
16. Lee A, O'Rourke J, De Ungria MC, *et al.* A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. *Gastroenterology* 1997;112:1386–1397.
17. Fu Y, Zheng S, Lin J, *et al.* Curcumin protects the rat liver from CCl₄-caused injury and fibrogenesis by attenuating oxidative stress and suppressing inflammation. *Mol Pharmacol* 2008;73:399–409.
18. Oriya K, Yoshikawa M, Ojui Y, *et al.* Embryonic stem cells reduce liver fibrosis in CCl₄-treated mice. *Int J Exp Pathol* 2008;89:401–409.
19. Zubakhin AA, Kutina SN, Maianaki DN. Functional state of the hematopoietic system in different stages of CCl₄-induced liver fibrosis in mice. *Biull Eksp Biol Med* 1992;114:22–24.
20. Mendler MH, Yashar B, Govindarajan S, *et al.* A novel semi-quantitative histological scoring system for nonalcoholic fatty liver disease: evaluation and clinical correlations. *Hepatology* 2002;36:407A.
21. Jeong DH, Lee GP, Jeong WI, *et al.* Alterations of mast cells and TGF-beta1 on the silymarin treatment for CCl₄-induced hepatic fibrosis. *World J Gastroenterol* 2005;11:1141–1148.
22. Goo MJ, Ki MR, Lee HR, *et al.* Primary biliary cirrhosis, similar to that in human beings, in a male C57BL/6 mouse infected with *Helicobacter pylori*. *Eur J Gastroenterol Hepatol* 2008;20:1045–1048.
23. Carmiel-Haggai M, Cederbaum AI, Nieto N. A high-fat diet leads to the progression of non-alcoholic fatty liver disease in obese rats. *FASEB J* 2005;19:136–138.
24. Powell EE, Jonsson JR, Clouston AD. Steatosis: co-factor in other liver diseases. *Hepatology* 2005;42:5–13.
25. Jeong WI, DO SH, Kim TH, *et al.* Acute effects of fast neutron irradiation on mouse liver. *J Radiat Res (Tokyo)* 2007;48:233–240.
26. Abdel-Hady H, Zaki A, Badra G, *et al.* *Helicobacter pylori* infection in hepatic encephalopathy: relationship to plasma endotoxins and blood ammonia. *Hepatol Res* 2007;37:1026–1033.
27. Engin A, Zemheri M, Bukan N, *et al.* Effect of nitric oxide on the hypoglycaemic phase of endotoxaemia. *ANZ J Surg* 2006;76:512–517.
28. Berry LJ, Smythe DS, Young LG. Effects of bacterial endotoxin on metabolism. I. Carbohydrate depletion and the protective role of cortisone. *J Exp Med* 1959;110:389–405.
29. Tahashi Y, Matsuzaki K, Date M, *et al.* Differential regulation of TGF-beta signal in hepatic stellate cells between acute and chronic rat liver injury. *Hepatology* 2002;35:49–61.
30. Dooley S, Hamzavi J, Breitkopf K, *et al.* Smad7 prevents activation of hepatic stellate cells and liver fibrosis in rats. *Gastroenterology* 2003;125:178–191.
31. Slater TF, Sawyer BC. The stimulatory effects of carbon tetrachloride and other halogenoalkanes on peroxidative reactions in rat liver fractions *in vitro*. General features of the systems used. *Biochem J* 1971;123:805–814.
32. Svegliati Baroni G, D'Ambrosio L, Ferretti G, *et al.* Fibrogenic effect of oxidative stress on rat hepatic stellate cells. *Hepatology* 1998;27:720–726.
33. Casini A, Cunningham M, Rojkind M, *et al.* Acetaldehyde increases procollagen type I and fibronectin gene transcription in cultured rat fat-storing cells through a protein synthesis-dependent mechanism. *Hepatology* 1991;13:758–765.
34. Sar P, Rath B, Subudhi U, *et al.* Alterations in expression of senescence marker protein-30 gene by 3,3',5-triiodo-L-tyrosine (T₃). *Mol Cell Biochem* 2007;303:239–242.
35. Ishigami A, Fujita T, Handa S, *et al.* Senescence marker protein-30 knockout mouse liver is highly susceptible to tumor necrosis factor-alpha- and Fas-mediated apoptosis. *Am J Pathol* 2002;161:1273–1281.
36. Wadström T, Ljungh A, Willén R. Primary biliary cirrhosis and primary sclerosing cholangitis are of infectious origin!. *Gut* 2001;49:454.

37. Francavilla A, Ierardi E, Francavilla R, *et al*. Immunohistochemical detection of *Helicobacter pylori* vacuolating cytotoxin in the hepatocytes of patients with isolated hypertransaminasaemia. *Ital J Gastroenterol Hepatol* 1999;3:675–676.
38. Figura N. Hypothesis: *Helicobacter* toxins and liver. *Helicobacter* 1996;1:187–189.
39. Day AS, Jones NL, Policova Z, *et al*. Characterization of virulence factors of mouse-adapted *Helicobacter pylori* strain SS1 and effects on gastric hydrophobicity. *Dig Dis Sci* 2001;46:1943–1951.
40. Jung KJ, Ishigami A, Maruyama N, *et al*. Modulation of gene expression of SMP-30 by LPS and calorie restriction during aging process. *Exp Gerontol* 2004;39:1169–1177.