

A role for thrombin in liver fibrosis

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Background/Aim: Several lines of evidence incriminate the serine proteinase thrombin in liver fibrogenesis either through its procoagulant function or its signaling via cell-surface receptors. The aim of this study was therefore to evaluate the effect of thrombin inhibition on experimental liver fibrosis.

Methods: Fibrosis was induced in rats by administration of CCl₄ for either three or seven weeks. Oral administration of the thrombin antagonist SSR182289 started one week after the start of CCl₄ intoxication. Fibrosis and the area occupied by alpha smooth muscle actin (ASMA) positive cells were quantified with histomorphometry. Expression of fibrosis related genes was measured by real time RT-PCR.

Results: After three weeks of CCl₄, treatment with SSR182289 did not significantly decrease the area of fibrosis but significantly decreased the area of ASMA positive cells by 22% (p=0.03) and the expression of TIMP-1 mRNA by 52% (p=0.02). There was no effect on gene expression of collagen I, MMP-2, or TIMP-2. After seven weeks of CCl₄, treatment with SSR182289 resulted in a significant decrease in fibrosis (–30%, p=0.04) and ASMA positive areas (–35%, p=0.05). SSR182289 alone had no effect on the measured parameters. Additionally, it did not alleviate the acute toxicity of CCl₄ as shown by measuring levels of serum aminotransferases and the area of necrosis.

Conclusions: These data provide evidence that thrombin antagonism can reduce liver fibrogenesis. The early effect of SSR182289 on ASMA and TIMP-1 expression suggests that it is beneficial in reducing fibrogenic cell activation.

Liver fibrosis is the main complication of chronic liver disease and leads eventually to cirrhosis. It is characterised by an excessive deposition of extracellular matrix components in the liver parenchyma. Synthesis of extracellular matrix components is performed by fibrogenic cells that derive from the activation of quiescent precursors such as hepatic stellate cells and portal fibroblasts.

During the past decade, a lot of attention has been given to the stimuli responsible for fibrogenic cell activation in the liver and a major focus has been on growth factors and oxidant stress.¹ The role of the coagulation cascade has been suggested by various observations but has not been formally proved. Activation of coagulation classically proceeds through binding of activated factor VII to the transmembrane protein tissue factor. This will allow cleavage of factor X to Xa. Factor Xa, in the presence of calcium and of factor Va will cleave prothrombin to thrombin. Thrombin is responsible for the conversion of fibrinogen to fibrin. Neubauer *et al* have shown that fibrin accumulates in the liver during acute as well as chronic experimental liver injury.² Similar results have been observed in models of pulmonary fibrosis. However it was reported that a total deficiency of fibrinogen did not modify the extent of bleomycin induced lung fibrosis.^{3,4} Such data have not been clearly reported in the liver although there is suggestion that mice deficient in fibrinogen are not protected against the acute or chronic effects of carbon tetrachloride.⁵ Recently, it was observed that thrombotic risk factors were frequently observed in patients with chronic hepatitis B or C and that patients with more than one risk factor had on average more severe fibrosis.⁶ The rate of fibrosis progression is also higher in hepatitis C patients that carry the prothrombotic factor V Leiden mutation.⁷

There are at least two mechanisms through which thrombin generation could be involved in fibrogenesis. Firstly, clot formation by itself leads to flow disturbance and local hypoxia which is a cofactor for fibrosis.⁸ Secondly,

thrombin signals via specific cellular receptors to regulate functions associated with tissue remodeling.^{9–11} Thrombin binds to several receptors that belong to the family of protease activated receptors (PAR).¹² The thrombin receptor PAR-1 is upregulated in human liver during acute and chronic injury.¹³ It has been reported that thrombin stimulates the proliferation of rat¹⁴ and human¹⁵ hepatic stellate cells. Thrombin also upregulates collagen synthesis in rat stellate cells.¹⁶

It has been shown that direct inhibition of thrombin with a synthetic antagonist decreased lung collagen accumulation in experimental pulmonary fibrosis.¹⁷ There are no clear data for liver fibrosis. Therefore, in this study, we investigated the effect of SSR182289, a synthetic thrombin inhibitor,^{18,19} on liver fibrosis induced by carbon tetrachloride in the rat.

