Fibromodulin, an Oxidative Stress-Sensitive Proteoglycan, Regulates the Fibrogenic Response to Liver Injury in Mice

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BACKGROUND & AIMS: Collagen I deposition contributes to liver fibrosis, yet little is known about other factors that mediate this process. Fibromodulin is a liver proteoglycan that regulates extracellular matrix organization and is induced by fibrogenic stimuli. We propose that fibromodulin contributes to the pathogenesis of fibrosis by regulating the fibrogenic phenotype of hepatic stellate cells (HSCs). **METHODS:** We analyzed liver samples from patients with hepatitis C–associated cirrhosis and healthy individuals (controls). We used a coculture model to study interactions among rat HSCs, hepatocytes, and sinusoidal endothelial cells. We induced fibrosis in livers of wild-type and *Fmod*$^{-/-}$ mice by bile duct ligation, injection of CCl_4, or administration of thioacetamide. **RESULTS:** Liver samples from patients with cirrhosis had higher levels of *fibromodulin* messenger RNA and protein than controls. Bile duct ligation, CCl_4, and thioacetamide each increased levels of fibromodulin protein in wild-type mice. HSCs, hepatocytes, and sinusoidal endothelial cells produced and secreted fibromodulin. Infection of HSCs with an adenovirus that expressed fibromodulin increased expression of collagen I and α–smooth muscle actin, indicating increased activation of HSCs and fibrogenic potential. Recombinant fibromodulin promoted proliferation, migration, and invasion of HSCs, contributing to their fibrogenic activity. Fibromodulin was sensitive to reactive oxygen species. HepG2 cells that express cytochrome P450 2E1 produced fibromodulin, and HSCs increased fibromodulin production in response to pro-oxidants. In mice, administration of an antioxidant prevented the increase in fibromodulin in response to CCl_4. Coculture of hepatocytes or sinusoidal endothelial cells with HSCs increased the levels of reactive oxygen species in the culture medium, along with collagen I and fibromodulin proteins; this increase was prevented by catalase. Fibromodulin bound to collagen I, but the binding did not prevent collagen I degradation by matrix metalloproteinase 13. Bile duct ligation caused liver fibrosis in wild-type but not *Fmod*$^{-/-}$ mice. **CONCLUSIONS: Fibromodulin levels are increased in livers of patients with cirrhosis. Hepatic fibromodulin activates HSCs and promotes collagen I deposition, which leads to liver fibrosis in mice.**

Keywords: Mouse Model; Liver Disease; Intercellular Communication; Fibers.

Although different cell types contribute to the increase in fibrillar collagen I levels during hepatic fibrogenesis, they all undergo a common process of differentiation and acquisition of a classic myofibroblast-like phenotype. Portal fibroblasts play a significant role in the development of portal fibrosis; however, hepatic stellate cells (HSCs) play a major role in collagen I deposition when hepatocellular injury is concentrated within the liver lobule and sinusoids.

Efforts to understand the pathogenesis of liver fibrosis focus on events that lead to the activation of profibrogenic cells and to early accumulation of scar (ie, fibrillar collagen I) to identify therapeutic targets to prevent its onset, slow its progression, or help its resolution.[1] Stimuli initiating activation, proliferation, migration, and invasion of HSCs derive from injured hepatocytes,[2,3] Kupffer cells,[4,5] sinusoidal endothelial cells (SECs),[6] and inflammatory cells[7] in addition to rapid changes in extracellular matrix (ECM) composition.[1]

Synthesis of collagen I is regulated by the ECM itself, and even though the basement membrane matrix preserves quiescence of HSCs, collagen I further enhances HSC activation in a paracrine manner. Increasingly, reactive oxygen species (ROS) are viewed as candidate drivers of HSC activation and collagen I up-regulation[3,4,8,9]; however, downstream mediators for the ROS effects on the activation of HSCs and the increase in collagen I levels require further study.

Synthesis and secretion of collagen I are a major focus of interest, during the onset of liver fibrosis; nevertheless, there is also considerable deposition of proteoglycans, glycoproteins, and glycosaminglycans into the space of Disse.[10] Fibromodulin (FMOD) is a small leucine-rich proteoglycan regulating ECM organization, which has been described as essential for tissue repair in multiple organs.[11] This family of proteoglycans is involved in cell metabolism via binding to growth factors as well as in matrix organization by interacting with various collagens.[12–15] *Fmod*$^{-/-}$ mice develop abnormal collagen fibril architecture in connective tissues in addition to showing increased age-dependent osteoarthritis and degenerative changes in cartilage structure[14,16,17]; thus, FMOD plays a significant role

Abbreviations used in this paper: α-SMA, α–smooth muscle actin; BDL, bile duct ligation; ECM, extracellular matrix; FMOD, fibromodulin; HSC, hepatic stellate cell; IHC, immunohistochemistry; rFMOD, recombinant fibromodulin; ROS, reactive oxygen species; SEC, sinusoidal endothelial cell; TAA, thioacetamide; WT, wild-type.

