Differences in circulating MMP-9 levels with regard to viral load and AST:ALT ratio between chronic hepatitis B and C patients

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ABSTRACT

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are the two major causes of chronic liver inflammation, fibrosis and cirrhosis. They have the ability to cause persistent infection in susceptible hosts and severely damage liver function. Matrix metalloproteinase-9 (MMP-9) is one of the gelatinases that may be important in liver fibrosis. This study aims to evaluate whether or not MMP-9 in relation to viral load is involved in the development of liver dysfunction in HBV and HCV. Blood samples from 20 patients chronically infected with HBV and 30 with HCV, along with 15 healthy individuals as controls, were investigated. Viral load was assessed by real-time polymerase chain reaction (PCR). Serum MMP-9 levels were evaluated by enzyme-linked immunosorbent assay (ELISA). Alanine transaminase and aspartate aminotransferase (ALT and AST) activities were measured spectrophotometrically. Levels of MMP-9 were significantly higher in HCV than in HBV patients ($P<0.01$), and positively correlated with HBV viral load ($r=0.842$, $P<0.01$) and AST:ALT ratio ($r=0.614$, $P<0.05$). Conversely, MMP-9 levels did not correlate with HCV viral load but did correlate with AST:ALT ratio ($r= 0.652$, $P<0.01$). Therefore, MMP-9 levels could reflect progressive liver damage in HBV and HCV infection. However, a distinction between the pathological mechanism of HCV and HBV is suggested, as HCV probably promotes hepatocyte damage and fibrosis through mechanisms other than replication. Continuous expression of the HBV genome through replication and secretion of viral antigens may contribute to the transcriptional regulation of MMP-9, thus promoting liver damage and fibrosis.

KEY WORDS: Alanine transaminase.
Aspartate aminotransferases.
Hepatitis B.
Hepatitis C.
Matrix metalloproteinase 9.

Introduction

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are major public health problems.[1] They represent the two foremost causes of chronic liver disease worldwide, and an important cause of morbidity and mortality from the sequelae of progressive liver injury resulting in cirrhosis and its complications.[2,3] Despite distinct virological features, both viruses are preferentially hepatotropic, not directly cytopathic, producing liver diseases that share several aspects of their natural history.[4]

In chronic liver disease, tissue injury induces a sustained wound-healing response.[5,6] This process is characterised by proliferation of myofibroblast-like cells mainly derived from hepatic stellate cells (HSCs), the principal fibrogenic cells in the liver. These stellate cells are considered to be the main source of extracellular matrix (ECM) proteins and are likely to play an essential role in liver fibrogenesis. This results in extensive remodelling of the hepatic ECM.[7–9] The mechanisms involved in activation of HSCs are not fully understood at the molecular level, but a number of different stimuli, including proteolytic remodeling of ECM proteins, are thought to be implicated.[10]

Liver fibrosis is characterised by a progressive accumulation of ECM, which reflects the imbalance between enhanced matrix synthesis and decreased breakdown of connective tissue proteins.[7] Matrix metalloproteinases are a family of at least 20 zinc-dependent proteolytic enzymes which regulate extracellular degradation of matrix proteins.[11] They play an important role in the fibrotic process, participating in the balance between collagen synthesis and degradation.[12] They are controlled by several mechanisms including regulation at the gene expression level, cleavage of the proenzyme to an active form, and specific inhibition of activated forms by tissue inhibitors of metalloproteinases.[13,14]

Among the MMP family, different groups can be distinguished according to their substrate specificity.[14] Gelatinases (type IV collagenases) may be especially important for the development of organ fibrosis because they degrade type IV (basal membrane) collagen and thus are involved in the early steps of tissue remodelling that characterise chronic liver diseases. Matrix metalloproteinase-9 (MMP-9 [gelatinase B, 92-kDa type IV collagenase]) is a gelatinase that may be particularly important in liver fibrosis,[15,16] as it has an important role in the pathogenesis of liver cirrhosis as well as in hepatocellular carcinoma.[17]

The aspartate aminotransferase:alanine transaminase

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Table 1. Mean values of the serum markers investigated in the three studied groups.