MATERIAL AND METHODS

Animals and experimental design

Male Wistar rats (Charles River, St Aubin-lès-Elbeuf, France) weighing 200–250 g were used in this study. The animals had free access to food and drinking water. This study was performed in accordance with the European Community Standards on the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of the University of Bordeaux.

Fibrosis was induced by giving carbon tetrachloride (CCl₄, Sigma, St Quentin-Fallavier, France) mixed with olive oil (1:5) at 0.375 ml/kg body weight by gavage, three times a week on Monday, Wednesday, and Friday morning during three or seven weeks.

Abbreviations: aPTT, activated partial thrombin time; ASMA, alpha smooth muscle actin; CCl₄, carbon tetrachloride; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; MMP, matrix metalloproteinase; PAR, protease activated receptor; TIMP, tissue inhibitor of matrix metalloproteinase.

The thrombin antagonist used in this model (SSR182289)^{18, 19} (Sanofi-Synthelabo-Recherche, Chilly-Mazarin, France) was administered daily on the evening by gavage, at the dose of 30 mg/kg body weight in distilled water. In order to avoid the risk of liver haemorrhage during the early phase of CCl₄ intoxication where necrosis is prominent, SSR182289 administration was delayed until the second week of CCl₄ treatment. Thus, the total length of SSR182289 treatment was either two or six weeks.

Control animals for CCl₄ and SSR182289 received the respective solvents. Another group of rats received only SSR182289 at the same dose during two or six weeks.

Thus, four groups each including nine animals could be distinguished. Group I, olive oil and distilled water alone; group II, olive oil and SSR182289 alone; group III, CCl₄ with distilled water; group IV, CCl₄ with SSR182289.

Finally, in order to evaluate the initial toxicity of CCl₄, a third group of animals received only two injections of CCl₄ before sacrifice at day four.

Animals were sacrificed at the designated time points (96 hours, three and seven weeks). Liver samples were taken from several lobes and either snap frozen in liquid nitrogen, cryopreserved in optimal cutting temperature (OCT) compound (Sakura, Torrance, CA) and snap frozen in liquid nitrogen cooled isopentane, or fixed in buffered formalin. Serum and plasma samples were also collected.

Liver function tests

Routine liver function blood tests (bilirubin, alkaline phosphatase, and transaminases) were performed on an automated analyser in the biochemistry department of Pellegrin Hospital in Bordeaux.

Blood clotting parameters

Venous blood anticoagulated with sodium citrate was withdrawn in Vacutainer tubes (Becton Dickinson, Plymouth, UK). Plasma was centrifuged twice at 2500 *g* for 10 minutes and rapidly frozen at -80°C. Activated partial thrombin time (aPTT), was determined by a conventional method.

Fibrosis assessment

This was done, basically as previously described, using a histomorphometric method that measures the surface of a liver section occupied by fibrous areas.²⁰ Sections 5 µm thick from formalin fixed, paraffin embedded liver tissue were prepared and were stained with Sirius red (saturated picric acid in distilled water containing 0.1% (w/v) Sirius red F3B (BDH Chemicals Ltd., Poole, UK) to allow visualisation of liver fibrosis.²¹ Slides were mounted with EUKITT (O Kindler GmbH & Co, Freiburg, Germany) and examined with a Zeiss Axioplan 2 microscope (Carl Zeiss Microscopy, Jena, Germany) by light microscopy. All samples from a series of experiments were stained simultaneously and evaluated in a blinded fashion. Images were acquired with an AxioCam camera (Carl Zeiss Vision, Hallbergmoos, Germany) by means of the Axiovision image processing and analysis system (Carl Zeiss Vision) and quantitative data of the Sirius red staining were obtained using a computerised image analysis system (KS300, Carl Zeiss Vision). Sampling corresponded to three sections randomly taken from left, median, and right lobes. The analysis was performed on an average of 50 fields per section using the ×10 objective. The whole surface of the sections was used for analysis with the exception of large centrilobular veins (diameter ≥150 µm) and large portal tracts. Fibrosis deposition was expressed as a percentage of Sirius red stained areas on the total area of the sections.

