

Factor VII activating protease (FSAP) exerts anti-inflammatory and anti-fibrotic effects in liver fibrosis in mice and men

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Background & Aims: Factor VII activating protease (FSAP) is a circulating serine protease produced in the liver. A single nucleotide polymorphism (G534E, Marburg I, MI-SNP) in the gene encoding FSAP (*HABP2*) leads to lower enzymatic activity and is associated with enhanced liver fibrosis in humans. FSAP is activated by damaged cells and its substrates include growth factors and hemostasis proteins.

Methods: We have investigated the progression of liver fibrosis in FSAP deficient mice and FSAP expression in human liver fibrosis.

Results: Serum FSAP concentrations declined in patients with end-stage liver disease, and hepatic FSAP expression was decreased in patients with advanced liver fibrosis and liver inflammation. Moreover, there was an inverse correlation between hepatic FSAP expression and inflammatory chemokines, chemokine receptors as well as pro-fibrotic mediators. Upon experimental bile duct ligation, *FSAP*^{-/-} mice showed enhanced liver fibrosis in comparison to wild type mice, alongside increased expression of α -smooth muscle actin, collagen type I and fibronectin that are markers of stellate cell activation. Microarray analyses indicated that FSAP modulates inflammatory pathways.

Conclusions: Lower FSAP expression is associated with enhanced liver fibrosis and inflammation in patients with chronic hepatic disorders and murine experimental liver injury. This strengthens the concept that FSAP is a “protective factor” in liver fibrosis and explains why carriers of the Marburg I SNP have more pronounced liver fibrosis.

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Introduction

Liver fibrosis is characterized by extensive extracellular matrix deposition. This scar tissue formation extensively alters the vasculature, affects the liver hemodynamic flow and depresses liver function. This state is initiated by diverse factors that include damage to parenchymal cells, hepatocytes, due to pathogens, alcoholism, toxins, and/or fatty diet [1]. Tissue injury recruits inflammatory cells and activates hepatic stellate cells (HSC) that further promote extracellular matrix remodeling. Under the influence of various regulatory factors, the balance between matrix synthesis and breakdown is shifted so that net extracellular matrix deposition results. Current therapeutic options are limited and only target the underlying pathology, while reversal of fibrosis is a major future goal [2].

The coagulation and fibrinolysis system is known to play a major role in the progression of liver fibrosis. This has been observed in patients with disorders of the hemostasis system such as Factor V Leiden mutation, protein C deficiency, and elevated FVIII [3]. Hypercoagulation state or depressed fibrinolysis is thought to regulate fibrin deposition in the liver and thereby regulating the fibrosis process. There is a correlation between the load and distribution of microthrombi, which cause parenchymal extinction, and the extent of hepatic fibrosis [3]. Another concept is that these factors directly regulate the cellular processes and proteolytic cascades in order to exert non-hemostasis-related effects [4,5]. In this scenario, coagulation factors Xa and thrombin can directly cleave and activate protease activated receptors (PAR's) on target cells and influence their proliferation, migration, and matrix remodeling properties related to fibrosis [3].

Factor VII activating protease (FSAP) is a circulating plasma serine protease that can activate Factor VII (FVII) and pro-urokinase (pro-uPA) and inactivate the tissue factor pathway inhibitor (TFPI), thus suggesting an involvement in the regulation of coagulation and fibrinolysis [6]. Population studies have shown that a single nucleotide polymorphism (SNP) in the FSAP gene (official gene name hyaluronic acid binding protein 2; *HABP2*), G534E, also called the Marburg I (MI) polymorphism, is found in about 5–8% of the European population and is associated with late complications of carotid stenosis [7], stroke [8], and severity of liver

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fibrosis [9]. MI-FSAP has about a 5-fold weaker proteolytic activity compared to wild type (WT) FSAP towards various substrates [10]. Hence, a change in a single amino acid in the protease domain may change the enzyme activity and specificity and seems to be linked to pathophysiological processes.

Circulating latent FSAP zymogen is activated by factors that are released from dead/injured or apoptotic cells, such as histones and nucleosomes [11,12]. This fits well with previous experimental results showing that acute application of the hepatotoxin carbon tetrachloride (CCl₄) leads to FSAP activation in mice, presumably through induction of cell death in the liver [13]. We have demonstrated that CCl₄-mediated liver injury is associated with an upregulation of FSAP expression [14]. Apart from its role in hemostasis, this protease is also known to inhibit the activity of growth factors, such as platelet-derived growth factor-BB (PDGF-BB) [10] and basic fibroblast growth factor (bFGF) [15]. Particularly, PDGF-BB is likely to be involved in liver fibrosis and we have demonstrated that the effects of PDGF-BB on HSC are inhibited by FSAP [9,14]. FSAP has also been shown to generate the anaphylatoxin C5a [16] and bradykinin [17] from respective precursor molecules. Cellular activation by FSAP is suggested to be mediated via PARs that are classically activated by hemostasis proteins such as thrombin and FXa [18].

