Clinical Significance of Serum Matrix Metalloproteinases and Tissue Inhibitors of Metalloproteinases in Chronic Liver Disease

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***Objective*: We have attempted to determine serum levels of type IV collagen (IV-C), laminin (LM), prolylhydroxylase (PH), metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) in chronic liver disease to elucidate the clinical significance of MMPs and TIMPs in the process of hepatic fibrosis.**
***Methods*: Serum samples were collected from 60 patients with chronic liver disease caused by hepatitis B or C. Serum levels of IV-C, LM, PH, MMP-1, 2 and 3, and TIMP-1 and 2 were measured by a one-step sandwich enzyme immunoassay using monoclonal antibodies. The values were correlated with Histology Activity Index (HAI) scores of liver biopsy specimens.**
***Results*: LM and IV-C levels markedly increased in parallel with the progression of the chronic liver disease. The MMP-2 and MMP-3 levels tended to increase in chronic active hepatitis (CAH), and significantly elevated in liver cirrhosis (LC). There was a positive correlation between the IV-C and MMP-2 levels, and the ratio of IV-C to MMP-2 levels was significantly elevated in LC. Both TIMP-1 and TIMP-2 levels were markedly increased in LC. The HAI scores were positively correlated with the serum IV-C and MMP-2 levels.**
***Conclusions*: Serum IV-C and MMP-2 levels may be useful diagnostic markers for hepatic fibrosis, since they increased in parallel with the progression of chronic liver disease. In addition, the imbalances between IV-C, LM, and TIMP-1 and 2 as fibrogenic factors and MMP-2 and 3 as fibrolytic factors may lead to fibrosis in chronic viral liver disease, especially in cirrhosis.**

Key words: hepatic fibrosis, MMP, TIMP, type IV collagen, chronic liver disease

ABBREVIATIONS
CIH, chronic inactive hepatitis; CAH, chronic active hepatitis; LC, liver cirrhosis; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; ECM, extracellular matrix proteins; IV-C, type IV collagen; LM, laminin; PH, prolyl hydroxylase

INTRODUCTION

In hepatic fibrosis, there is excessive accumulation of extracellular matrix proteins (ECMs), as a result of an imbalance between synthesis and degradation [1]. Hepatic fibrosis is characterized by the deposition of ECMs, especially collagens, in the extracellular space. Type I collagen (I-C) and type III collagen (III-C) increase in portal areas, forming the bulk of the septa as chronic liver disease progresses. Type IV collagen (IV-C) as one of the basement membrane proteins is widely represented in fibrotic liver tissue. Recent immunohistochemical and molecular biology studies have indicated that hepatic stellate cells and myofibroblasts may be responsible for accumulation of ECMs in hepatic fibrogenesis [2-4].

The matrix metalloproteinases (MMPs), a family of neutral proteinases that require zinc and calcium ions for their activity, have the ability to degrade ECMs, while tissue inhibitors of metalloproteinases (TIMPs) are regulatory factors in the degradation of ECMs [5, 6]. Thus far, 17 MMPs have been identified by DNA cloning, and each MMP degrades specific collagen as a substrate. They play an important role in a number of biological processes, such as wound healing/repair, fibrosis, and metastasis [7]. MMP-1, or interstitial collagenase, produced by connective tissue cells cleaves the triple helix of I-C, II-C, and III-C. MMP-2 or gelatinase has been found to degrade IV-C, V-C, VII-C, X-C. MMP-3 or stromelysin-1 cleaves proteoglycan, IV-C, and various stromal components.

In this study we determined the serum levels of IV-C and laminin (LM) as ECMs, prolyl hydroxylase (PH) as a collagen synthesize, MMPs as a degrading enzyme, and TIMPs in patients with chronic viral hepatitis and liver cirrhosis (LC) by one-step sandwich enzyme immunoassay (EIA) using monoclonal antibodies. The correlation between the serum levels and the histological findings in biopsy specimens were assessed to elucidate the clinical significance of MMPs and TIMPs in hepatic fibrogenesis.