Immunolabeling for alpha smooth muscle actin (ASMA) was performed on serial sections of paraffin embedded

hepatic tissue with a anti-ASMA monoclonal antibody (Dako A/S, Glostrup, Denmark, clone 1A4) diluted to 1/400, amplified with the EnVision+ system HRP detection kit (Dako A/S, Glostrup, Denmark) and revealed with liquid diaminobenzidine substrate (Dako A/S, Glostrup, Denmark). Results were analysed with the same method.

Quantitative measurements of collagen type I, MMP-2, TIMP-1, and TIMP-2 mRNAs by RT-PCR

After disruption and homogenisation in lysis buffer with the Mixer Mill MM300 (Qiagen SA, Courtaboeuf, France) of approximately 50 mg snap frozen liver, total RNA was isolated using the RNeasy Mini kit (Qiagen).

Transcripts of tissue inhibitor of matrix metalloproteinases (TIMP)-1, type I collagen (α(I)1 chain), matrix metalloproteinase-2 (MMP-2), and TIMP-2 were quantified by a technique of quantitative RT-PCR, that has previously been described in detail.²² Each sample was normalised on the basis of its expression of the RPLP0 gene (also known as 36B4, encoding acidic ribosomal phosphoprotein P0).

Radical scavenging activity

Radical scavenging properties of SSR182289 were determined according to Blois²³ by measuring reduction of the stable radical 1,1-Diphenyl-2-picrylhydrazyl (DPPH). Various concentrations of compounds diluted in dimethyl sulphoxide (10 µl) were added to a 1 ml solution of Tris/HCl (10 mM), pH 7.2/MeOH (2/1, v/v) containing DPPH (20 µM). Absorbance at 517 nm was measured after 20 minutes.

Statistical analysis

All values are expressed as mean (standard deviation). Comparison of multiple means was performed with the Kruskal Wallis non-parametric test followed by the Mann-Whitney test, using the StatView 2.0 software (Abacus Concepts Inc, Berkeley, CA, USA).

RESULTS

Effect of SSR182289 on blood coagulation

Control aPTT was 33 s. One hour after the oral administration of 5, 10, 20, or 30 mg/kg of SSR182289, aPTT increased to 44.5, 50.7, 55.7, or 67.6 s, respectively. The 30 mg/kg dose was then selected for further studies.

Effects of SSR182289 on CCl₄ induced initial toxicity

Because the induction of CCl₄ induced fibrosis depends on the initial induction of hepatocyte necrosis, we examined the effect of SSR182289 on the toxicity of CCl₄. For that purpose, two groups of six rats treated with two administrations of CCl₄ with or without two administrations of SSR182289 were killed 24 hours after the second CCl₄ dose and evaluated for acute toxicity of CCl₄ by serum tests and measurement of the area of necrosis on liver sections. As shown in table 1, treatment with CCl₄ resulted in an increase in the serum level of transaminases. Although there was a trend towards higher values in animals receiving the thrombin antagonist, the differences were not significant. As expected, treatment with CCl₄ induced a significant centrilobular necrosis as shown on tissue sections. The necrosis area was however not statistically different between the two groups.

In addition, SR182289 did not display any antioxidant ability in a DPPH scavenging assay (1% inhibition, data not shown). The same assay clearly demonstrated the expected antioxidant activity of BHT and vitamin E^{24, 25} (IC₅₀ = 4 (SD 0.8) and 6 (SD1.4) µM respectively).

Vital parameters and liver function tests

After three weeks of CCl₄, liver weight increased significantly as shown by the increase in liver weight to body weight ratio.