Collectively, evidence from human genetics with respect to MI-SNP, as well as changes in FSAP expression in mouse liver fibrosis model, and the effects of FSAP on HSC in cell culture indicate that FSAP might be an important regulator in liver fibrosis. To further define the role of FSAP in liver fibrosis, we have measured FSAP mRNA expression in the liver of patients with a varying progression of disease, and found a significant negative correlation between fibrosis/inflammation and FSAP expression. In line with this finding, FSAP serum concentration declined in patients with advanced liver cirrhosis. Experiments with *FSAP*^{-/-} mice also confirmed an influence of FSAP on inflammation and liver fibrosis. Furthermore, microarray analyses revealed a change in the gene expression pattern related to inflammation in the fibrotic liver of *FSAP*^{-/-} mice. These studies demonstrate an important role for FSAP in regulating the inflammatory and fibrotic adaptation to liver injury.

Material and methods

Human serum and liver samples

Serum samples of 85 patients with chronic liver disease and 15 healthy controls were collected and stored at -80 °C until further assessment. Each patient provided informed, written consent and the study was approved by the local ethics committee. Liver fibrosis/cirrhosis was diagnosed on the basis of liver histology, imaging studies and/or the presence of cirrhosis-related complications [19]. Patients fulfilling criteria of systemic inflammation syndrome (SIRS) or sepsis were excluded from the present study. Patients with established cirrhosis were stratified according to the Child-Pugh score (Table 1 contains detailed information on the study cohort). FSAP antigen in serum was measured as previously described [20]. Liver tissue was acquired either from biopsies for routine clinical purposes or explants of cirrhotic livers obtained during liver transplantation. An independent pathologist, blind to experimental data, performed grading (grade of inflammation) and staging (stage of fibrosis) according to the Desmet-Scheuer score [19]. FSAP mRNA expression was analyzed in a total of 58 liver specimens from varying etiologies encompassing n = 15 hepatitis C (HCV), n = 12 hepatitis B (HBV), n = 11 alcoholic liver disease, n = 11 primary biliary cirrhosis (PBC), n = 9 miscellaneous. Tissue from tumor-free margins of resected hepatic metastasis within normal liver parenchyma (n = 5) served as control.

FSAP^{-/-} mice

The mice were obtained from the Texas Institute for Genomic Medicine (Houston, Texas). The retroviral vector (pKOS-3) was used to generate a library of randomly targeted embryonic stem (ES) cells [21]. Using this random approach, an ES line with an insert in the *HABP2* gene was identified as described before [21]. Further sequencing showed that a 65 bp fragment of exon 1 starting at ATG was replaced with the promoter-less gene expressing galactosidase (*lacZ*) and neomycin resistance gene. For PCR-based genotyping, a combination of primers specific for *HABP2* and the inserted sequence were used to amplify the corresponding WT or mutant alleles. Mice were backcrossed for 3 generations into the BalbC background (F3). Mice from heterozygous crosses between 10–14 weeks of age were used, and all experiments were performed on littermate controls. All experiments reported here were approved by the local committee for care and use of laboratory animals as well as the Landesamt für Natur, Umwelt und Verbraucherschutz NRW (LANUV), and performed according to strict governmental and international guidelines on animal experimentation.

Statistical analysis

For the experiments with experimental animals, data between the groups were analyzed with ANOVA followed by pair-wise comparison with Bonferroni's multiple test. In the figures, only the statistical significance between WT and *FSAP*^{-/-} mice is shown where relevant. Human data were analyzed using Mann-Whitney test or Kruskal-Wallis test and Dunn's test for *post hoc* analysis when comparing more than 2 groups (Graphpad Prism). Correlation analyses were performed by Spearman's non-parametric rank correlation test with SPSS V13.0 (SPSS Inc, Chicago, IL, USA). A *p* value <0.05 was considered statistically significant.

Results

Expression of FSAP in patients with liver fibrosis and cirrhosis

Hepatic FSAP (*HABP2*) mRNA expression was analyzed in 64 liver samples (n = 58 patients, n = 5 controls). Compared with the control liver, the diseased liver without scarring tended to display increased FSAP expression, though statistical significance was not reached. In patients with moderate to advanced fibrosis (F1–3), FSAP gene expression was diminished and even further decreased in patients with established liver cirrhosis (F4) (Fig. 1A) compared to non-fibrotic livers. Of note, decrease of hepatic FSAP expression was also associated with increased hepatic inflammation (Fig. 1B). While patients with low to absent inflammation (G0–G1) showed increased FSAP gene transcription in contrast to control livers, FSAP expression was lower in livers with higher inflammation scores. Moreover, FSAP mRNA gene expression inversely correlated with hepatic expression of many inflammatory chemokines and their cognate receptors (e.g., CCL2/CCR2; CCL5/CCR5, CCL20/CCR6), suggesting that FSAP might negatively regulate inflammatory pathways and immune cell dynamics in patients (Table 2). Interestingly, we noted a positive correlation between FSAP expression and the fractalkine system (CX₃CL1 and its receptor CX₃CR1), which has been previously shown to exert protective roles in liver fibrosis in mice and humans [22]. Moreover, low FSAP expression was associated with high MMP-1 and high TGF-β, the latter representing a prototypical pro-fibrotic mediator. When normalized to stage of liver fibrosis, there was no significant difference between different etiologies, indicating that hepatic FSAP regulation mirrors common uniform pathogenic pathways of liver fibrosis (Fig. 1C).