Parameter	Group		
	Controls (n=15)	HBV patients (n=20)	HCV patients (n=30)
Viral load (iu/mL)		$1.19x10^4 \pm 7.8 \times 10^3$	$2.89 \times 10^5 \pm 1.3 \times 10^5$
MMP-9 (ng/mL)	43.23±4.79	106.11±26.56	203.32±23.62
*AST (U/L)		29.29±5.77	28.62±3.46
*ALT (U/L)		15.32±1.82	17.54±2.35

Values expressed as mean±SEM.
MMP-9: matrix metalloproteinase-9; AST: aspartate aminotransferase; ALT: alanine transaminase.
*Normal range: up to 12 U/L.

(AST:ALT) ratio has been used to assess non-invasively the severity of disease in patients with chronic liver disease, as it reflects progressive liver functional impairment and fibrosis.[18] The AST:ALT ratio has been evaluated in previous studies[18–20] performed on patients with chronic liver disease. In these patients, the AST:ALT ratio provided useful clinical information on the severity of liver disease as progressive liver functional impairment is associated with an increase in the AST:ALT ratio[21] and is considered one of the best predictors of fibrosis progression in chronic hepatitis C. The elevation of AST might be due to the reduction in the clearance of AST and mitochondrial injury.[22]

Currently available data suggest that MMPs may have a role in the development of fibrosis and cirrhosis. However, there is a lack of information about differential MMP regulation in human chronic liver disease. The present work aims to evaluate whether or not MMP-9, in relation to viral load and AST:ALT ratio as a parameter of hepatic dysfunction, is involved in a similar way in the development of HBV and HCV liver disease.

Materials and methods

Study participants included 55 patients with either chronic HBV (n=20; 12 male, 8 female; mean age: 42.95±12.1 years) or chronic HCV (n=30; 18 male, 12 female; mean age: 45.97±8.2 years). Fifteen healthy blood donors comparable for gender and age and negative for both HBsAg and HCV antibodies served as controls. The inclusion criteria for HBV and HCV patients were absence of drug or alcohol abuse, autoimmune diseases, human immunodeficiency virus (HIV) and HBV/HCV co-infection, neoplasia or other serious illness. Blood samples were obtained after overnight fasting. Viraemia was quantified using a real-time quantitative PCR technique using TaqMan technology.

Real-time quantitative PCR

Hepatitis B DNA was extracted from 200 µL serum using the QIAamp DNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. Viral load was assessed quantitatively by the Artus HBV PCR kit (Qiagen), a commercial ready-to-use system for the detection of HBV DNA using real-time PCR. Thermal cycling was performed using the Mx3000P real-time PCR system (Stratagene) as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles each of denaturation at 95°C for 15 sec, and annealing and extension at 60°C for 1 min.

Hepatitis C RNA was extracted from 140 µL serum using the QIAamp viral RNA mini kit (Qiagen) according to the manufacturer's instructions. Viraemia was analysed and quantified by real-time PCR (Mx3000P, Stratagene) using TaqMan probe technology. The HCV RNA was amplified as follows: one cycle each of 48°C for 30 min to transcribe viral RNA to complementary DNA (cDNA) by reverse transcriptase, and 95°C for 10 min for AmpliTaq gold activation, followed by 40 cycles for two PCR-step amplification, of denaturation at 95°C for 15 sec, and of annealing and extension at 60°C for 1 min.

Serum MMP-9 levels were quantified by the commercially available solid-phase MMP-9 sandwich enzyme-linked immunosorbent assay (ELISA) kit (BMS2016, Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions. The concentrations were determined by interpolation from a standard curve. Liver biomarker (ALT and AST) activities were measured spectrophotometrically[23] using commercially available kits (Randox Laboratories, UK).