Table 1 Liver function tests and area of necrosis in rats after 96 hours of CCl₄ treatment with or without SSR182289

Treatment	Aspartate aminotransferase (IU/l)	Alanine aminotransferase (IU/l)	Necrosis area (%)
CCl ₄	255.8 (87.0)	117.2 (65.7)	10.2 (4.8)
CCl ₄ +SSR182289	346.2 (194.0)	206.4 (149.8)	11.9 (5.0)

Groups of six rats were treated with two administrations of CCl₄ with or without two administrations of SSR182289 and were killed 24 hours after the second CCl₄ dose. Values are mean (standard deviation). No difference reached statistical significance.

Treatment with SSR182289 significantly decreased this ratio (table 2). Treatment with CCl₄ resulted in increased values of serum transaminases and alkaline phosphatase. The administration of SSR182289 did not significantly decrease these parameters (table 2).

After seven weeks of CCl₄, no clinical or biological parameter was significantly different between rats receiving or not SSR182289 (table 3).

No bleeding episode was observed during the course of these experiments.

Fibrosis measurement

CCl₄ treatment for three weeks induced fibrosis deposition predominantly located around centrolobular hepatic veins, as shown by Sirius red staining (fig 1C). The area of fibrosis increased about tenfold from 0.285 (SD 0.05) % of field area in control animals which received only olive oil (fig 1A and B), to 2.62 (SD 0.65) % in CCl₄ treated animals.

SSR182289 administered alone had no effect on the fibrosis area (0.296 (SD 0.04) %). Treatment with SSR182289 together with CCl₄ slightly decreased the fibrosis area to 2.26 (SD 0.49) % (fig 1G). This decrease did not however reach statistical significance.

ASMA staining was restricted to vessel walls as expected in control animals (fig 2A and B). Following CCl₄ treatment, many ASMA positive cells were found in the diseased areas around centrolobular veins. Concurrent treatment with SSR182289 resulted in a significant 22% relative decrease in the ASMA positive areas (from 3.71 (SD 1.11) % in the CCl₄ group down to 2.89 (SD 0.65) % in the CCl₄ + SSR182289 group ($p = 0.03$) (fig 2C, D, G).

The longer, seven weeks CCl₄ treatment induced a more severe fibrosis with larger septae arising from the hepatic veins that sometimes encircled nodules as shown on Sirius

Table 2 Vital parameters and liver function tests in rats after 3 weeks of treatment

Parameters	Control	SSR182289	CCl ₄	CCl ₄ +SSR182289
Body weight (g)	374.4 (29.1)	354.9 (15.7)	325.8 (24.3)	317.2 (12.8)
Liver weight (g)	14.4 (2.4)	13.5 (0.9)	15.3 (2.5)	13.2 (1.3)*
LW:BW ratio (×100)	3.8 (0.4)	3.8 (0.1)	4.7 (0.6)	4.2 (0.3)*
ASAT (IU/l)	89.9 (17.5)	113.2 (29.0)	269.3 (133.9)	205.0 (98.2)
ALAT (IU/l)	48.6 (8.2)	46.2 (6.6)	285.7 (173.0)	202.6 (129.2)
AP (IU/l)	168.1 (47.6)	188.1 (63.8)	300.0 (50.2)	247.8 (68.9)
Bilirubin (μM/l)	2.6 (1.0)	2.3 (0.7)	2.4 (1.6)	2.7 (0.7)

* $p \leq 0.05$ compared with CCl₄ group. Values are mean (standard deviation).

