

Associations among serum concentrations of interleukin-18, matrix metalloproteinase-2 (MMP-2), tissue inhibitor of metalloproteinase-1 (TIMP-1) and METAVIR fibrosis score in patients with chronic hepatitis

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Abstract: During fibrogenesis, the quantity, proportion, and composition of matrix proteins in the liver change due to the activation of hepatic stellate cells (HSCs) resulting in excessive accumulation of fibrous tissue. The exact mechanisms of these changes are not fully known. In this study, we postulated that IL-18 may upregulate matrix metalloproteinase-2 (MMP-2) and its tissue inhibitor (TIMP-1) in such proportions that will encourage fibrogenesis. To test this hypothesis we estimated the serum concentrations of IL-18, MMP-2, TIMP-1 in 56 patients with chronic hepatitis C virus (HCV), 28 patients with hepatitis B virus (HBV), 16 patients with non-alcoholic steatohepatitis (NASH) and 100 healthy controls using commercially available ELISA kits. The METAVIR activity and fibrosis scores of the liver biopsies from the patients were determined histologically. We found that IL-18 concentrations were significantly higher among HCV, HBV, and NASH patients than in healthy controls. We also found that IL-18 increased progressively from patients with no fibrosis (397.46 ± 73.54 pg/mL) to patients with cirrhosis (1384.11 ± 526.60 pg/mL). Although IL-18 is associated with minimal production of MMP-2 throughout the period of fibrosis from 199.48 ± 18.62 ng/mL at F0 to 225.25 ± 14.75 ng/mL at F4, it is associated with two-fold increase of TIMP-1 from 225.25 ± 14.75 ng/mL to 500.77 ± 30.50 ng/mL. We suggested that the high concentration of TIMP-1 inhibits the relatively low concentration of MMP-2 thus promoting the continuous deposition of collagen fibers in the liver. We concluded that IL-18 and TIMP-1 may be more important than MMP-2 in hepatic fibrogenesis.

Keywords: IL-18, MMP-2, TIMP-1, liver fibrosis

Introduction

The activated hepatic stellate cells (HSCs) play a central role in the pathogenesis of liver fibrosis, a characteristic feature of most types of liver disease such as chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, alcohol abuse, and non-alcoholic steatohepatitis (NASH). Following liver injury and stimulation by cytokines, HSCs are activated and they proliferate into profibrogenic myofibroblastic phenotypes which secrete a wide range of matrix metalloproteinases (MMPs) and their specific tissue inhibitors of matrix metalloproteinases (TIMPs). MMPs and TIMPs play an important role in hepatic fibrosis.¹⁻³ In particular, serum MMP-2 concentration has been reported to correlate with the degree of periportal necrosis and fibrosis

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presented and the total histological activity score⁴⁻⁶ while serum TIMP-1 has been reported to correlate with liver fibrosis in a cohort of HIV-HCV co-infected patients,^{6,7} in HCV infection,⁸⁻¹⁰ in alcoholic liver disease,¹¹ in HBV infection¹² and in parasitic infestation.¹³ What all these studies would seem to indicate is that whatever might be the cause of liver damage, the mechanism of fibrogenesis would appear to be the same. There is an imbalance in the concentrations of MMPs and TIMPs leading to excessive matrix apposition and more liver fibrosis.

The MMP-TIMP axis is highly regulated to avoid tissue damage in normal tissues. Under pathological conditions, activated Kupffer cells and lymphocytes produce profibrotic cytokines that regulate the MMP-TIMP axis. Our hypothesis is that interleukin-18 (IL-18), an inducing interferon γ (IFN- γ) inducing cytokine may play an important role in the MMP-TIMP axis in a manner that would encourage an MMP-TIMP imbalance and cause fibrosis. Although there are several reports in the literature on the serum concentrations of IL-18 in patients with chronic liver disease,¹⁴⁻¹⁸ to the best of our knowledge, there has been no report on its association, if any, with MMP-2 or TIMP-1. Our aim in this study was to investigate the association of IL-18 with MMP-2 and TIMP-1 and to see if this could partly help us to understand the mechanism of liver fibrogenesis in patients with chronic hepatitis.

