Evidence for altered hepatic matrix degradation in genetic haemochromatosis

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Abstract

Background—Altered matrix degradation contributes to fibrosis in some liver diseases but the role of matrix degradation in fibrogenesis associated with genetic haemochromatosis has not previously been addressed.

Aims—To measure serum concentrations of tissue inhibitor of metalloproteinase 1 (TIMP-1) and matrix metalloproteinases (MMP), MMP-1, MMP-2, and MMP-3 in patients with haemochromatosis and control subjects.

Patients—Forty patients with haemochromatosis and 19 healthy control subjects. Ten of the 40 patients were studied before and after venesection therapy.

Methods—Serum levels of TIMP-1, MMP-1, MMP-2, and MMP-3 were measured by enzyme immunoassay and correlated to hepatic iron concentration and degree of histological fibrosis.

Results—Serum TIMP-1 was increased in patients with haemochromatosis compared with controls (163 (30) versus 123 (28) ng/ml, $p < 0.0002$). Mean serum TIMP-1 concentration of patients with haemochromatosis without fibrosis was significantly higher than in controls (153 (16) versus 123 (28) ng/ml, $p = 0.03$). Serum TIMP-1 concentration correlated with both hepatic iron concentration and hepatic iron index ($r = 0.42$, $p < 0.01$; $r = 0.42$, $p < 0.01$). Serum MMP-2 concentrations correlated with increasing degree of fibrosis in patients with haemochromatosis ($r = 0.38$, $p = 0.01$). The mean MMP-1:TIMP-1, MMP-2:TIMP-1 and age/sex matched MMP-3:TIMP-1 ratios were significantly lower in patients with haemochromatosis than controls (0.11 (0.06) versus 0.2 (0.14), $p = 0.02$; 3.32 (0.9) versus 3.91 (0.81), $p = 0.05$; and 0.26 (0.12) versus 0.47 (0.27), $p = 0.007$, respectively). Following venesection, MMP-2 and MMP-3 concentrations increased by 11% ($p = 0.03$) and 19% ($p = 0.03$), respectively.

Conclusions—This study provides the first evidence of an alteration in matrix degradation in haemochromatosis that may be a contributing factor to hepatic fibrogenesis in this disease.

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Keywords: hepatic stellate cell; hepatic fibrosis; matrix metalloproteinase; tissue inhibitor of metalloproteinase-1; genetic haemochromatosis

Haemochromatosis is an inherited disorder of iron metabolism affecting approximately 1 in 300 Caucasians. The disorder is characterised by an inappropriate increase in iron absorption. Excess iron is deposited in the parenchymal cells of many organs but the liver is the major site of iron accumulation.¹ Hepatic fibrosis and cirrhosis may develop and cause significant morbidity and early death.² In contrast to most other liver diseases, hepatic fibrosis complicating haemochromatosis usually occurs in the absence of significant histological necrosis or inflammation.³ The hepatic iron concentration (HIC) usually associated with hepatic fibrosis is 250 $\mu\text{mol/g}^4$ to 400 $\mu\text{mol/g}^5$. Venesection treatment, if instituted early, will prevent cirrhosis² and may result in regression of pre-existing fibrosis.^{6,7}

Liver fibrosis occurs due to the net accumulation of extracellular matrix. Hepatic stellate cells (HSC), formerly known as lipocytes, Ito cells, or fat storing cells,⁸ are the major source of collagens and other components of extracellular matrix in many types of liver injury.⁹ Following liver injury, HSC become "activated", develop a myofibroblast-like phenotype, and express markers such as α -smooth muscle actin (SMA).⁹ In animal models of iron overload, HSC assume an activated phenotype,¹⁰ and are the major source of collagen type I.^{10,11} In a recent study we showed the presence of activated HSC in haemochromatosis and showed that increasing HIC correlated with increasing HSC activation.¹²

In addition to increased matrix formation, alterations in the degradation of extracellular matrix may also contribute to the development of hepatic fibrosis.¹³ The matrix metalloproteinases (MMPs) are a family of related enzymes that degrade a variety of extracellular matrix proteins. Their activity is closely regulated at many levels including stoichiometric binding by specific tissue inhibitors of metalloproteinases (TIMPs).¹³ Activated HSC produce MMP-1 (interstitial collagenase),¹⁴ MMP-2 (gelatinase A),¹⁵ MMP-3 (stromelysin 1),¹⁶ and their inhibitor, TIMP-1.¹⁷