PATIENTS AND METHODS

Patients

The subjects were a total of 60 patients (37 men and 23 women) with chronic liver disease caused by hepatitis B or C infection. There were seven cases of chronic inactive hepatitis (CIH), 36 cases of chronic active hepatitis (CAH), and 17 cases of LC (Table 1). There were no significant differences between the clinical data in these groups, except that age and total bilirubin were higher in LC than in CIH and CAH. All patients were

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Table 1 Clinical data of the patients with chronic inactive hepatitis (CIH), chronic active hepatitis (CAH) and liver cirrhosis (LC).

	CIH	CAH	LC
No. of patients	7	36	17
hepatitis B	2	15	3
hepatitis C	5	21	14
male / female	4 / 3	26 / 10	7 / 10
age (years)	39 ± 5	47 ± 3	58 ± 10
ALT (U/l)	61 ± 36	122 ± 24	77 ± 16
γ - GTP (U/l)	21 ± 3	60 ± 10	129 ± 64
Total Bil (mg/dl)	0.5 ± 0.1	0.6 ± 0.1	1.8 ± 0.4

Values are expressed as means ± SE.

diagnosed according to both of biochemical data and the histological findings in liver specimens obtained blindly by needle biopsy, or laparoscopically. None of the patients had alcoholic liver disease, autoimmune liver disease or drug-induced liver disease. The aliquots of serum for determination of ECM, MMPs, and TIMPs were stored at −80 ˚C until used.

Measurements of ECM, MMPs, and TIMPs

Serum levels of human MMP-1, MMP-2, and MMP-3 were determined by one-step sandwich enzyme immunoassay (EIA) using monoclonal antibodies against the individual MMPs (Fuji Chemical Industries, Takaoka, Japan) [8, 9]. The monoclonal antibodies against individual MMPs detected both precursor and active forms of MMPs, but did not cross-react with each other. The assay system employed two simultaneous immunoreactions that used a solid-phase monoclonal antibody and a horseradish peroxidase-labeled monoclonal antibody (Fab').

Serum TIMP-1 and TIMP-2 levels were also determined by the same one-step sandwich EIA described above using monoclonal antibodies [10, 11]. The antibodies recognized a free form of TIMP and a form complexed with MMPs. Serum IV-C (helical domain), LM, and PH levels were measured by EIA (Fuji Chemical Industries, Takaoka, Japan) [12, 13].

Calculation of Histology Activity Index (HAI) Scores

Liver specimens obtained by needle biopsy from all of the patients with chronic liver disease were fixed in 10% formalin, embedded in paraffin, and routinely stained with hematoxylin-eosin (H&E) and Azan-Mallory stain. The liver biopsy specimens were examined by two different hepatologists and scored according to the method of Knodell *et al.* [14]. Total scores and scores in each of four categories, i.e., I) periportal necrosis, II) intralobular degeneration and focal necrosis, III) portal inflammation and IV) fibrosis were calculated in all the liver tissues.

Statistical Analysis

All the data were expressed as means ± SE. Statistical comparisons between two groups were made by ANOVA. Correlations between the two parametric data were examined by Pearson standard linear regression analysis or Spearman rank correlation analysis.

RESULTS

Serum levels of IV-C tended to be elevated as chronic liver disease progressed. In CIH, the levels was 118.6 ± 9.9 ng/ml (mean ± SE), but they were elevated in CAH (152.7 ± 9.2 ng/ml), and significantly increased in LC to be 309.3 ± 21.8 ng/ml, compared with CIH and CAH (Fig. 1). The LM levels were elevated to be 190.0 ± 19.1 ng/ml in CIH, 222.2 ± 6.7 ng/ml in CAH and 315.9 ± 16.7 ng/ml in LC. The serum LM levels were significantly ($p < 0.001$) increased in LC, compared with CIH and CAH. On the other hand, the PH levels were tended to increase, but the differences between the liver diseases were not marked at least in serum.

The serum MMP-1 levels differed in CIH (7.8 ± 2.2 ng/ml), CAH (10.5 ± 1.7 ng/ml), and LC (5.9 ± 0.8 ng/ml), as shown in Fig. 2. It was of particular interest that the levels of MMP-2, which degrades IV-C, were 704.3 ± 89.7 ng/ml in CIH and 751.7 ± 20.9 ng/ml in CAH, but they significantly increased in LC (1204.1 ± 97.3 ng/ml). Serum MMP-3 levels tended to increase in parallel with the progression of chronic liver disease, and they were markedly increased in LC (77.6 ± 21.0 ng/ml) compared with CIH (35.1 ± 5.4 ng/ml) and CAH (43.9 ± 4.1 ng/ml).

