Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

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.

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

Keywords: Liver fibrosis; HABP2; Marburg I SNP.
Received 9 May 2012; received in revised form 8 August 2012; accepted 3 September 2012; available online 16 September 2012

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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 (CCl_4) leads to FSAP activation in mice, presumably through induction of cell death in the liver [13]. We have demonstrated that CCl_4-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 (Table1 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 (CX_3CL1 and its receptor CX_3CR1), 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 (Table1). Despite a slight increase when comparing all patients to normal