Serum TIMP-1 concentrations have been measured in a variety of different liver diseases,¹⁸⁻²¹ and serum TIMP-1 was positively correlated with the degree of histological fibrosis in patients with chronic viral hepatitis,^{18-19,21} and alcoholic liver disease,^{18,20,21} thus suggesting a pathogenic role for TIMP-1 in fibrosis associated with these disorders. However, these studies have shown that serum TIMP-1 correlates equally well with the histological

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Table 1 Characteristics of controls and patients with haemochromatosis

Patient data	Control	Haemochromatosis
Age (y)	33 (7)	39 (12)
Sex (M:F)	11:8	30:10
Serum ferritin (ng/ml)	95 (82)	1104 (750)
HIC (mmol/g dry weight)	NA	243 (149)
Abnormal LFT (%)	0	33

Results are presented as mean (SD). Normal range for HIC is <40 µmol/g dry weight and for serum ferritin is <250 ng/ml. HIC, hepatic iron concentration; LFT, liver function tests; NA, not assessed.

inflammation invariably associated with these diseases. Whether changes in matrix degradation contribute to hepatic fibrosis in haemochromatosis, a disease in which hepatic inflammation is not prominent, is unknown as there are no studies of matrix degrading systems in this important liver disease.

In this study, serum concentrations of MMP-1, MMP-2, MMP-3, and TIMP-1 were measured in a series of haemochromatosis patients with different levels of hepatic iron concentration and liver fibrosis to provide evidence for altered matrix degradation. In addition, patients' sera were examined before and after venesection therapy to determine changes in TIMP-1 and MMPs.

Materials and methods

PATIENTS AND CONTROLS

Forty patients with haemochromatosis were studied. The diagnosis of haemochromatosis was confirmed by histological and biochemical assessment of liver biopsy specimens. The hepatic iron index (HII) was greater than 2.0 in 38 of the 40 patients. One patient did not have an HIC measurement but had grade 4 hepatocyte iron staining and was HLA identical to a sibling with known haemochromatosis. Another patient had a hepatic iron index of 1.8 but was iron deficient due to menorrhagia until a hysterectomy five years prior to diagnosis. This patient, and all others tested to date (32 of 40), were homozygous for the recently described haemochromatosis (C282Y) mutation.²² All patients were negative for hepatitis B surface antigen, hepatitis C antibody, antismooth muscle antibody, and antimitochondrial antibody. No patient had evidence of hepatocellular carcinoma as suggested by ultrasound scanning or elevated α -fetoprotein concentrations. Blood was collected from patients within two weeks of liver biopsy and prior to venesection, and serum was stored at -20°C until further analysis. All patients were treated by regular venesection (usually 500 ml of blood removed weekly) until iron stores were depleted. Ten patients (five with and five without fibrosis) were studied before and after iron depletion. Control serum was obtained from 19 healthy Caucasian laboratory volunteers who had normal liver function tests and iron indexes (table 1). The protocols used in this investigation were approved by the ethics committees of the Royal Brisbane Hospital and The Queensland Institute of Medical Research and informed consent was obtained from each patient.

LIVER HISTOLOGY AND MEASUREMENT OF HEPATIC IRON CONCENTRATION

Biopsy specimens were fixed in buffered formalin and embedded in paraffin wax. Sections were stained with haematoxylin and eosin for morphological evaluation, Perls' Prussian blue stain for assessment of iron loading, and Gordon and Sweet's reticulin, Masson's trichrome, or haematoxylin Van Gieson stains for assessment of fibrosis. The degree of inflammation and fibrosis was graded according to the method of Scheuer,²³ by a single pathologist (NIW), without knowledge of the patients' clinical or laboratory data. HIC was measured by atomic absorption spectrophotometry as previously described.⁵

SERUM ASSAYS FOR TIMP-1, MMP-1, MMP-2, AND MMP-3

Serum TIMP-1 concentrations were measured with a one step enzyme immunoassay (EIA) based on the method of Kodama *et al*,²⁴ but using an anti-TIMP-1 antibody coated microtitre plate (Fuji Chemical Industries Ltd, Takaoka, Japan). Serum MMP-1,²⁵ MMP-2,²⁶ and MMP-3²⁷ concentrations were measured individually with a one step EIA using specific antibody coated polystyrene balls (Fuji Chemical Industries Ltd). Individual serum samples were incubated with a single antibody coated polystyrene ball. After washing, the ball was incubated with a second, peroxidase labelled antibody and the absorbance measured at 450 nm (MMP-1, MMP-2) or 492 nm (MMP-3). The interassay and intra-assay variability for these EIA kits was reported as having a coefficient of variation which was less than 12%. The sensitivity of the TIMP-1, MMP-1, MMP-2, and MMP-3 EIAs was 1.25, 1.0, 6.3, and 12.5 ng/ml, respectively.