In order to elucidate whether FSAP was also regulated on a systemic level, we measured FSAP concentration in a well-defined cohort of 85 patients with chronic liver disease (Table 1). Despite a slight increase when comparing all patients to normal

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Table 1. Patient characteristics and FSAP serum concentrations.

	All patients	Stage of cirrhosis			
		No cirrhosis	Child-Pugh A	Child-Pugh B	Child-Pugh C
n	85	41	17	18	9
Sex male/female, n	51/34	25/16	8/9	11/7	7/2
Age, yr	58	43	62	63.50	58
Median (range)	(17-77)	(17-73)	(37-75)	(38-77)	(21-76)
Disease etiology, n					
Virus hepatitis	36	25	4	5	2
Biliary/autoimmune	10	6	2	2	4
Alcohol	18	1	6	7	3
Other origins	21	9	5	4	9
Ascites, n (%)	19 (22)	1 (2)	3 (18)	8 (44)	7 (78)
Esophageal varices, n (%)	29 (34)	0 (0)	9 (53)	12 (67)	8 (89)
Hepatocellular carcinoma, n (%)	11 (13)	0 (0)	4 (31)	4 (22)	3 (33)
MELD score	10	n.a.	7	12	15
Median (range)	(6-24)		(6-11)	(8-17)	(9-24)
ALT, U/L	46	75	25	29	29
Median (range)	(8-2103)	(10-2103)	(8-125)	(12-189)	(12-215)
GFR, ml/min/1.73 m ²	99	126	72	59	72
Median (range)	(20-291)	(66-291)	(25-189)	(26-121)	(20-147)
Bilirubin, mg/dl	0.9	0.7	0.9	1.9	3.8
Median (range)	(0.2-28.2)	(0.2-2.1)	(0.2-2.6)	(0.4-12.2)	(0.6-28.2)
Albumin, mg/dl	40.5	45	37.4	29.85	28.45
Median (range)	(12-50)	(35-50)	(28-46)	(12-43)	(25-37)
INR	1.08	1.015	1.04	1.185	1.29
Median (range)	(0.93-3.95)	(0.93-1.28)	(0.93-3.95)	(1.04-1.46)	(1.12-1.61)
Factor V, %	121	128	132	86	57
Median (range)	(29-164)	(71-147)	(68-164)	(51-147)	(29-132)
Hyaluronic acid	180	31	225	520	580
Median (range)	(10-800)	(10-250)	(54-770)	(200-800)	(55-800)
Platelets, G/L	193	242	268	102	97
Median (range)	(44-638)	(112-538)	(53-638)	(44-611)	(54-259)
FSAP antigen, ng/ml	6090	6766.515	6695.259	5587.762	3710.195
Median (range)	(2086-12,964)	(3438.9-11,977)	(3438.9-12,964)	(2086-9835.5)	(2576.2-8671.1)

Clinical and laboratory characteristics of 85 patients with chronic liver diseases are presented. Patients were categorized according to disease severity assessed by the Child-Pugh's criteria. For quantitative values the median is given with the range in parenthesis. ALT, alanine aminotransferase; GFR, glomerular filtration rate; INR, international normalized ratio; MELD, model of end-stage liver disease; n.a., not available.

controls (Fig. 1D), dissecting the different stages of liver cirrhosis revealed that the total incline could be attributed to a substantial rise in FSAP serum concentration in early stages of chronic liver disease (no cirrhosis, Child A), whereas it dropped in advanced Child C cirrhosis (Fig. 1E). Furthermore, FSAP inversely correlated with approved non-invasive fibrosis tests such as aspartate aminotransferase/platelet index (APRI) and hyaluronic acid (data not shown), indicating that FSAP decline in serum is linked to fibrosis progression. Again, after normalizing to the stage of disease, no relation to the underlying etiology could be observed (Fig. 1F). However, we were able to demonstrate an inverse correlation with pro-collagen-III-peptide levels (Fig. 1G), again demonstrating that FSAP has anti-fibrotic potential. We also tried to elucidate whether serum FSAP levels directly related to hepatocyte demise. This analysis did not reveal any correlation with transaminases and glutamate dehydrogenase (GLDH) (ALT $r = -0.109$, $p = 0.348$; AST $r = 0.041$, $p = 0.715$, GLDH $r = 0.046$, $p = 0.716$). Together, our data from patients with chronic liver diseases revealed that disease progression was associated with a significant decrease in both hepatic and circulating FSAP, and the

reduced hepatic FSAP expression was closely linked to the increased inflammatory and pro-fibrotic mediator expression but not to hepatic cell death.