Since both IV-C and MMP-2 increased in chronic liver disease, the relation between the serum levels of the two was assessed. Interestingly, a positive correlation was found between IV-C and MMP-2 levels in chronic liver disease (Fig. 3), and the ratio of IV-C to MMP-2 levels was markedly elevated in LC, compared with CIH and CAH (Fig. 4).

The serum level of TIMP-1 was elevated in CAH (208.9 ± 12.4 ng/ml), and significantly increased in LC (249.1 ± 26.1 ng/ml), compared with CIH (153.7 ± 13.4 ng/ml). On the other hand, the serum levels of TIMP-2 were unchanged in CIH (53.1 ± 17.1 ng/ml) and CAH (46.9 ± 4.2 ng/ml). They tended to be increased in LC (81.9 ± 18.4 ng/ml), but the difference was not significant (Fig. 5). The ratio of MMP-2 to TIMP-2 was approximately 20 in each disease, and there were no obvious differences among chronic liver diseases.

The relation between the serum levels of IV-C, LM, MMP-1, 2, and 3, TIMP-1 and 2, and the HAI scores of biopsy specimens was assessed in the chronic liver diseases. There were positive correlations between serum IV-C and both the total HAI score and the cat-

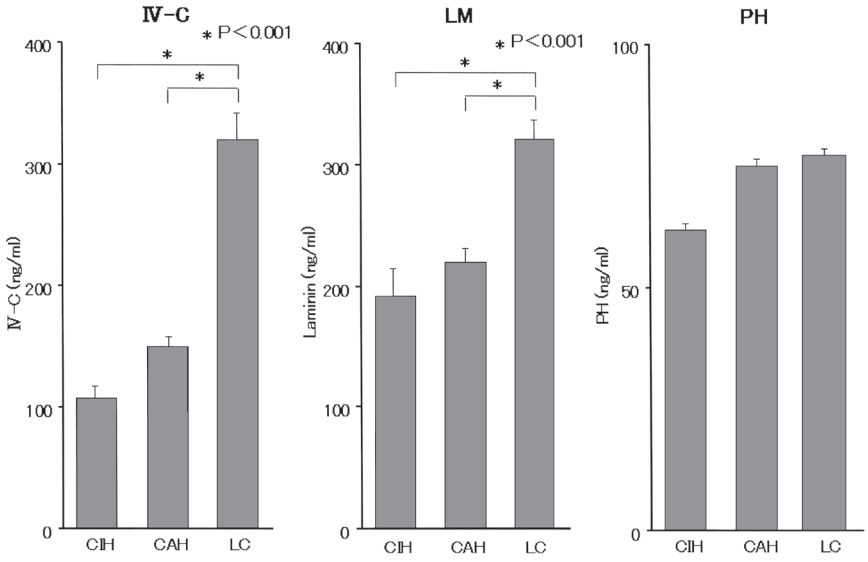

Fig. 1 Serum type IV collagen (IV-C), laminin (LM) and proly hydroxylase (PH) levels in chronic liver diseases.
Both IV-C and LM levels increased as chronic liver disease progressed, and then significantly increased in LC. PH also tended to increase.