STATISTICAL ANALYSIS

Results are expressed as mean (SD). Differences between groups were analysed using analysis of variance and unpaired Student's *t* test. Differences before and after venesection therapy were analysed using paired Student's *t* test. Correlations were calculated using Pearson's correlation coefficient for continuous variables and Kendall's rank correlation coefficient for discontinuous variables. Results were considered to be statistically significant if *p* was less than 0.05.

Results

HISTOLOGY

Table 1 presents patient details. All patients had histological evidence of iron overload as assessed by Perls' Prussian blue stain. Mild portal and/or acinar inflammation was present in 14 patients (all but one being Scheuer grade 1). Eight patients had no histological evidence of fibrosis, whereas 32 had Scheuer grade 1–4 fibrosis (eight, grade 1; 15, grade 2; four, grade 3; five, grade 4).

SERUM TIMP-1

Serum TIMP-1 concentrations in patients with haemochromatosis were significantly higher than those in control subjects (163 (30) versus

Table 2 Serum TIMP-1 and MMP in controls and patients with haemochromatosis

	Control	Haemochromatosis	p Value
TIMP-1	123 (28)	163 (30)	0.0002
MMP-1	22 (14)	19 (11)	NS
MMP-2	460 (62)	494 (74)	NS
MMP-3	53 (28)	56 (26)	NS
MMP-3*	58 (34)	39 (19)	0.03

Results are presented in ng/ml as mean (SD). *MMP-3 concentrations were also calculated for age and sex matched control and haemochromatotic subjects (n=15).

Figure 1 Serum TIMP-1 concentrations in control subjects, and in patients with haemochromatosis graded for the degree of fibrosis (Scheuer grades 0–4).

123 (28) ng/ml, $p<0.0002$ (table 2). Serum TIMP-1 concentrations in patients with haemochromatosis without fibrosis (Scheuer grade 0) were significantly higher than those in control subjects (153 (16) versus 123 (28) ng/ml, $p=0.03$) (fig 1), showing that increased TIMP-1 concentrations precede the development of fibrosis in haemochromatosis. Serum TIMP-1 was also significantly elevated in patients with cirrhosis (Scheuer grade 4) (210 (43) versus 123 (28) ng/ml, $p=0.0001$). TIMP-1 concentrations correlated with both HIC ($r=0.42$, $p<0.01$) (fig 2) and HII ($r=0.42$,

Figure 2 Linear regression analysis of the correlation between serum TIMP-1 concentrations and HIC in all patients with haemochromatosis (all circles, continuous line). The broken line represents analysis after exclusion of the 14 patients with mild acinar and/or portal inflammation (open circles).

$p<0.01$) (results not shown), but not with the grade of fibrosis or serum ferritin concentration. When the 14 patients with mild acinar and/or portal inflammation were excluded from the analysis (as hepatic inflammation is known to influence serum TIMP-1 concentrations), there was an increase in the correlation between serum TIMP-1 and HIC ($r=0.60$, $p=0.002$) (fig 2).

SERUM MMP

There were no significant differences between the absolute serum concentrations of MMP-1 and MMP-3 in controls and patients with haemochromatosis (table 2). However, in the normal population, serum MMP-3 concentrations (but not other MMPs or TIMP-1), increase with age and male sex.²⁸ Thus the older mean age and greater male preponderance in the haemochromatosis group may obscure changes in this group compared with control subjects. When MMP-3 concentrations of patients with haemochromatosis closely matched for age and sex to controls were analysed (n=15), there was a significant reduction in concentrations in patients with haemochromatosis compared with controls (58 (34) versus 34 (19) ng/ml, $p=0.03$).

Although there was no significant difference in the mean serum MMP-2 concentration of patients with haemochromatosis and controls, there was a significant positive correlation between serum MMP-2 concentrations in patients with haemochromatosis and the degree of fibrosis ($r=0.38$, $p<0.01$). The mean MMP-2 concentration in patients with haemochromatosis with cirrhosis was significantly greater than in patients with fibrosis only (661 (87) versus 486 (57) ng/ml, Scheuer grade 4 versus Scheuer grades 1–3, respectively; $p=0.003$) (fig 3A). There was a significant correlation between MMP-2 and HIC ($r=0.40$, $p=0.04$) which was not altered when the 14 patients with inflammation were excluded from the analysis (fig 3B). There was no significant correlation between either serum MMP-1 or MMP-3 concentrations and HIC, HII, or serum ferritin (results not shown).