Materials and methods

Patients

This study is a continuation of our ongoing study in relation to which other results, including demographic and biochemical parameters, have been published.^{19,20} Out of the 140 liver biopsies that have been collected so far, only those with HCV, HBV, and NASH were included in the present study. The methods for METAVIR activity and fibrosis scores have been previously described in one of our publications.¹⁹

Measurement of serum IL-18 concentrations

Serum IL-18 concentrations in patients with HCV, HBV, NASH, and 100 healthy controls were measured using commercially available ready-to-use sandwich Human IL-18 Platinum ELISA kits (Bender Med Systems GmbH, Vienna, Austria). The manufacturer's instructions were followed closely. The sensitivity of the assay (mean of six independent assays) in our laboratory was 10 pg/mL. The intra-assay coefficient of variation was 6.0% and the inter-assay coefficient of variation was 8.0%.

Measurement of serum MMP-2 concentrations

Serum concentrations of MMP-2 in patients and controls were measured using the commercially available Quantikine[®] Human MMP-2 (total) ELISA kits (R&D Systems Inc, Minneapolis, MN). The Quantikine[®] MMP-2 immunoassay is a 4.5-hour solid phase ELISA designed to measure total MMP-2 (pro and active MMP-2 in human serum, plasma, and other fluids). The manufacturer's instructions were followed carefully. The sensitivity of the assay was 0.16 ng/mL while the intra-assay and inter-assay coefficients of variations were 5% and 7% respectively.

Measurement of serum TIMP-1 concentrations

The serum concentrations of TIMP-1 in the patients and controls were measured using the commercially available Quantikine[®] Human TIMP-1 ELISA kits (R&D Systems Inc). The Quantikine[®] Human TIMP-1 immunoassay is a 3.5-hour solid phase ELISA designed to measure TIMP-1 concentrations in human fluid. The manufacturer's instructions for carrying out the assay were carefully followed. The intra-assay and inter-assay precisions were 5.0% and 4.9% respectively.

Statistics

Data were analyzed using statistical software SPSS for Windows (v16; IBM Corporation, Armonk, NY). Values were expressed as mean \pm standard error of mean. Correlation coefficients were calculated according to Spearman's rank correlation coefficient (r). Differences were considered to be significant for a value of $P < 0.05$.

Results

Demographics

Out of a total of 140 liver biopsies examined histologically, 56 had HCV, 28 HBV, and 16 NASH. These 100 were the patients whose results were analyzed in this work.

Serum concentrations of IL-18

Table 1 shows that the mean serum concentration of IL-18 in patients with HCV 499.97 ± 47.74 pg/mL was not significantly different ($P > 0.05$) from that of patients with HBV (450.95 ± 42.71 pg/mL) nor that of patients with NASH (409.42 ± 23.23 pg/mL) but was significantly higher ($P = 0.000$) than the mean for healthy controls (284.27 ± 14.22 pg/mL). Patients with HBV and NASH also had significantly higher mean serum concentrations of IL-18 than that of the control.

Table 1 Serum concentrations of IL-18, MMP-2, and TIMP-1 in patients with chronic hepatitis due to HCV, HBV, NASH and healthy controls

Parameter	HCV n = 56	HBV n = 28	NASH n = 16	Healthy controls n = 100
IL-18 (Mean ± SEM) pg/mL	499.79 ± 47.74	450.95 ± 42.71	409.42 ± 23.23	284.27 ± 14.22
MMP-2 (Mean ± SEM) ng/mL	228.81 ± 10.64	201.22 ± 10.45	192.60 ± 14.19	142.98 ± 2.19
TIMP-1 (Mean ± SEM) ng/mL	355.82 ± 20.33	324.99 ± 32.67	376.07 ± 45.32	254.78 ± 19.94

Notes: Interleukin-18: HCV vs control $P < 0.000$; HBV vs control $P < 0.001$; NASH vs control $P < 0.005$; MMP-2: HCV vs control $P < 0.000$; HBV vs control $P < 0.000$; NASH vs control $P < 0.010$. TIMP-1: HCV vs control $P < 0.001$; HBV vs control $P < 0.05$; NASH vs control $P < 0.03$.