The study was carried out according to the principles of the Declaration of Helsinki and approved by the institutional ethics committee. Informed consent was obtained from all study subjects. Statistical analysis was carried out using the Statistical Program for Social Sciences (SPSS version 14). Data are presented as mean±SE. Student's t-test was used to compare groups. Pearson's correlation coefficients were calculated between the investigated markers. $P<0.05$ was regarded as significant.

Results

Table 1 shows the mean values for investigated serum markers. The HCV-infected patients had significantly higher serum MMP-9 values than did chronic HBV-infected patients (t=2.72, $P<0.01$). In the HCV-infected group, circulating MMP-9 concentration was higher than in the control group (t=4.74, $P<0.01$). Similarly, chronic HBV-infected patients displayed significantly higher serum MMP-9 levels (t=2.42, $P<0.05$).

A significant positive correlation was found between serum MMP-9 level and HBV viral load (r=0.842, $P<0.01$). However, HCV viral load did not correlate with MMP-9 concentration. Viral load correlated strongly with AST and AST:ALT ratio (r=0.764, r=0.666; $P<0.01$, respectively) and correlated weakly with ALT (r=0.474, $P<0.05$). However,

Table 2. Correlation (r) between MMP-9 level, viral load and liver dysfunction parameters among the HBV- and HCV infected groups.

Group	Parameter	Viral load	ALT	AST	AST:ALT ratio
HBV	MMP-9	0.842†	0.433*	0.698†	0.614*
	Viral load		0.474*	0.764†	0.666†
HCV	MMP-9	0.046	0.104	0.264	0.652†
	Viral load		0.190	0.651†	0.210

*$P<0.05$, †$P<0.01$.
MMP-9: matrix metalloproteinase-9; AST: aspartate aminotransferase; ALT: alanine transaminase.

HCV RNA viral load correlated strongly with AST ($r=0.651$, $P<0.001$) but not with ALT. No correlation was seen between HCV viral load and AST:ALT ratio (Table 2).

Among chronic HBV infected patients, a weak positive correlation was observed between MMP-9 and ALT activities ($r=0.433$, $P<0.05$). Furthermore, a strong positive correlation was detected between MMP-9, AST and AST:ALT ratio ($r=0.698$, $P<0.01$; $r=0.614$, $P<0.05$, respectively). In the HCV-infected group, a strong positive correlation was found between MMP-9 and AST:ALT ratio ($r=0.652$, $P<0.01$); however, no correlation was seen between MMP-9 and either ALT or AST (Table 2).

Discussion

In the present work, patients with chronic HBV and HCV infection demonstrated significantly higher serum MMP-9 levels than did healthy controls. Previous reports have shown that serum MMP-9 is elevated in patients with chronic hepatitis.[17,24] Kim *et al*.[25] indicated that the over-expression of MMP-9 mRNA is the result of HBV transfection. In addition, Chung *et al*.[26] reported that mean serum MMP-9 concentration is significantly increased in chronic HBV patients.

The present results point to the role of HBV and HCV infection in the up-regulation of MMP-9 expression and supports work by Marinosci *et al*.,[27] who stated that plasma and tissue MMP-9 levels are decreased in chronic hepatitis C patients at the end of the follow-up period after ribavirin plus interferon-α2b treatment in sustained virological responders but not in non- responders. Type IV collagenases are important for the development of organ fibrosis and involved in the early steps of tissue remodelling that characterises chronic liver diseases.[15,16] Conversely, Mangoud *et al*.[20] described an inverse correlation between MMP-9 and fibrosis. Lichtinghagen *et al*.[16] found that circulating MMP-9 was lower in patients with chronic active hepatitis than in healthy controls, and that it declined in patients with cirrhosis, despite increased expression in circulating peripheral blood cells. However, they concluded that these alterations in expression do not explain the changes in circulating protein concentration and do not mirror the progression of liver disease.