Table 3 Vital parameters and liver function tests in rats after 7 weeks of treatment with or without SSR182289

Parameters	Control	SSR182289	CCl ₄	CCl ₄ +SSR182289
Body weight (g)	404.3 (29.5)	388.6 (28.5)	341.1 (27.0)	315.3 (24.5)
Liver weight (g)	14.5 (0.7)	14.0 (1.9)	14.1 (3.0)	13.5 (1.4)
LW:BW ratio (×100)	3.6 (0.4)	3.6 (0.2)	4.2 (0.7)	4.4 (0.4)
ASAT (IU/l)	94.3 (5.8)	112.9 (22.9)	440.0 (264.8)	919.0 (645.8)
ALAT (IU/l)	50.7 (8.3)	48.7 (9.1)	286.4 (209.8)	505.0 (295.9)
AP (IU/l)	144.2 (40.0)	116.3 (35.0)	315.2 (160.0)	485.6 (238.2)
Bilirubin (μM/l)	2.6 (0.9)	3.3 (1.6)	4.9 (5.6)	4.7 (5.9)

red stained sections (fig 1E). Treatment with SSR182289 resulted in a 30% decrease in fibrosis area from 6.56 (SD 2.77) % of field area in CCl₄ treated animals down to 4.56 (SD 1.65) % ($p = 0.04$) (fig 1F and G).

SSR182289 administered alone had no effect on the area of fibrosis (0.58 (SD 0.08) %) compared with untreated controls (0.49 (SD 0.12) %).

The ASMA positive area increased greatly after seven weeks of treatment with CCl₄ from 0.55 (SD 0.08) % in controls with olive oil (or 0.48 (SD 0.10) % in rats with SSR182289 alone), up to 11.57 (SD 6.42) % of field area (fig 2E). When the CCl₄ treated animals also received SSR182289, the ASMA positive area was decreased by 35% down to 7.56 (SD 3.94) % of field area ($p = 0.05$) (fig 2F and G).

Expression of fibrosis related genes

Fibrosis deposition results from an imbalance between synthesis and degradation of its components. During liver fibrosis, failure of degradation is associated with an upregulation of the expression of the matrix metalloproteinase inhibitors TIMP-1 and TIMP-2.^{26, 27} We thus evaluated the mRNA levels of collagen $\alpha 1(I)$, a major component of liver fibrosis and of TIMP-1 and TIMP-2 by real time RT-PCR in total RNA extracted from rat livers. We also measured the levels of MMP-2 transcripts because although MMP-2 degrades several matrix components, MMP-2 overexpression is also associated with liver fibrosis.²⁸ After three weeks, the administration of SSR182289 in CCl₄ treated animals resulted in a significant 52.0% decrease in TIMP-1 expression ($p = 0.02$) (fig 3). On the other hand, whereas expression of transcripts for type I collagen, MMP-2, and TIMP-2 greatly increased with CCl₄ treatment, they were not reduced by treatment with SSR182289 (fig 3). Administration of SSR182289 alone had no effect on gene expression.

DISCUSSION

In this study, we have evaluated the effect of chronic thrombin inhibition on the extent of fibrosis deposition in the CCl₄ model of liver fibrosis in the rat. We show that a daily administration of SSR182289, a novel, selective, orally active synthetic thrombin inhibitor,^{18, 19} decreased liver fibrogenesis induced by CCl₄ after either three or seven weeks of intoxication. Although at three weeks the decrease in fibrosis deposition failed to reach statistical significance, there was already a significant decrease in the area occupied by ASMA positive cells and in TIMP-1 expression. This probably indicates a reduced extent of activation of fibrogenic cells in animals receiving the thrombin antagonist. After seven weeks of SSR182289, fibrosis area and ASMA positive