Characterization of FSAP^{-/-} mice

In order to functionally characterize the potential role of FSAP expression in liver disease progression, FSAP-deficient mice were generated and further subjected to experimental liver injury. Heterozygous crosses produced offspring of different genotypes in the normal expected Mendelian ratio under standard breeding conditions, in a specific pathogen-free barrier facility. Homozygous FSAP^{-/-} mice, up to 40 weeks of age, showed no obvious phenotype. Size and weight of mice as well as organ anatomy were normal. Blood cell counts were normal as were concentrations of various enzymes and metabolites, indicating normal liver and kidney function (data not shown). Using two different exon-spanning primer pairs no evidence for FSAP mRNA in the liver was found in FSAP^{-/-} mice (Supplementary Fig. 1A). Similarly, Western blotting with two different rabbit polyclonal antibodies

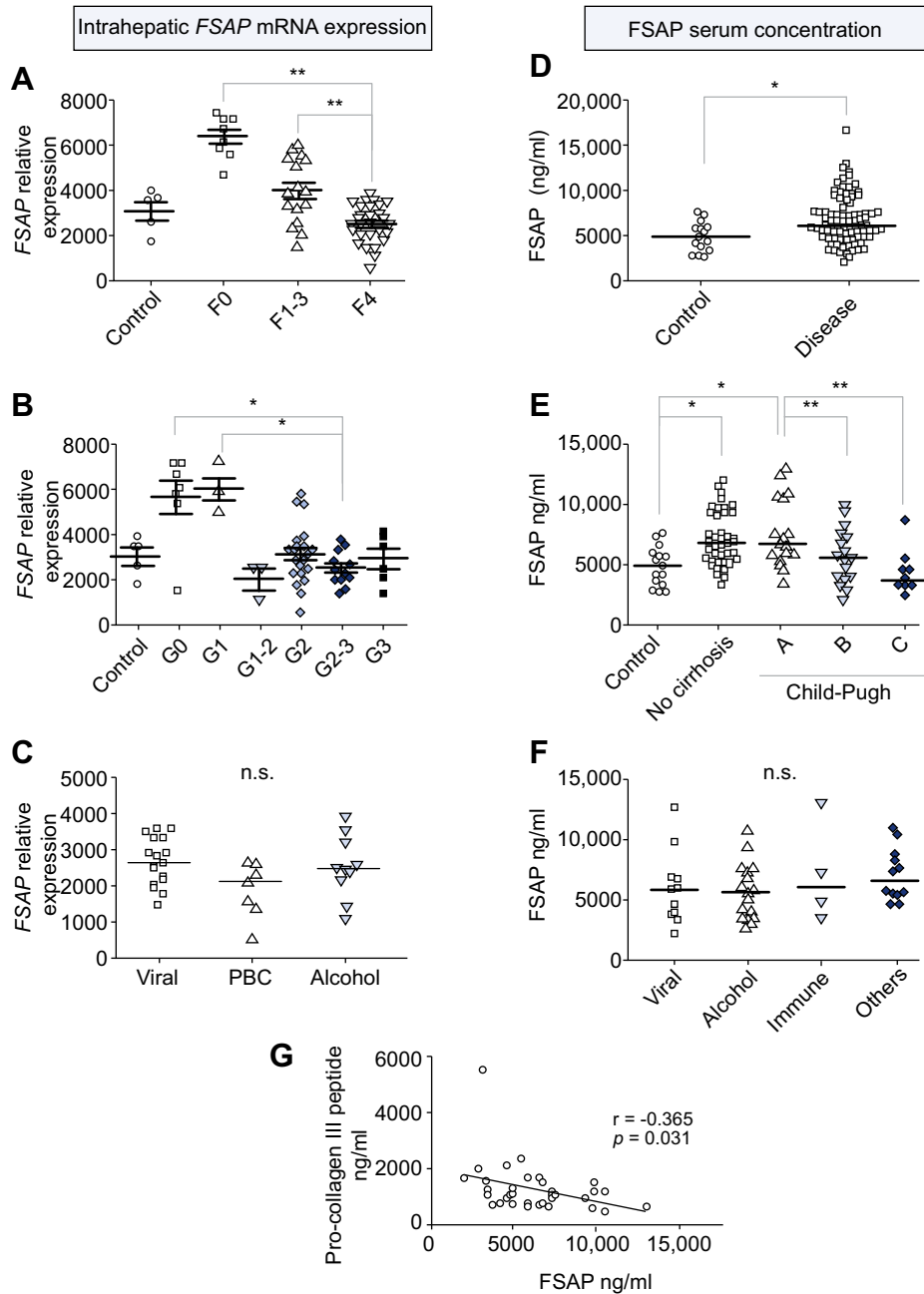


Fig. 1. Expression of FSAP mRNA in human liver fibrosis and systemic FSAP serum levels. (A–C) FSAP gene transcripts were measured by qRT-PCR in RNA isolated from human liver biopsies or explants, and normalized to β -actin as housekeeping gene. Individual relative expression (arbitrary values) and medians are indicated comparing FSAP expression in (A) stage of fibrosis (F0–F4), (B) grade of inflammation (G0–G3) and (C) etiology of underlying liver disease (viral, primary biliary cirrhosis (PBC), alcohol). (D–F) FSAP serum concentration was evaluated in a cohort of 85 patients with chronic liver disease and 15 healthy controls. Individual values and medians are depicted, comparing FSAP serum concentration according to (D) disease status, (E) stage of chronic liver cirrhosis stratified by the Child-Pugh score and (F) etiology (viral, alcohol, immune, others). Statistical significances are calculated using Mann–Whitney test (two groups) or Kruskal–Wallis test (>2 groups) and Dunn’s test for *post hoc* analysis (n.s., not significant; * $p < 0.05$; ** $p < 0.01$). HC, healthy controls; F, stage of fibrosis (ranging from 0 = no fibrosis to 4 = severe fibrosis/cirrhosis); G, grade of inflammation (ranging from 0 = no inflammation to 4 = severe inflammation); PBC, primary biliary cirrhosis. (G) Correlation of serum pro-collagen-III-peptide concentrations with FSAP serum levels. Statistical analysis was performed using Spearman-rank correlation test. r , correlation coefficient.