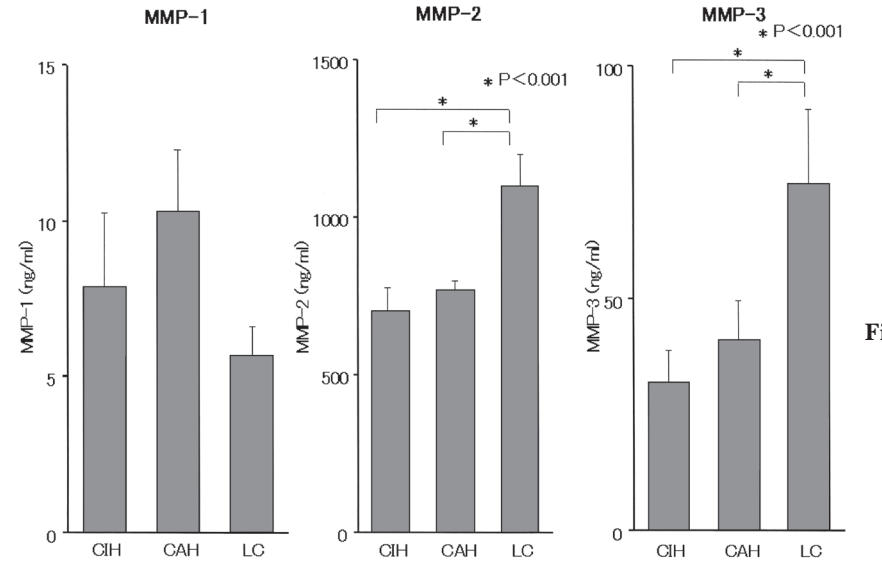

Fig. 2 Serum matrix metalloproteinase (MMP) levels in chronic liver diseases.
MMP-1 levels varied in chronic liver disease, whereas MMP-2 and MMP-3 levels increased with the progression of chronic liver disease, and were markedly increased in LC.

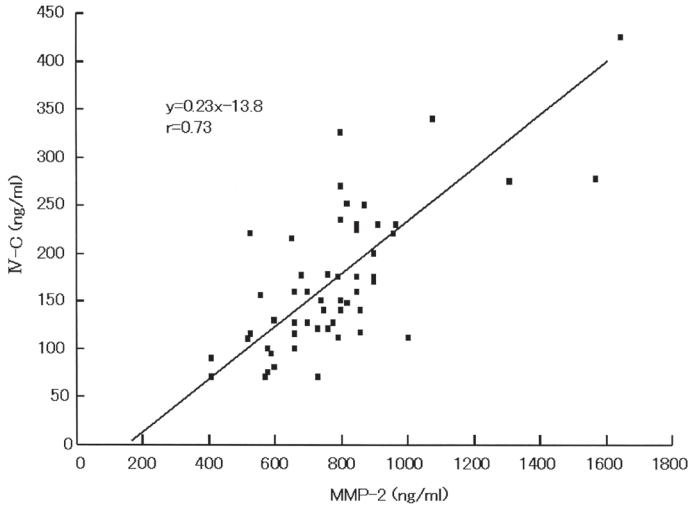

Fig. 3 Correlation between serum type IV collagen (IV-C) and matrix metalloproteinase (MMP)-2 levels.
There was a significant correlation between the IV-C and MMP-2 levels in chronic liver disease.

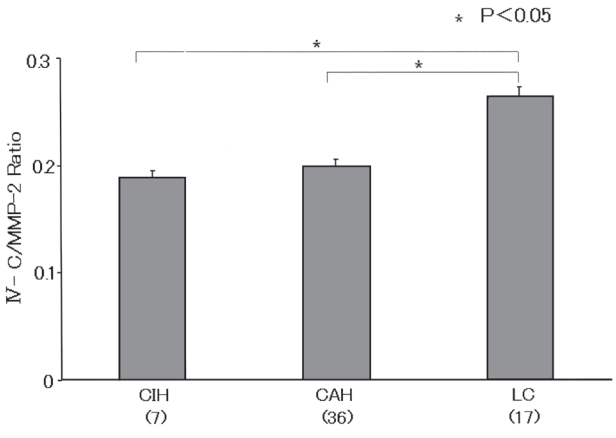

Fig. 4 Relative changes in serum type IV collagen (IV-C) and matrix metalloproteinase (MMP)-2 levels.
The ratio of IV-C to MMP-2 levels was significantly higher in LC than in CIH and CAH.