The correlation coefficients between serum MMP-2 and both the degree of fibrosis and HIC were not altered when the 14 patients with inflammation were excluded from the analysis.

SERUM MMP:TIMP-1 RATIO

Although both serum TIMP-1 and MMP-2 concentrations were increased in patients with fibrosis and cirrhosis, the MMP-2:TIMP-1 ratio of patients with haemochromatosis was significantly lower than that of control subjects ($p=0.05$). MMP-3:TIMP-1 (when adjusted for age and sex) and MMP-1:TIMP-1 were also significantly lower in patients with haemochromatosis than in controls (table 3).

EFFECT OF IRON DEPLETION

Table 4 shows the serum TIMP-1, MMP-1, MMP-2, and MMP-3 concentrations in 10 patients with haemochromatosis before venesection therapy and after iron depletion. Serum MMP-2 and MMP-3 concentrations

Figure 3 Linear regression analysis of the correlation between serum MMP-2 concentrations and (A) the degree of fibrosis and (B) HIC, in patients with haemochromatosis.

Table 3 Serum MMP:TIMP-1 ratios in controls and patients with haemochromatosis

	Control	Haemochromatosis	p Value
MMP-1:TIMP-1	0.20 (0.14)	0.11 (0.06)	0.02
MMP-2:TIMP-1	3.91 (0.81)	3.32 (0.90)	0.05
MMP-3:TIMP-1	0.43 (0.23)	0.39 (0.19)	NS
MMP-3:TIMP-1*	0.47 (0.27)	0.26 (0.12)	0.007

Results are presented as mean (SD). *MMP-3 concentrations were also calculated for age and sex matched control and haemochromatotic subjects (n=15).

Table 4 Change in serum TIMP-1 and MMP (in ng/ml) after venesection therapy

	Before venesection	After venesection	p Value
TIMP-1	164 (17)	169 (49)	NS
MMP-1	22 (19)	22 (16)	NS
MMP-2	453 (59)	501 (70)	0.03
MMP-3	55 (36)	65 (48)	0.03

Results are expressed as mean (SD).

Table 5 Change in MMP:TIMP-1 ratio after venesection therapy

	Before venesection	After venesection	p Value
MMP-1:TIMP-1	0.13 (0.07)	0.15 (0.10)	NS
MMP-2:TIMP-1	2.79 (0.37)	3.27 (0.93)	NS
MMP-3:TIMP-1	0.34 (0.16)	0.42 (0.21)	0.08

Results are presented as mean (SD).

were significantly increased by 11% and 19%, respectively, following iron depletion but there were no significant changes in serum TIMP-1 or MMP-1 concentrations. Serum MMP-1:TIMP-1, MMP-2:TIMP-1, and MMP-3:TIMP-1 ratios were all increased following iron depletion (table 5) but this increase did not reach statistical significance.

Discussion

This study provides serological evidence of altered matrix degradation in haemochromatosis and we postulate that these changes may contribute to hepatic fibrosis complicating progressive iron overload. Serum TIMP-1 concentration was significantly increased in patients with haemochromatosis compared with controls and there was a significant correlation between serum TIMP-1 concentration and HIC. Furthermore, patients with haemochromatosis without fibrosis had significantly higher serum TIMP-1 concentrations than

control subjects, showing that elevated TIMP-1 precedes, and may be a contributory factor to, the development of fibrosis in haemochromatosis.

TIMP-1 is a 29 kDa glycoprotein produced in the liver by activated HSC.¹⁷ It is a logical assumption that the serum TIMP-1 concentration reflects hepatic production in haemochromatosis as HSC become activated in iron overload,¹² the liver is the site of maximal iron deposition and organ damage in haemochromatosis, and the serum TIMP-1 concentration is not increased in conditions such as osteoarthritis²⁹ and non-insulin dependent diabetes mellitus,²⁸ which are other common complications of haemochromatosis. Furthermore, the changes in serum concentrations of TIMP-1 reflect the levels of hepatic TIMP-1 and mRNA in a variety of other chronic liver diseases.³⁰⁻³¹ The requirement of at least 10 mg wet weight of tissue to assay hepatic TIMP-1 makes it virtually impossible to measure simultaneously hepatic TIMP-1 and serum concentrations in a disease such as haemochromatosis when liver tissue must be available for both biochemical and histological evaluation of the disease.