(in house preparations) showed that there was no FSAP protein in the circulation of $FSAP^{-/-}$ mice (Supplementary Fig. 1B). In an FSAP activity assay performed with immunocaptured FSAP, there was no activity in plasma of $FSAP^{-/-}$ mice (Supplementary Fig. 1C).

Mice with genetic deletion of FSAP display exacerbated liver fibrosis

To assess the functional relevance of FSAP in liver fibrosis, $FSAP^{-/-}$ and littermate WT mice were subjected to bile duct ligation (BDL), which is an established model of chronic liver injury. The

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Table 2. Correlation analysis of intrahepatic FSAP mRNA expression.

	FSAP relative expression	
	r	p value
Chemokines		
CCL2	-0.682	<0.001
CCL3	-0.536	<0.001
CCL5	-0.372	0.001
CCL20	-0.663	<0.001
CXCL5	-0.389	0.007
CXCL16	-0.268	0.034
CX3CL1	0.399	0.001
Chemokine receptors		
CCR1	-0.648	<0.001
CCR2	-0.488	<0.001
CCR5	-0.420	0.001
CCR6	-0.494	<0.001
CX3CR1	0.644	<0.001
Fibrosis		
TGF- β	-0.324	0.026
MMP-1	-0.416	0.004

Intrahepatic FSAP mRNA expression was analyzed by qRT-PCR and correlated with the mRNA expression of various chemokines, chemokine receptors, cytokines and metalloproteinases in 64 liver samples using a non-parametric correlation rank-test (r, Spearman's rank correlation coefficient).

mortality due to BDL was 6/11 for the WT group and 1/11 for the FSAP^{-/-} group, possibly demonstrating that FSAP has initially beneficial effects on the inflammatory response induced by surgery. Mice were sacrificed at 2 or 3 weeks after BDL, but only data for the 2-week time point are shown, since these tended to show more significant differences in the two mouse strains. Liver fibrosis was determined histologically and by hydroxyproline content, which is the amino acid most abundantly found in extracellular collagen matrices. FSAP^{-/-} mice showed a significantly increased fibrogenic response as determined by Sirius red positive areas (Fig. 2A and B) as well as hydroxyproline content (Fig. 2C). BDL in WT mice lowered FSAP mRNA in the liver and FSAP activity in the circulation, but the changes were not significant (data not shown).

In order to test the universality of these findings, we performed further experiments with the CCl₄ model of liver fibrosis. We found increased fibrosis as determined by Sirius red staining and also higher expression of the extracellular matrix protein collagen α 1 (Supplementary Fig. 2).

Liver injury and hepatic inflammation in mice without endogenous FSAP

Plasma levels of parameters indicating liver damage, such as bilirubin, AST, ALP, and ALT, were within the normal range in sham-operated control mice, but were highly elevated in mice with BDL ligation. There was no statistically significant difference in the two strains of mice with BDL (data not shown).

In FSAP^{-/-} mice with BDL, portal fields were more injured, as shown by an increase of connective tissue, proliferation of bile ducts, and an increased number of infiltrating neutrophils, upon