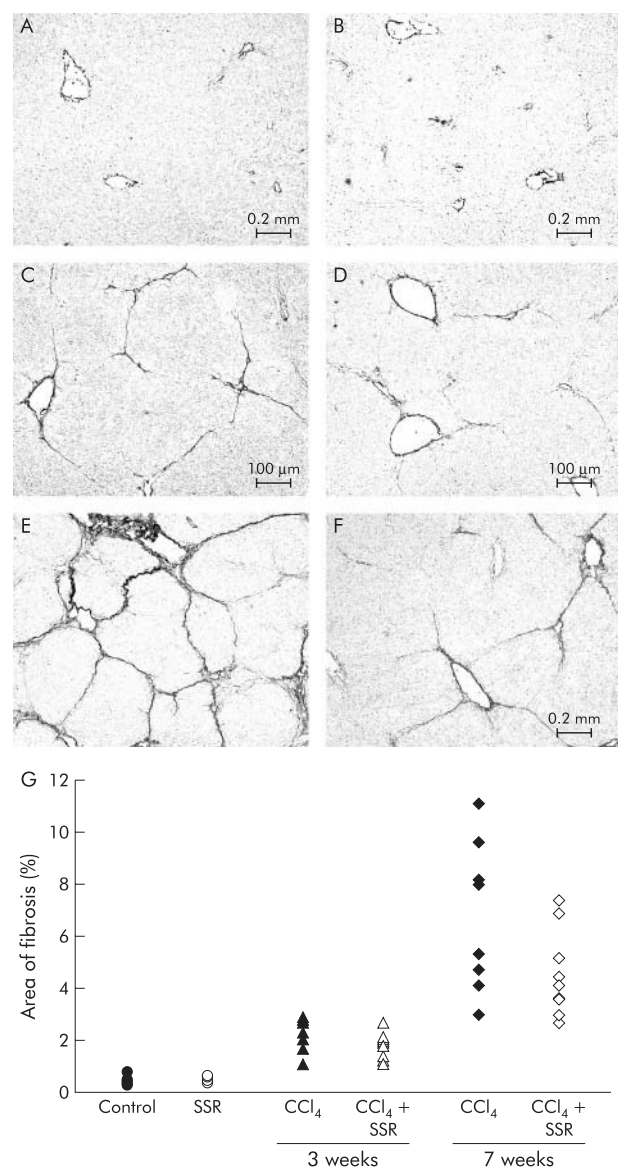


Figure 1 Staining of fibrosis with Sirius red. Representative sections from animals of every group: (A) control, (B) SSR182289 for three weeks alone, (C) CCl₄ for three weeks, (D) CCl₄ with SSR182289 for three weeks, (E) CCl₄ for seven weeks, (F) CCl₄ and SSR182289 for seven weeks. (G) Values for individual animals. Note that although there were nine animals per group, some points are not visible because of superimposition.

area were both significantly reduced in animals treated with the thrombin antagonist. Inhibition of thrombin has been shown to offer protection against the acute toxicity of lipopolysaccharide to the liver.²⁹ It is thus important to stress that SSR182289 had no protective effects in the early events of liver necrosis elicited by CCl₄, which would have complicated the data interpretation. In addition, as the toxicity of CCl₄ relies on oxidative stress, it was important to demonstrate that SSR182289 does not behave as an antioxidant *in vitro*.

Altogether, our data suggest that thrombin plays a detrimental role in chronic liver injury. This is also supported by recent data showing that mice genetically deficient in the fibrinogen related procoagulant Fgl2, an alternative prothrombin activator, are protected against liver injury from mouse hepatitis 3 virus; moreover, a high liver Fgl2