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; IL-18, interleukin-18; NASH, non-alcoholic steatohepatitis; MMP-2, matrix metalloproteinase-2; SEM, standard error of the mean; TIMP-1, tissue inhibitor of metalloproteinase-1.

Serum concentrations of MMP-2

Table 1 shows that the mean serum concentration of MMP-2 in patients with HCV (228.1 ± 10.64 ng/mL) was not significantly ($P > 0.05$) different from the mean of patients with HBV (201.22 ± 10.45 ng/mL) nor that of patients with NASH (192.60 ± 14.19 ng/mL), but was significantly higher ($P = 0.000$) than the mean in healthy controls (142.98 ± 2.19 ng/mL). Patients with HBV and NASH also had significantly higher mean serum MMP-2 concentrations than the healthy controls.

Serum concentrations of TIMP-1 (Table 1)

The mean serum concentration of TIMP-1 (355.82 ± 20.33 ng/mL) in patients with HCV was significantly higher than that of healthy controls (254.78 ± 19.94 ng/mL) but was not significantly different from that of patients with HBV (324.99 ± 32.67 ng/mL) or NASH (376.07 ± 45.32 ng/mL). Patients with HBV and HCV also had significantly higher TIMP-1 concentrations than healthy controls.

Variations of serum markers according to METAVIR activity score (Table 2)

The serum IL-18 data from HBV, HCV, and NASH patients were pooled for these results. Serum IL-18 concentrations at A2 (901.16 ± 283.62 pg/mL) and A3 (1495.63 ± 638.15 pg/mL) were significantly higher ($P < 0.05$) than those at A0

(397.57 ± 78.74 pg/mL) and A1 (500.56 ± 49.27 pg/mL). Serum IL-18 concentration could not differentiate patients with an A0 METAVIR activity score from those with A1 but could differentiate those with A0 from A2 and A3. Table 2, however, shows that there were no significant differences between the serum concentrations of MMP-2 or TIMP-1 and METAVIR activity scores.

Variations of serum markers with METAVIR fibrosis scores (Table 3)

There were no significant differences ($P > 0.05$) in the mean serum concentration of IL-18 between F0 (397.36 ± 73.54 pg/mL) and F1 (405.75 ± 48.57 pg/mL) or F2 (450.75 ± 74.67 pg/mL). However, the values of IL-18 at F3 (971.96 ± 346.11 pg/mL) and F4 (1384.11 ± 526.60 pg/mL) were significantly higher than those at F0 or F1. In other words, the serum IL-18 concentrations rose in a significant progressive manner from F1 to F4 (Figure 1). All of the 24 patients with cirrhosis (F4) had mean serum IL-18 levels higher than those of healthy controls (500.77 ± 30.50 ng/ml) or patients with no fibrosis. The area under the curve of a receiver operating characteristic (ROC) of IL-18 for the diagnosis of cirrhosis was 0.61 giving a specificity and sensitivity of 60% respectively. Table 3 also shows that there were no significant differences in serum MMP-2 concentrations among the METAVIR fibrosis scores. The mean serum TIMP-1 concentrations in patients with cirrhosis (500.74 ± 30 ng/mL) and those

Table 2 Mean serum concentrations of IL-18, MMP-2, and TIMP-1 according to METAVIR activity scores in patients with chronic hepatitis

Degree of activity	n	Mean IL-18 (pg/mL) (Mean ± SEM)	Mean MMP-2 (ng/mL) (Mean ± SEM)	Mean TIMP-1 (ng/mL) (Mean ± SEM)
A0	27	397.57 ± 78.74	190.53 ± 25.27	379.16 ± 64.23
A1	15	500.56 ± 49.27	207.29 ± 10.91	392.03 ± 34.44
A2	34	901.16 ± 283.62	211.18 ± 10.20	434.55 ± 34.75
A3	24	1495.63 ± 638.15	228.62 ± 16.48	440.5 ± 49.03

Notes: Interleukin-18: A0 vs A2 $P < 0.05$; A0 vs A3 $P < 0.05$; A1 vs A3 $P < 0.05$; A2 vs A3 $P < 0.05$.