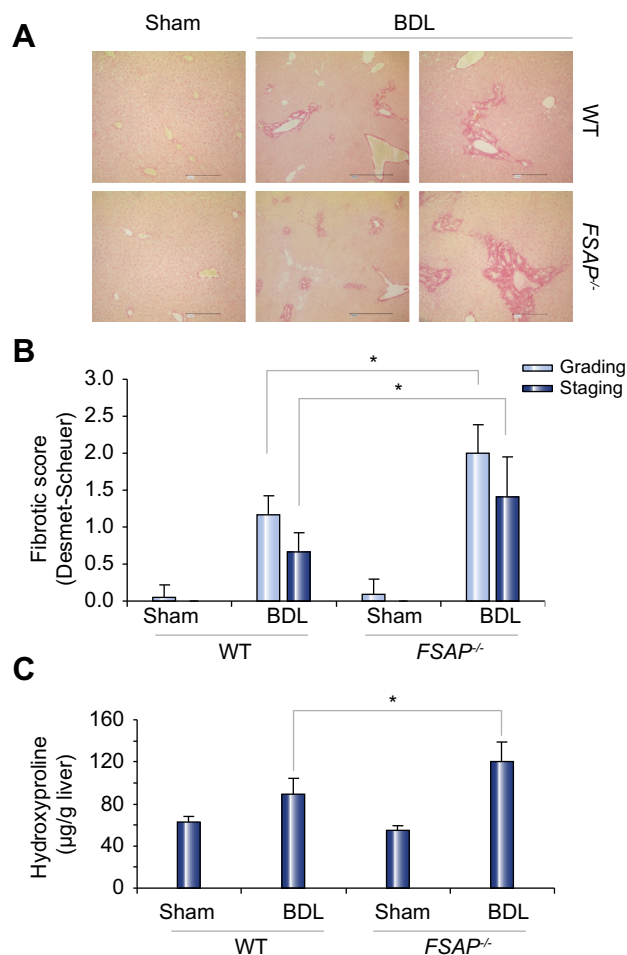


Fig. 2. Enhanced liver fibrosis in FSAP^{-/-} mice after BDL. (A) Two weeks after BDL, the livers of FSAP^{-/-} and WT mice were subjected to Sirius red staining to define the areas of fibrosis. (B) Sirius red staining was scored by a blinded observer (mean \pm SEM). (C) Fibrosis in the same mice was also quantitated by measurement of hydroxyproline. Statistical significance using ANOVA and Bonferroni multiple post test is shown (* p < 0.05). (This figure appears in color on the web.)

histomorphological analysis (Fig. 3). Necrosis of single liver epithelia or small cell groups was found. In FSAP^{-/-} mice with BDL, connective tissue deposits were preferentially found in portal areas. Frequently, portal fibrosis included establishment of septa, sometimes with confluence of portal areas and mild disturbance of the liver tissue architecture. Occasionally, fibers surrounding the bile duct proliferated, forming lamellar structures. In such cases, duct epithelia displayed aspects of epithelial atrophy.

Neutrophil and monocyte infiltration is a significant factor contributing to liver injury after BDL. No neutrophil or monocyte accumulation was observed in sham operated mice. However, in BDL mice, accumulation of neutrophils was found, preferentially localized in portal fields. The fibrotic portal areas were enlarged and displayed bile duct proliferation. In liver sinus, an increase of inflammatory cells, including monocytes, was visible. The cells were diffusely distributed or associated with damaged liver cells (liver cell necrosis) (Fig. 3). Detailed statistical analysis of neutrophil accumulation revealed no differences between WT and FSAP^{-/-} mice.

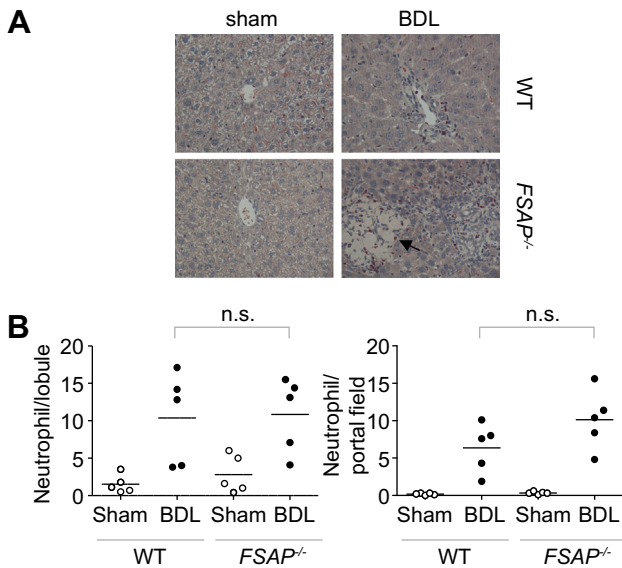


Fig. 3. Necroinflammatory changes in FSAP^{-/-} mice after BDL. (A) Two weeks after BDL, the livers of FSAP^{-/-} and WT mice were subjected to neutrophil staining with chloroacetate esterase. BDL-treated mice display fibrosis, influx of inflammatory cells, preferentially neutrophils (red), bile duct proliferates, and death of liver cells (arrow). (B) Quantification of neutrophil accumulation in a blinded manner does not show any statistically significant differences between WT and FSAP^{-/-} mice after BDL. n.s., not significant. (This figure appears in color on the web.)

Enhanced fibrosis is associated with hepatic stellate cell (HSC) activation

In the BDL model of liver fibrosis, there is an extensive activation of HSC, which was also analyzed. HSC activation was determined by measuring the expression of α -smooth muscle actin (α -SMA) by immunohistochemistry (Fig. 4A). Western blot and subsequent densitometric analysis indicated that there was a higher expression of α -SMA in FSAP^{-/-} mice compared to FSAP^{+/+} mice (Fig. 4B). The same pattern was also observed for fibronectin and collagen I as well as LCN, which are all markers of liver fibrosis or inflammation. Therefore, altered HSC activation correlated with enhanced fibrosis response in mice lacking FSAP.