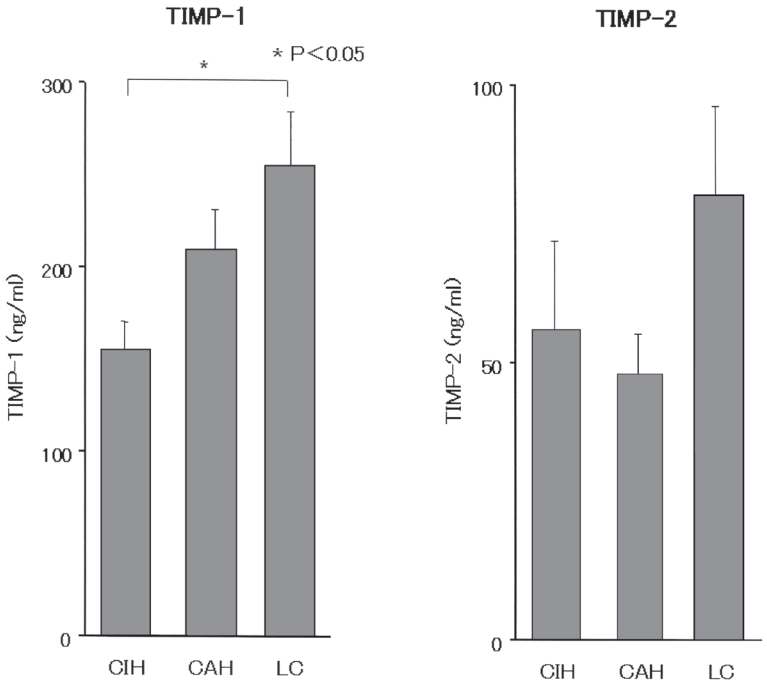

Fig. 5 Serum tissue inhibitor of metalloproteinase (TIMP) levels in chronic liver disease.
TIMP-1 levels increased with the progression of chronic liver disease, and TIMP-2 levels tended to be higher in LC.