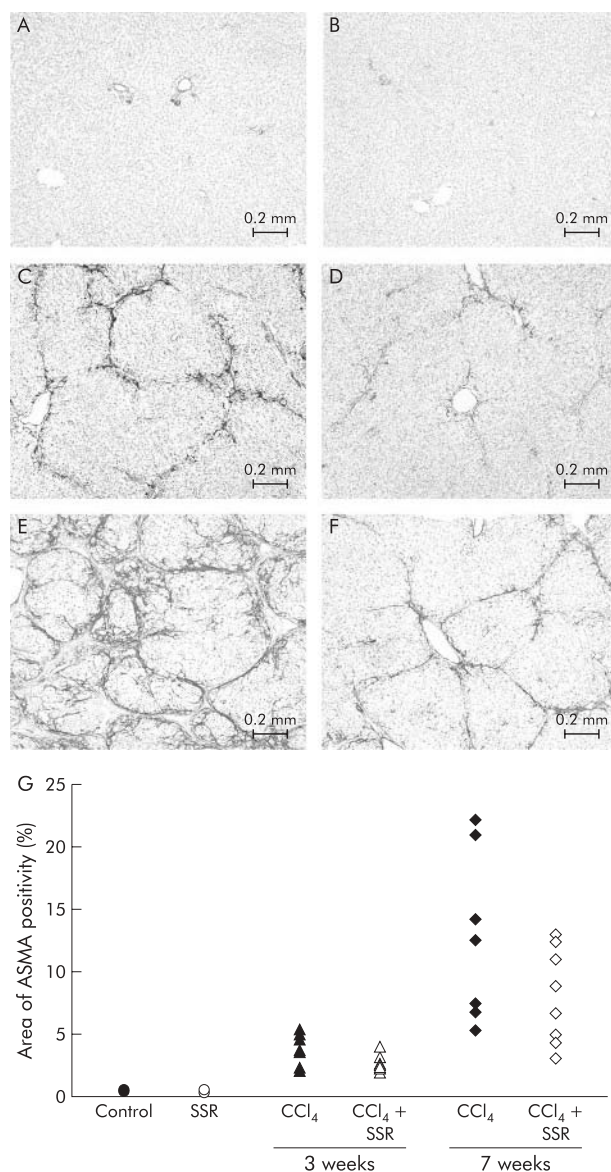


Figure 2 Immunostaining of alpha smooth muscle actin. Representative sections from animals of every group: (A) control, (B) SSR182289 for three weeks alone, (C) CCl₄ for three weeks, (D) CCl₄ with SSR182289 for three weeks, (E) CCl₄ for seven weeks, (F) CCl₄ and SSR182289 for seven weeks. (G) Values for individual animals. Note that although there were nine animals per group, some points are not visible because of superimposition.

expression was associated with fibrin deposition and severe forms of chronic viral hepatitis B in human.³⁰

The mechanisms by which thrombin could aggravate liver diseases remain to be further elucidated. Because of the well known role of thrombin in fibrinogen cleavage, the first hypothesis to consider is the procoagulant function of thrombin. Fibrin deposition is indeed associated with both experimental and human liver fibrosis. It is not restricted to acute inflammatory liver disease but can also be found in chronic lesions.²⁻³⁰ Fibrin deposition in vascular beds would lead to occlusive thrombi contributing to downstream liver injury. However, indirect data point against this mechanism. Firstly, bleomycin induced lung fibrosis, which shares many mechanistic features with experimental liver fibrosis, is not reduced in animals genetically deficient in fibrinogen.³⁻⁴ Secondly, mice with a combined deficiency in fibrinogen and plasminogen are quite sensitive to the profibrogenic