Abbreviations: IL-18, interleukin-18; MMP-2, matrix metalloproteinase-2; SEM, standard error of the mean; TIMP-1, tissue inhibitor of metalloproteinase-1.

Table 3 Mean serum concentrations of IL-18, MMP-2, and TIMP-1 according to METAVIR fibrosis scores in patients with chronic hepatitis

Degree of fibrosis	n	Mean IL-18 (pg/mL) (Mean \pm SEM)	Mean MMP-2 (ng/mL) (Mean \pm SEM)	Mean TIMP-1 (ng/mL) (Mean \pm SEM)
F0	16	397.46 \pm 73.54	199.48 \pm 18.62	225.25 \pm 14.75
F1	11	405.75 \pm 48.57	210.68 \pm 11.52	243.52 \pm 13.90
F2	15	450.56 \pm 74.67	216.63 \pm 14.18	367.57 \pm 28.46
F3	34	971.96 \pm 346.11	217.50 \pm 16.67	490.36 \pm 61.51
F4	24	1384.11 \pm 526.60	225.25 \pm 14.75	500.77 \pm 30.50

Notes: Interleukin-18: F0 vs F3 $P < 0.05$; F0 vs F4 $P < 0.001$; F1 vs F3 $P < 0.005$; F1 vs F4 $P < 0.001$; F2 vs F3 $P < 0.05$; F3 vs F4 $P < 0.05$. TIMP-1: F0 vs F3 $P < 0.05$; F0 vs F4 $P < 0.001$; F1 vs F3 $P < 0.05$; F1 vs F4 $P < 0.001$.

Abbreviations: IL-18, interleukin-18; MMP-2, matrix metalloproteinase-2; SEM, standard error of the mean; TIMP-1, tissue inhibitor of metalloproteinase-1.

with F3 fibrosis (490.36 \pm 61.51 ng/mL) were significantly higher ($P < 0.001$) than the mean at F0 (225.25 \pm 14.75 ng/mL) or F1 (243.52 \pm 13.90 ng/mL). All of the 24 patients that had cirrhosis (F4) had serum TIMP-1 levels higher than the healthy controls (225.25 \pm 14.75 ng/mL) or patients with no fibrosis. Serum TIMP-1 levels showed a progressive rise with the degree of fibrosis. The ROC of TIMP-1 for the diagnosis of cirrhosis gave 60% sensitivity and specificity.

Relationship between serum IL-18 and MMP-2 in patients with HBV, HCV, and NASH with chronic hepatitis

Since there were no significant differences in mean serum IL-18 concentrations among the patients with HCV, HBV, and NASH, the data were pooled for these statistics. Figure 2 shows

that there is a significantly positive ($r = 0.253$, $P = 0.01$) association between serum IL-18 and MMP-2 in patients with chronic hepatitis due to HBV, HCV, and NASH.

Relationship between serum IL-18 and TIMP-1 in patients with chronic hepatitis due to HBV, HCV, and NASH

Figure 3 shows the significantly positive ($r = 0.239$; $P = 0.01$) association between serum IL-18 and TIMP-1 in these patients.

Relationship between serum TIMP-1 and MMP-2

Figure 4 shows the significantly positive ($r = 0.338$; $P = 0.001$) association between serum TIMP-1 and MMP-2 in patients with chronic hepatitis due to HBV, HCV, and NASH.