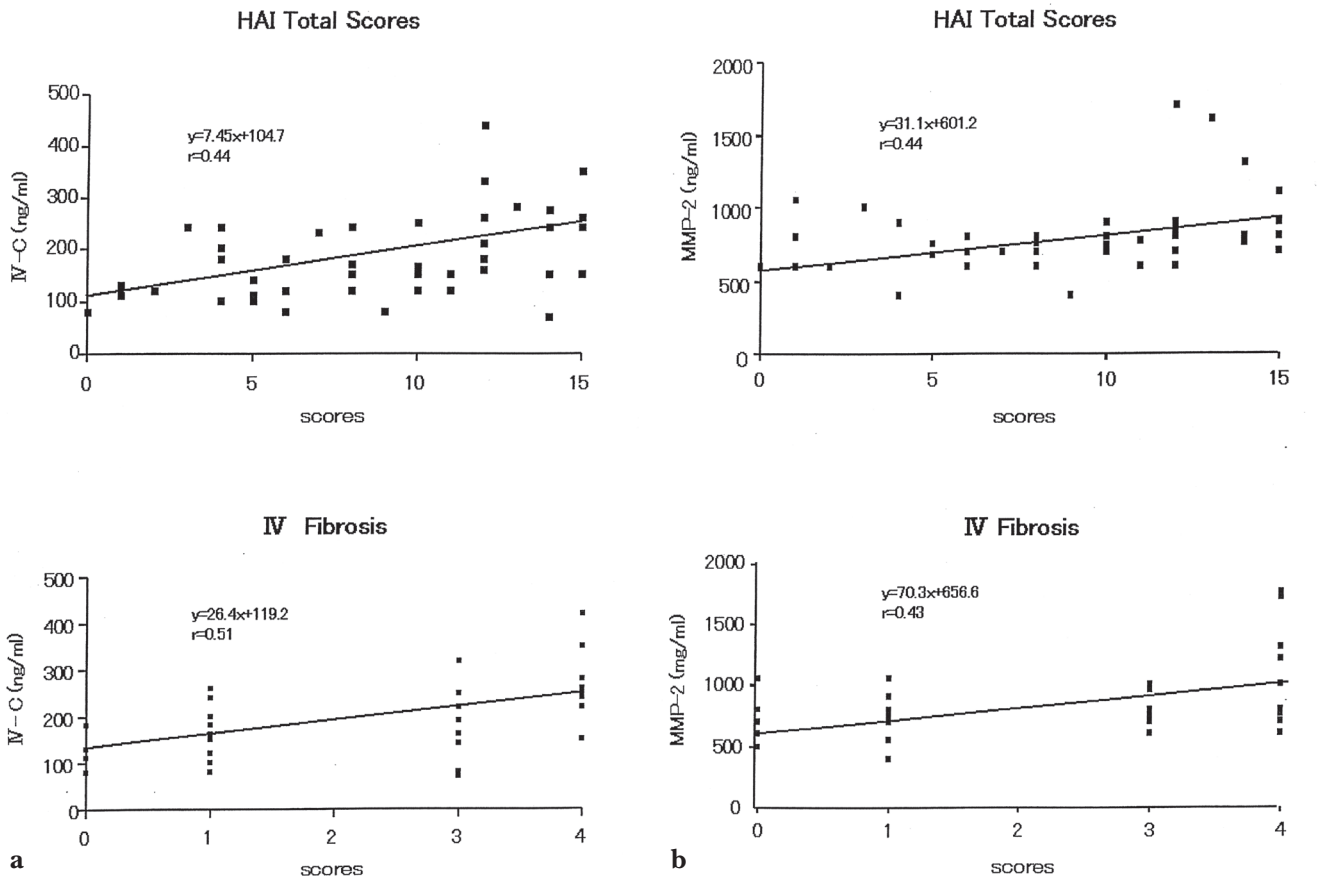

Fig. 6 Correlation between serum type IV collagen (IV-C) and matrix metalloproteinase (MMP)-2 levels and histology activity index (HAI) scores in chronic liver disease.
a. Correlation between serum IV-C levels and HAI scores. b. Correlation between serum MMP-2 levels and HAI scores.
Both IV-C and MMP-2 levels were positively correlated with total HAI scores and hepatic fibrosis scores.

egory 4 score, i.e., hepatic fibrosis. The serum MMP-2 levels were also positively correlated with the HAI scores (Fig. 6), but the MMP-1, MMP-3, TIMP-1 and TIMP-2 levels were not correlated with any of the HAI scores.

DISCUSSION

The deposition of ECMs including collagens caused by the overproduction exceeding the degradation is one of the most important phenomena in hepatic fibrogenesis. In the present study, we determined the serum levels of IV-C, LM, PH, and TIMPs, as fibrogenic factors and MMPs, as fibrolytic factors in chronic liver diseases caused by viral infections, and correlated them with the histological findings in liver biopsy specimens. Serum levels of IV-C in CIH were as high as those in healthy subjects [12]. They tended to elevate in CAH and significantly increased in LC, suggesting that IV-C may increase in serum as chronic liver diseases progressed. In addition, there was a positive correlation between serum IV-C and the total HAI scores and hepatic fibrosis scores of the liver biopsy tissue. These findings are consistent with the results of previous studies, which indicate that serum levels of IV-C are increased with progression of hepatic fibrosis in chronic viral disease [15] and alcoholic liver disease [16]. Serum IV-C levels are more prominently increased in alcoholic liver disease than in chronic viral disease, and then well correlated with their tissue levels in the liver. It is generally known that increases in IV-C and LM lead to the appearance of basement membranes along the sinusoidal wall, so-called sinusoidal capillarization, in LC. Takahara *et al.* reported that IV-C gene expression is activated in the early stage of hepatic fibrosis induced by carbon tetrachloride in rats [17], and they concluded that IV-C is increased at the gene and protein levels in the fibrotic process of the liver to be elevated in serum levels in LC.

A number of enzymes are involved in the degradation and the synthesis of ECMs in the liver, and progression of hepatic fibrosis may be associated with the imbalance between the activities of MMPs and TIMPs. MMPs are a family of Zn-end peptidases, and divided into three broad categories: 1) MMP-1, or interstitial collagenase, 2) MMP-2 and MMP-9, or gelatinase, and 3) MMP-3, or stromelysin. In this paper, we have reported the serum levels of MMP-1, 2, and 3 and TIMP-1 and 2 in the process of chronic liver diseases caused by viral infection. Serum MMP-2 levels did not markedly increase in CIH (704.3 ± 89.7 ng/ml), when compared with those of healthy subjects (570.0 ± 118.0 ng/ml) [9]. It is noteworthy that the serum MMP-2 levels were elevated in CAH (751.7 ± 20.9 ng/ml), and significantly increased in LC (1204.1 ± 97.3 ng/ml). Both IV-C and MMP-2 levels were increased in the chronic liver diseases, and there was a significant correlation between them, as shown in Fig. 3. When the relations between the serum IV-C and MMP-2 levels and the histological findings in the liver biopsy specimens were assessed, both were found to be positively correlated with the HAI scores, especially the hepatic fibrosis scores. It is therefore concluded that increases in serum IV-C and MMP-2 levels in serum are closely associated with the progress of hepatic fibrosis in chronic liver disease. Since the ratio of IV-C to MMP-2 levels was markedly elevated in LC, compared with CIH and CAH, presumably, production of IV-C exceeds degradation in LC, leading to accumulation of extracellular matrix during hepatic fibrogenesis.