Changes in expression of PDGF-B and its receptor PDGFR in mice

We previously showed that FSAP inhibits PDGF-BB-mediated activation of HSC [14,23] and that this is related to the proteolytic degradation of this growth factor [10]. The expression of PDGF-B and PDGFR mRNA was increased in both mouse strains after BDL ligation. Although the increase was more pronounced in FSAP^{-/-} mice, this was not statistically significant (Supplementary Fig. 3A). We extended these studies by measuring both proteins and phospho-PDGFR using Western blotting. PDGF-B expression at the protein level could not be detected using 3 different antibodies, but PDGFR expression was strongly increased in FSAP^{-/-} mice after BDL (Supplementary Fig. 3B). Phospho-PDGFR was also increased, indicating that a combination of higher expression of PDGF-B and its receptor led to a stronger receptor phosphorylation. Increased expression of PDGF-B and its lower proteolysis in FSAP^{-/-} mice would also contribute to the enhanced fibrosis.

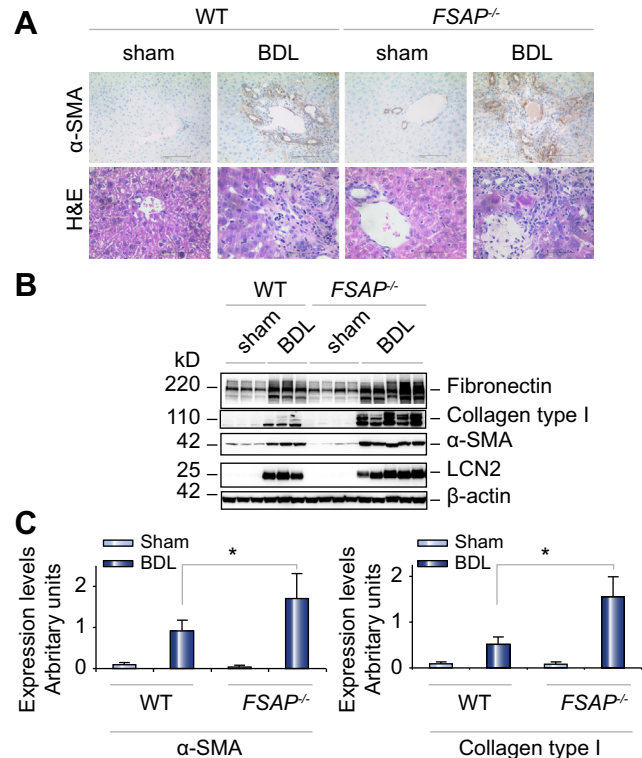


Fig. 4. Increased expression of α -SMA and other markers of HSC activation in FSAP^{-/-} mice with BDL. (A) BDL in FSAP^{-/-} mice leads to more fibrosis and HSC accumulation than in WT mice, as determined by immunostaining for α -SMA actin. For comparison, H&E staining is shown to illustrate infiltration of cells and tissue damage. (B) Western blot analysis of fibronectin, collagen I, α -SMA, and LCN-2 was performed to measure the quantitative difference between the two mouse strains. (C) Expression levels are normalized to β -actin by densitometric analysis. Statistical significance using ANOVA and Bonferroni multiple post test is indicated (* $p < 0.05$). (This figure appears in color on the web.)

Microarray-based comparison of gene expression profiles in FSAP^{-/-} and WT mice

To identify late molecular mechanisms associated with liver fibrosis, microarray analysis was performed on liver tissue, 2 weeks after BDL. In sham operated mice, using the cut-off of a 2-fold change, 212 genes were downregulated in FSAP^{-/-} mice compared to WT mice, while 248 genes were upregulated. With BDL, 233 genes were downregulated in FSAP^{-/-} mice compared to WT mice, while 256 genes were upregulated (Supplementary Table 2). For detailed information, the data have been deposited in the NCBI Geo repository (GEO, accession number GSE36066).

A functional grouping analysis of the 256 upregulated genes, after BDL in FSAP^{-/-} mice, revealed a significant enrichment of biological processes related to B-cell and T-cell immunity, innate immunity, receptor signaling, cell death, response to toxins and cell adhesion (Supplementary Fig. 4). Thus, analysis of the overall pattern of gene expression using gene ontology terms indicates that FSAP influences the gene expression pattern in relation to the immune system. Independently of the microarray data, various genes of interest in relation to liver fibrosis were further quantified by quantitative PCR. Compared to the WT-BDL group, in FSAP^{-/-} mice with BDL there was a lower expression of FSAP

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and *TIMP-4* and a higher expression of α -SMA and *TNF α* but no differences in *collagen I*, *IL-6*, and *IL-1* (data not shown).

Discussion

The Marburg I SNP of FSAP results in a protein with a lower enzymatic activity, and its association with enhanced liver fibrosis indicates that FSAP is involved in the aetiology of liver fibrosis in humans [9]. We herein report that FSAP expression in the human liver is reduced in conditions of advanced fibrosis and inflammation. Modeling this in an animal experiment, we demonstrate that FSAP-deficient mice exhibit enhanced fibrosis and inflammation, indicating that FSAP fulfils a protective function in the liver. This congruence of results from the human and mouse system strengthens our hypothesis that FSAP plays an important role in liver fibrosis.