Of particular interest, our data have shown that serum TIMP-1 concentrations were increased in patients with haemochromatosis even without histological evidence of hepatic fibrosis. As previously stated, TIMP-1 is produced by activated HSC and the finding of increased TIMP-1 prior to the development of hepatic fibrosis supports our previous observations that HSC are activated in haemochromatosis at a relatively low HIC as evidenced by increased α -SMA expression, even before the development of histological fibrosis.¹² In view of the proposed role of TIMP-1 in fibrogenesis in other liver diseases,¹³⁻³⁰ it seems likely that increased TIMP-1 may contribute at an early stage in the fibrogenic process associated with haemochromatosis.

It is unclear whether liver cells other than HSC synthesise TIMP-1 in iron overload. It has been postulated that hepatocytes can also produce TIMP-1. TIMP-1 mRNA and protein have been detected in hepatocytes in livers with hepatocellular carcinoma or cholangiocarcinoma,³² although another study has failed to confirm this finding.³³ Hepatocyte cultures

have been shown to produce TIMP-1 in response to the proinflammatory cytokine interleukin 6,³⁴ but contaminating HSC have been proposed as the source of TIMP-1 in these cultures.³⁵ However, hepatocyte TIMP-1 protein expression has recently been shown by immunohistochemistry in chronic liver diseases such as chronic viral hepatitis, in the absence of hepatocellular carcinoma.³¹ It is unknown whether iron loaded hepatocytes in haemochromatotic livers are a cellular source of TIMP-1. However, as the results of our study show a significant correlation between TIMP-1 and HIC, we propose that either (1) iron or iron containing compounds may act to increase TIMP-1 production by HSC via increasing activation, or alternatively, (2) iron loaded hepatocytes may also produce TIMP-1.

Previous studies have shown that MMP-2 can degrade basement membrane and result in HSC activation as the maintenance of the quiescent phenotype is dependent on the presence of an intact matrix.¹³ In the present study, serum MMP-2 concentrations were significantly increased in patients with haemochromatosis with cirrhosis. While there was no difference in the MMP-2 concentrations of patients with haemochromatosis with fibrosis and control subjects, there was a significant correlation between serum MMP-2 and the degree of fibrosis. A similar correlation between serum MMP-2 and fibrosis has recently been described in patients with chronic hepatitis C.³⁶ An increase in MMP-2 mRNA with increasing fibrosis has been shown in other fibrotic liver diseases,³⁰ and increased MMP-2 may play a role in the perpetuation of fibrogenesis in iron overload.

Our study has shown no significant difference between the MMP-3 concentrations of patients with haemochromatosis and all control subjects. However, when the MMP-3 concentrations of patients with haemochromatosis were compared with appropriately age and sex matched controls, we found a significant reduction in the patients with haemochromatosis. A decrease in hepatic MMP-3 concentrations could contribute to hepatic fibrogenesis, as the complete activation of interstitial collagenase, MMP-1, requires the presence of active MMP-3.¹³ The mean MMP-3:TIMP-1 ratio (when matched for age and sex) was also significantly lower in patients with haemochromatosis compared with controls.

Our study has shown no significant difference between the mean serum MMP-1 concentration in patients with haemochromatosis and control subjects. This again reflects the absence of an increase in MMP-1 mRNA in other fibrotic liver diseases.³⁰ However, the mean MMP-1:TIMP-1 ratio (when matched for age and sex) was significantly lower in patients with haemochromatosis compared with controls. In fact, the mean ratios of each MMP to TIMP-1 were all decreased in patients with haemochromatosis compared with controls, emphasising that the increase in serum TIMP-1 is the dominant change in this disease.

It is known that fibrosis may regress after iron depletion in some patients with haemochromatosis.^{6,7} We have shown that serum MMP-2 and MMP-3 concentrations increased following iron depletion by venesection therapy, but serum TIMP-1 and MMP-1 concentrations did not change. We have previously shown, however, in patients with liver biopsy before and after iron depletion, that the number of activated HSC are decreased after excess iron is removed.¹² The biological relevance of these small but statistically significant increases in MMP-2 and MMP-3 concentrations, to the resolution of iron induced hepatic fibrosis in postvenesection therapy, remains to be determined.

In summary, we have shown significant changes in serum TIMP-1 and MMP concentrations in haemochromatosis which show for the first time that matrix degradation is altered in haemochromatosis, and may contribute to hepatic fibrosis with progressive iron loading.

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