It has been reported that the activity of MMPs in liver homogenates elevates in parallel with the increased collagen accumulation at the early stage of hepatic fibrosis in alcoholic liver disease, but that increased MMP activity is reduced in the late stage [18]. In this study, serum MMP-1 levels differed in CIH, CAH, and LC, but they remained within normal limits [8]. Both MMP-2 and MMP-3 levels tended to increase in chronic hepatitis, and markedly elevated in LC. These findings are consistent with the previous report by Maruyama *et al.* [18], except that MMPs increased in LC. This discrepancy may be due to differences in patient background between alcoholic and viral liver disease. It should also be borne in mind that the one-step sandwich enzyme immunoassay used here determines both precursor and active forms of MMPs. Takahara *et al.* reported that expression of MMP-2 increased as fibrosis progressed in experimental liver fibrosis induced by carbon tetrachloride [3], and that the expression of both active and latent forms of MMP-2 detected by zymography is elevated 15- fold in the early reversible stage, but decreases gradually after discontinuation of intoxication. Recently, membrane-type MMPs were identified as a unique activator of proMMP-2 [19, 20]. It has been reported that the expression of membrane type1-MMP as well as MMP-2 is increased in human livers of chronic hepatitis and cirrhosis, and that dual overexpression is found in stellate cells and fibroblasts, suggesting that activated MMP-2 may play an important role in remodeling during the process of hepatic fibrosis [4]. MMP-2 and membrane type1-MMP have been reported to be localized on hepatic stellate cells in the lobules and periportal areas and on fibroblasts in the fibrous septa, indicating that MMP-2 and membrane type1-MMP may be produced by these cells. It is of particular interest that activated hepatic stellate cells have the potential to remodel ECM as they produce MMP-2, MMP-3 [21] and TIMP-1 [22].

TIMPs are synthesized by a variety of connective tissue cells and specifically inhibit MMPs. TIMP-1 and TIMP-3 are biologically similar in function and distribution, but they are independently regulated [23, 24]. Our study indicated that there were no significant differences of serum TIMP-1 levels between CIH and healthy subjects [10]. However, they increased as chronic liver disease progresses, and they are significantly elevated in LC, compared with CIH. By contrast, the serum levels of TIMP-2 did not change so much in CIH and CAH, but they tended to increase in LC. Serum TIMP-2 levels were varied in chronic liver disease, but they stayed within normal limits [11]. There have been several reports on TIMP-1 in hepatic fibrosis showing that TIMP-1 concentrations increase with the progression of chronic liver disease and correlate with the serum levels of procollagen III peptides in chronic viral liver disease [25] and alcoholic liver disease [26]. Therefore, TIMP-1 has been considered as a useful marker for hepatic fibrosis. It is clinically noted that there is a tendency towards either an increase in serum

MMP-1 levels or a decrease in serum TIMP-1 levels in responders of interferon therapy for chronic hepatitis C [27]. Murawaki *et al.* provided additional evidence, indicating that serum TIMP-1 may reflect the change in liver TIMP-1 in chronic liver disease, since the serum and liver TIMP-1 levels are well correlated, and that both are increased in LC [28]. Recent molecular studies [29, 30] have demonstrated that increased expression of TIMP-1 and TIMP-2 messenger RNA in fibrotic livers in humans and rats, especially in hepatic stellate cells, promotes the progression of hepatic fibrosis by preventing the collagen degradation.

Finally, serum levels of IV-C and MMP-2 as well as TIMP-1 are considered to be useful diagnostic markers for LC, since all of them significantly increased in LC. It is also noted that increases in serum IV-C and MMP-2 levels may be closely associated with the histological progression of hepatic fibrosis in chronic liver disease due to viral infection. In addition, the imbalances between IV-C, LM and TIMP-1 and 2 as fibrogenic factors and MMP-2 and 3 as fibrolytic factors may lead to hepatic fibrosis in chronic liver disease, especially in cirrhosis.

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