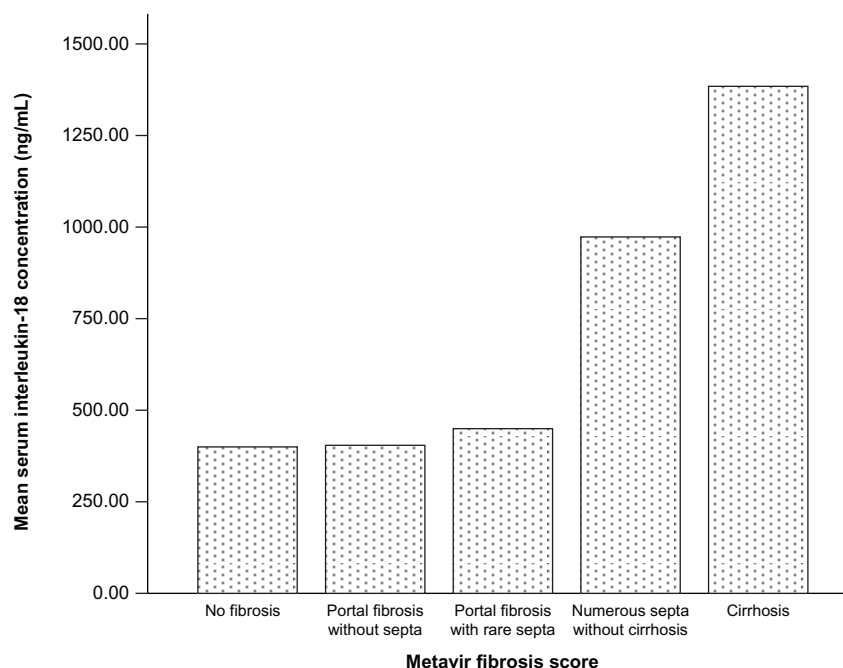


Figure 1 Bar diagram of the mean serum IL-18 concentrations according to METAVIR fibrosis scores in patients with chronic hepatitis due to HBV, HCV, and NASH.

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; IL-18, interleukin-18; NASH, non-alcoholic steatohepatitis.

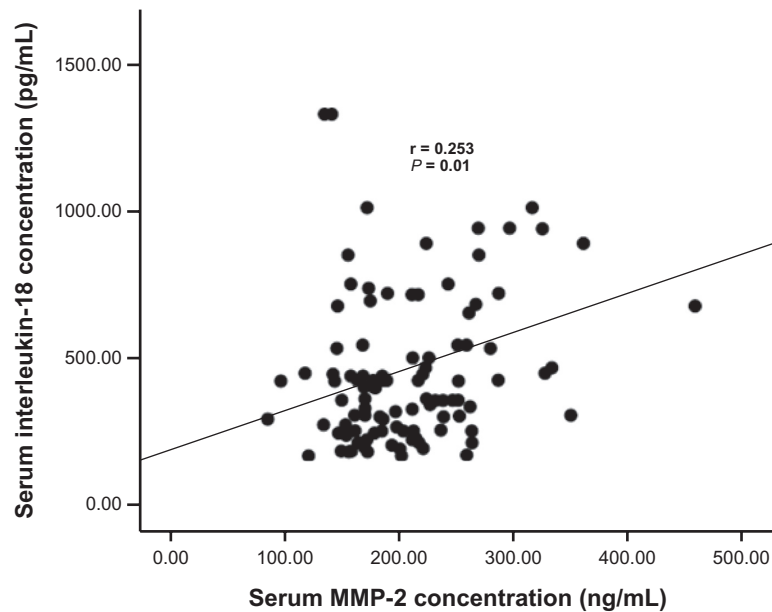


Figure 2 Correlation between serum IL-18 and serum MMP-2 concentrations in patients with chronic hepatitis due to HBV, HCV, and NASH.
Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; IL-18, interleukin-18; MMP-2, matrix metalloproteinase-2; NASH, non-alcoholic steatohepatitis.