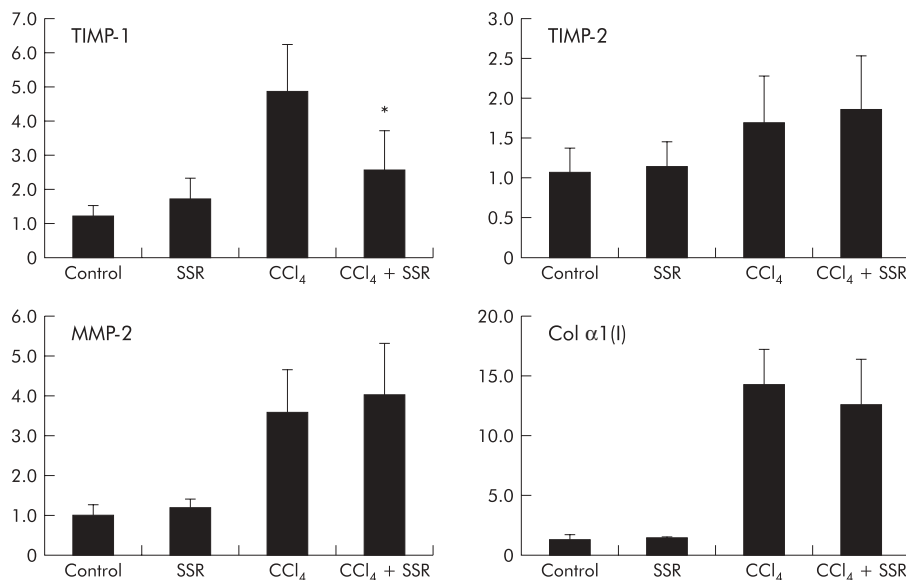


Figure 3 Expression of fibrosis related genes. The expression of the indicated transcripts was measured by real time RT-PCR on total liver RNA extracted from the livers of animals treated for three weeks in the indicated conditions. Transcripts levels were normalised on the basis of the expression of the RPLP0 gene. Data are shown as mean (SEM) (n = 9) and are in arbitrary units. The asterisk indicates p=0.02. No other differences between the CCl₄ and CCl₄+SSR182289 groups were statistically significant.

effects of CCl₄.⁵ There are however no published data on liver fibrosis in animals with a single deficiency in fibrinogen. Other mechanisms must also be considered. Indeed, besides its procoagulant effect, thrombin also acts on cell surface receptors from the PAR family to induce intracellular signaling. Many signals relevant to liver fibrogenesis have been described. These include the stimulation of expression of the profibrogenic mediator connective tissue growth factor,^{10–31} of type I collagen,⁹ or of TIMP-1.³² Thrombin can also induce the activation of the proform of MMP-2.³³ More specifically, thrombin has been shown to stimulate proliferation¹⁴ and matrix synthesis¹⁶ of cultured rat hepatic stellate cells and human liver myofibroblasts.¹⁵ Although there are no data for hepatic stellate cells, it has been shown that thrombin can induce the differentiation of lung fibroblasts into myofibroblasts.³⁴ Finally, the thrombin receptor PAR-1 is overexpressed during human liver fibrogenesis in a location consistent with that of fibrogenic cells.¹³ Thus, circumstantial evidence suggests that the detrimental effects of thrombin on liver fibrogenesis could be mediated by its cell surface receptors. Our data provide a clue as to the mechanism by which thrombin inhibition may be protective. Indeed, we found that treatment with SSR182289 greatly reduced the expression of TIMP-1 transcripts. This appeared quite specific, as no differences were seen for other fibrosis related genes such as collagen α1(I), MMP-2, or TIMP-2. These data are in keeping with the reported stimulating effect of thrombin on TIMP-1 expression in glomerular mesangial cells³² and in human liver myofibroblasts (J Gillibert Duplantier and J Rosenbaum, unpublished observations). TIMP-1 is a major actor of liver fibrogenesis. This has been notably shown in a study where overexpression of TIMP-1 by transgenesis strongly potentiated liver fibrosis.³⁵ This is also suggested by the study by Raetsch *et al*³⁶ in bile duct ligated rats, where treatment with pentoxifylline had only a limited effect on liver fibrosis although it profoundly decreased expression of collagen I mRNA, most likely because it increased TIMP-1 expression. The deleterious effects of TIMP-1 may be related not only to its inhibitory effect on matrix degradation, but also to its antiapoptotic effect on fibrogenic cells.³⁷ Thus, altogether, it is likely that the specific decrease in TIMP-1 expression may explain the antifibrotic effect of thrombin inhibition seen in this study.

While this manuscript was being submitted, Fiorucci *et al* showed that a PAR-1 antagonist strongly reduced liver fibrosis in the bile duct ligation model.³⁸ In addition to thrombin, PAR-1 is also a receptor for other coagulation proteases including factor Xa^{39–40} and activated protein C⁴¹ and it is thus possible that the benefits of antagonising PAR-1 relate in part to the inhibition of the signalling of these proteins. However, altogether, the case is strong for a role of coagulation proteases in liver fibrogenesis.

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