In the present study, HCV patients had significantly higher serum MMP-9 levels, which could be because chronic hepatitis B and C have different pathways leading to significant fibrosis and cirrhosis. A significant positive correlation was found between MMP-9 and HBV viral load, and viral load correlated strongly with AST and AST:ALT ratio. This can be explained in terms of HBV replication, and therefore expression and secretion of viral antigens may contribute to transcriptional regulation of MMP-9, thus promoting necroinflammatory activity and liver fibrosis. These results are supported by Chung *et al*.[28] who found that continuous expression of integrated HBV genomic DNA was associated with expression of high levels of proMMP-9, and they concluded that HBV infection of hepatocytes affected the up-regulation of MMP-9 expression. Another study carried out by Chung *et al*.[29] suggested that the X protein (HBx) of HBV, shown to be essential for the development of hepatocellular carcinoma, contributes to the transcriptional regulation of MMP-9.

In contrast, HCV viral load did not correlate with serum MMP-9 concentration, but HCV RNA viral load did correlate with AST. It is widely reported[30–32] that, in the course of chronic hepatitis C, cytokines released by inflammatory cells and damaged hepatocytes play a key role in liver inflammation and in the wound healing process. These cytokines are involved in modulation of the inflammatory response and, moreover, can up-regulate the expression of matrix metalloproteinases and ECM turnover.[33] Consequently, in patients who have chronic HCV infection, viral burden does not necessarily predict the natural history of clinical disease and the liver reacts to viral aggression through an inflammatory response, which is a component of fibrogenesis.

In the present study, a strong positive correlation was found between MMP-9 and AST:ALT ratio in the HCV group. In the HBV group, however, a weak positive correlation was observed between MMP-9 and ALT, but a strong positive correlation was seen between MMP-9 and AST and MMP-9 and AST:ALT ratio. Previous studies[18,19] report that the AST:ALT ratio correlates with histological stage in liver disease, including chronic hepatitis B and C. Therefore, the present results support the benefit of MMP-9 determination in evaluating disease activity and progression, as the fluctuating pattern of AST and ALT activity in patients with chronic viral hepatitis may be an important limitation to their use in assessing liver injury. Thus, a combination of these assays will prove more beneficial.

The present results are supported by the findings of Reif *et al.*,[24] who declared that serum MMP-9 values correlate with grade of liver inflammation, as the highest serum MMP-9 levels were observed between grades 2 and 3, and was more useful than the rise in transaminase level. Hence, it could serve as marker of disease activity in chronic HCV patients.

Although liver biopsy is currently considered the gold standard for staging hepatic fibrosis, it carries a small but significant risk of morbidity and mortality. In addition, a

single liver biopsy provides no information on fibrogenesis and fibrolysis that characterise the dynamic processes related to ECM metabolism.[34] As chronic hepatitis B and C, like other chronic liver diseases, is characterised by continuing hepatocellular injury, inflammation and fibrosis, repeat liver biopsy and histological examination is necessary to follow the course of the disease. As chronic hepatitis B and C are slowly progressive diseases, a non-invasive alternative to liver biopsy would be welcomed.

In conclusion, the results presented here show that increasing serum MMP-9 levels reflect progressive liver damage in HBV and HCV infection. Consequently, the use of MMP-9 measurement in conjunction with liver enzymes might serve as a useful non-invasive alternative in monitoring the progression of liver disease as well as in guiding therapy and assessing response to therapy. The expression of MMP-9 during the course of chronic hepatitis B and C appears to be a closely regulated process. The present findings suggest that the influence on MMP-9 level by the virus might affect subsequent disease progression differently, indicating a distinction between the pathological mechanisms of HCV and HBV. However, further investigation is need into the effect of proinflammatory cytokines in HCV infection as well as the effect of different HBV protein expression in HBV infection. In addition, the up-regulation of MMP-9 in hepatitis B and C requires further study. □

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