The fact that FSAP gene transcription was lower in control livers from a non-liver disease background compared to early stages of fibrosis and inflammation, may indicate counter-regulatory mechanisms involving potentially beneficial properties of FSAP activation. However, this interpretation is subject to the assumption that the tumor-adjacent tissue is a valid control. The decreased hepatic FSAP expression inversely correlates with the expression of many chemokines and chemokine receptors that govern recruitment of monocytes (CCR1, CCL2/CCR2), T cells (CCL5/CCR5, CCR6/CCL20) and B cells (CXCR5) to the injured liver. This would indirectly suggest that FSAP is involved in the regulation of inflammation, a contention which is also supported by microarray analysis indicating increased gene expression in the immune/inflammatory pathways in FSAP deficient mice.

Besides local regulation, systemic FSAP levels are also tightly associated with the stage of liver disease. Decreased FSAP serum concentrations correlate well with clinical progression of liver cirrhosis, and therefore might be useful to assess the severity of liver decompensation. Serum FSAP inversely correlates with circulating markers of fibrosis, such as pro-collagen-III peptide, but not with markers of hepatocyte cell death, such as GLDH and ALT. This indicates a specific regulation of FSAP expression in fibrosis, which potentially modifies disease progression, and is not just a consequence of loss of hepatocyte function. It should be noted that there is no information available to date about factors that influence FSAP turnover in the circulation.

We chose to apply surgical ligation of the common bile duct to induce a reliable and consistent experimental murine model of hepatic fibrosis. In the BDL model, the ligation of the biliary duct leads to the damage of the biliary epithelium and progressively to the destruction of hepatocytes [24]. This in turn leads to the activation of the inflammatory response that results in leukocyte recruitment [25]. The extent of injury also depends on factors, such as bile salt homeostasis [26], and finally there are manifested fibrotic changes. All fibrosis-related parameters are increased to a greater extent in *FSAP*^{-/-} mice. These results could be recapitulated in another mouse model of liver fibrosis based on CCL₄ application.

Subsequently, we concentrated on the mechanisms that lead to enhanced hepatic fibrosis in animals lacking FSAP. It is well accepted that fibrogenesis is a process that is triggered by many soluble factors. Among the numerous pro-fibrogenic mediators, a functional hierarchy might exist, which points to transforming growth factor- β 1 (TGF- β 1) and PDGF-BB as the most effective

ones. While PDGF mainly triggers cellular activation and proliferation, TGF- β prominently induces the synthesis of extracellular matrix components including collagen [27]. We previously showed that FSAP can inhibit the activity of growth factors such as PDGF-BB, and that this effect is specific for PDGF-BB but not PDGF-AA and that the Marburg I isoform of FSAP cannot mediate this cleavage [28]. Considering the previous observations regarding FSAP in the vascular system relating to neointima formation [10] and activation of HSC [9], we predicted that in the BDL model we would observe maximal differences in this final fibrotic phase of disease. Indeed, in *FSAP*^{-/-} mice, all markers of fibrosis such as deposition of fibronectin and collagen in the matrix, hydroxyproline levels and markers of HSC activation, such as α -SMA and markers of hepatic inflammation, including LCN-2, were elevated to a greater extent. The increased fibrosis in *FSAP*^{-/-} mice could be due to increased levels of PDGF-BB that in turn lead to higher levels of PDGFR activation.

In the BDL model, the second wave of changes is related to cellular damage, increased proliferation of bile ducts, portal fibrosis, and infiltration of leucocytes, in the first line neutrophils. In *FSAP*^{-/-} mice, the extent of neutrophil infiltration is similar to that of WT mice. However, transcriptional profiling analysis also indicates that the lack of FSAP influences pathways related to the immune system. Analysis of the upregulated genes in *FSAP*^{-/-} mice in BDL, showed an upregulation of genes related to T- and B-cell immunity as well as innate immunity, and more general processes such as receptor signaling, cell death and cell adhesion.

It is well known that the inflammatory response in the injured liver is tightly regulated and characterized by distinct recruitment and activation of various cells from the innate (neutrophils, monocytes/macrophages, NKT and gamma-delta T cells) and adaptive (mainly T lymphocytes) immunity [29]. Further studies are needed to dissect the exact mechanisms by which FSAP controls activation of various immune cells in liver injury.

Hepatocyte damage by multiple mechanisms can lead to the release of various intracellular components generally termed as DAMPs or danger-associated molecular patterns. Some of the DAMPs include molecules such as histones, nucleosomes and nucleic acids, which can activate FSAP from the zymogen form to the active enzyme [11,12]. This activation could in turn dampen the inflammatory system, which would have the dual effect of protecting the host and weakening the host response, in removing and recovering from the damage-causing factors. At least in human carriers of Marburg I SNP and in *FSAP*^{-/-} mice, the prevailing consequence seems to be a loss of protective effect in liver fibrosis/inflammation. Among the various known possibilities: which process is regulated by FSAP that in turn alters fibrosis progression? We find evidence of changes in known pathways based on PDGF, and microarray data provide a novel concept for evaluating the role of FSAP in inflammation. Regulation of the hemostasis pathway and PAR may also play a contributing role. Although it is unlikely that the exogenous FSAP protein could be used as a therapeutic agent to treat liver fibrosis, it is possible that agents could be developed that would specifically activate circulating FSAP zymogen, which would then achieve the same final result.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jhep.2012.09.007>.

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