Ratios among various serum markers (Table 4)

Table 4 shows that IL-18 to MMP-2 ratios were significantly higher at F3 and F4 than at F0–F2. The serum IL-18 to MMP-2 ratio differentiated F3 and cirrhosis from F0 suggesting that this ratio might be useful in the diagnosis of cirrhosis. The IL-18 to TIMP-1 ratio could only differentiate F4 from F0. The MMP-2 to TIMP-1 ratio was 0.560 among the healthy control but rose to about 1.0 at F0 and then fell again

to 0.58 at F2 and to 0.44 at F3 and F4. The rise and fall of this ratio might be important to fibrogenesis (see below).

Discussion

Sharma et al¹⁶ showed a close relationship between elevated circulating plasma levels of IL-18 and the severity of HCV infection. They also showed a higher concentration of IL-18 in patients with cirrhosis. In this study, we found elevated serum IL-18 concentrations in patients with HBV,

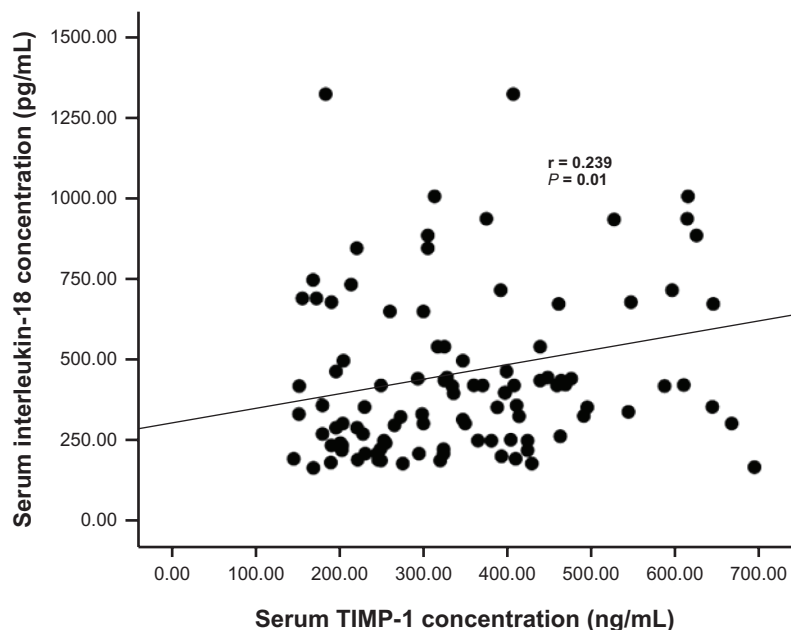


Figure 3 Correlation between serum IL-18 and serum TIMP-1 concentrations in patients with chronic hepatitis due to HBV, HCV, and NASH.
Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; IL-18, interleukin-18; NASH, non-alcoholic steatohepatitis; TIMP-1, tissue inhibitor of metalloproteinase-1.

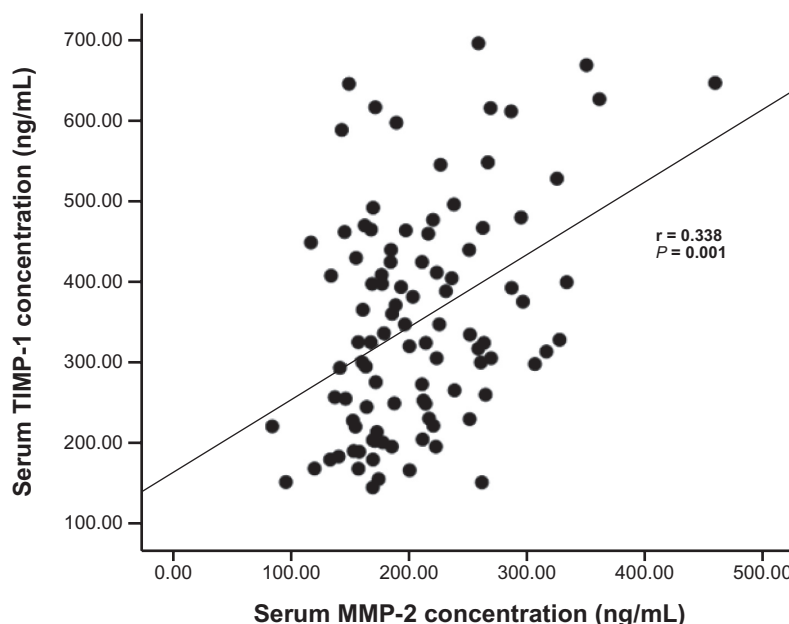


Figure 4 Correlation between serum TIMP-1 and serum MMP-2 concentrations in patients with chronic hepatitis due to HBV, HCV and NASH.

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; IL-18, interleukin-18; MMP-2, matrix metalloproteinase-2; NASH, non-alcoholic steatohepatitis; TIMP-1, tissue inhibitor of metalloproteinase-1.

HCV, and NASH. All our patients with cirrhosis had serum IL-18 concentrations higher than healthy controls. Our finding that elevated IL-18 is not limited only to HCV was also confirmed by Ludwiczek et al¹⁸ who showed increased plasma levels of IL-18 in chronic liver disease with cirrhosis and no cirrhosis without any regard to the etiology of the underlying liver disease. Our finding that elevated IL-18 could be due to causes apart from HCV is in contrast with the work of Mihm et al²¹ who showed that expression of interferon-gamma-inducible protein-10 (IP-10), a chemokine that recruits activated T lymphocytes was associated strongly with the accumulation of IFN- γ and IL-18 mRNA in the liver in patients with chronic hepatitis C, but not in those with chronic hepatitis B or those with non-alcoholic

steatohepatitis. The difference between our finding and that of Mihm et al could be due to the fact that while we measured systemic levels of IL-18, Mihm et al measured hepatic levels. Our finding that serum IL-18 is elevated in chronic hepatitis C is in contrast with that of Schvoerer et al²² who reported lower levels of IL-18 in plasma and supernatants of stimulated peripheral blood mononuclear cells from patients with genotype 1 HCV infection than in those from normal controls. There are probably many mechanisms by which IL-18 can cause liver fibrosis. Liver fibrosis occurs as a consequence of net accumulation of matrix proteins in response to liver injury. It is underpinned by the activation of HSCs to a myofibroblast-like phenotype with a consequent increase in their synthesis of matrix proteins, such as interstitial collagens that characterize fibrosis.²³ IL-18 originally designated as an IFN- γ inducing factor is a cytokine produced by activated Kupffer cells, monocytes, and dendrite cells during innate immune responses.^{24,25} It induces immunogenetic hepatic fibrosis by activating CD4⁺ T cells, which then secrete large amounts of tumor necrosis factor-alpha (TNF- α) and IFN- γ . These cytokines then activate HSCs to proliferate into myofibroblasts, which then secrete myofibrils, thus forming hepatic fibrosis.

The second mechanism by which IL-18 may cause liver fibrosis is by upregulating matrix metalloproteinases and their tissue inhibitors. To the best of our knowledge, this study would be the first to report that serum IL-18 is associated positively with both MMP-2 and TIMP-1.

Table 4 Ratios among various serum markers according to METAVIR fibrosis scores in patients with chronic hepatitis and healthy controls

Category	IL-18 to MMP-2 ratio	IL-18 to TIMP-1 ratio	MMP-2 to TIMP-1 ratio
Healthy controls	1.986	1.11	0.560
F0 fibrosis	1.985	1.76	0.888
F1 fibrosis	1.933	1.00	0.864
F2 fibrosis	1.933	1.22	0.588
F3 fibrosis	4.46**	1.98	0.444
F4 fibrosis	6.151**	2.76*	0.449

Notes: * $P < 0.01$ compared with F0; ** $P < 0.000$ compared with F0.

Abbreviations: IL-18, interleukin-18; MMP-2, matrix metalloproteinase-2; SEM, standard error of the mean; TIMP-1, tissue inhibitor of metalloproteinase-1.

The intricate network formed by the metalloproteinases and their tissue inhibitors determine the integrity of the extracellular matrix. Fibroproliferation in chronic hepatitis can be viewed as a process of tissue remodeling. It is characterized by breakdown of preformed tissue structures, increased de novo synthesis, and turnover of collagen and, in later stages, inhibition of collagen breakdown. These effects in the human liver are accompanied by changing concentrations of MMPs and TIMPs at different stages of the liver.

MMP-2, the gelatinase, is secreted by activated HSCs under stimulation by different cytokines. Our study showed that its serum concentration is associated with the serum concentration of IL-18. When formed, it promotes the remodeling of extra cellular matrix (a characteristic of chronic liver diseases), by degrading type IV collagen, one of the components of extra cellular matrices. In our study, there was significant difference in serum MMP-2 concentration between patients with chronic hepatitis and healthy controls. Our findings agreed with those of Murawaki et al²⁶ who found elevated levels in cirrhosis and Boeker et al.¹⁰ Serum MMP-2 in our study did not correlate with either hepatic inflammatory activity or fibrosis. This was in agreement with the work of Walsh et al²⁷ who found no correlation with the degree of fibrosis or the histologic activity index, and Kasahara et al²⁸ who reported a weak correlation between circulating MMP-2 and fibrosis but did not agree with that of Murawaki et al²⁶ and Ebata et al.²⁹ The differences among these reports could be due to the fact that different authors were estimating different forms of MMP-2 or using different types of antibodies in their ELISA assays.

TIMPs inhibit the action of MMPs. The most important in the liver in this respect is TIMP-1. Unlike MMP-2, however, we found that serum TIMP-1 levels increased significantly with METAVIR activity and fibrosis scores. This is in agreement with those of other authors⁶⁻¹³ but did not agree with that of Lichtinghagen et al.³⁰ Using the ROC curve, the specificity and sensitivity of TIMP-1 for diagnosing cirrhosis were 62% and 65%, respectively. This was in contrast to the work of Boeker et al¹⁰ who reported high specificities and sensitivities for TIMP-1 in detecting cirrhosis.

One of the most important observations in this study was that although serum MMP-2 concentration was strongly positively associated with TIMP-1 ($r = 0.338$; $P = 0.001$) in patients with chronic hepatitis, the serum concentrations of MMP-2 remained relatively unchanged (from 199.48 ± 18.62 ng/mL at F0 to 225.25 ± 14.75 ng/mL at F4) while that of TIMP-1 more than doubled (from 225.25 ± 14.75 ng/mL to 500.77 ± 30.50 ng/mL). Our findings of differences in MMP-2 to TIMP-1 ratios during

the different stages of fibrosis may partially explain the mechanism of hepatic fibrogenesis. Our results would appear to suggest that in the normal state, the ratio of MMP-2 to TIMP-1 was maintained at about 0.5 to maintain a balance between the MMP-2 and TIMP-1 axis. At the beginning of fibrogenesis (F0 and F1) the ratios of MMP-2 to TIMP-1 rose close to one. This relatively higher ratio is to allow for the degradation of cell membranes by MMP-2 to enable the activated HSCs to escape to the tissues. Once the activated HSCs have escaped and started to secrete fibrils, the ratio of MMP-2 to TIMP-1 falls from F2 through F4. This is to allow the relatively higher concentrations of TIMP-1 to inhibit MMP-2, and to promote continuous deposition of collagen fibers in the liver which finally results in hepatic fibrosis.

Conclusion

The main emphasis of this work is to report that IL-18 is positively associated with MMP-2 and TIMP-1 concentrations in patients with chronic hepatitis and to explain how the differing ratios of MMP-2 to TIMP-1 concentrations may partially explain the process of fibrogenesis in the liver of patients with chronic hepatitis. We also found that IL-18 and TIMP-1 may be more important in hepatic fibrogenesis than MMP-2.

Disclosure

The authors report no conflict of interest in this work. This work was not funded or supported by any company, apart from Kuwait University.

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