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in defining tissue integrity.[14] Moreover, in vivo both collagen and FMOD are likely to have an important functional role in tissues where they are coexpressed due to potential physical interaction between both proteins.[18]

Thus far, there is no information on whether FMOD is present in the liver, which cells express it, and where specifically it is induced upon the establishment of hepatic injury.[19] In addition, little is known about its potential role in the development of liver fibrosis,[19] whether induction of FMOD in liver cells could contribute to the profibrogenic potential of HSCs, and the molecular mechanism involved in these events.

Identifying if FMOD stimulates the profibrogenic response to hepatic damage could be central for understanding the pathogenesis of liver fibrosis. Thus, the aim of this study was to explore how FMOD regulates the HSC profibrogenic phenotype, a key event in liver fibrosis, as well as the consequences of in vivo ablation of the *Fmod* gene for the fibrogenic response to liver injury under a profibrogenic stimuli. To investigate this, we used a coculture model that resembles aspects of the interplay between liver cells in addition to inducing liver fibrosis in vivo using wild-type (WT) and *Fmod*$^{-/-}$ mice. The results suggest that hepatic FMOD, sensitive to oxidant stress, contributes to HSC activation and collagen I deposition, thus participating in the pathogenesis of liver fibrosis.

Materials and Methods

Cell Treatments

Rat HSCs (250,000 cells/well) were seeded on 6-well plates in Dulbecco's modified Eagle medium/F12 with 10% fetal bovine serum. Primary cells were cultured using Dulbecco's modified Eagle medium/F12 for 4 to 7 days, which was replaced by serum-deprived Dulbecco's modified Eagle medium/F12 before treatment with 50 nmol/L endotoxin-free human recombinant FMOD (rFMOD) for 24 hours (donated by Dr Ake Oldberg, Lund University, Lund, Sweden). Cells were infected with Ad-LUC or Ad-FMOD at a multiplicity of infection of 50 for 48 hours. The adenoviruses were a gift from Dr David T. Curiel (Washington University, St Louis, MO). H_2O_2 (25 μmol/L) and catalase (200 U/mL) were added to the cells for 24 hours (both from Sigma, St Louis, MO).

Mice

Fmod$^{-/-}$ mice and their WT littermates (C57BL/6J) were obtained from Dr Marian Young (National Institutes of Health, Bethesda, MD).[20,21] These mice were backcrossed for at least 10 generations. Colonies were established by intercrossing *Fmod*$^{+/-}$ mice, and littermates were used in all experiments. *Fmod*$^{-/-}$ mice have normal heart, liver, lung, skin, and cartilage; however, they show abnormal tissue organization, collagen fiber bundles, and fiber architecture.[22]

Induction of Liver Injury

Ten-week-old male WT mice and their *Fmod*$^{-/-}$ littermates were used in all experiments. To induce liver injury, 3 in vivo models were used. In the first model, cholestasis was induced by placing a ligature around the common bile duct while controls were sham operated. All mice were killed 3 weeks later. In the second model, mice were intraperitoneally injected twice a week with 0.5 mL/kg body wt CCl_4 (Sigma) or an equal volume of mineral oil for 1 month and killed 48 hours after the last injection of CCl_4. In the third model, mice were treated with thioacetamide (TAA) (300 mg/L; Sigma) in the drinking water or received an equal volume of water for 4 months. Mice were killed 48 hours after withdrawal of TAA. Blood was collected by orbital venous plexus bleeding. Each liver was excised into fragments by using the same liver lobe for biochemical assays and paraffin embedding for staining. All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health.

Human Samples

Dr Andrea D. Branch (Mount Sinai School of Medicine, New York, NY) provided the human liver protein lysates and RNA from resections from de-identified controls and subjects with biopsy-proven stage 3 hepatitis C virus (HCV) cirrhosis. Samples were scored according to the Scheuer/Ludwig Batts classification.[23,24] These samples were exempt from institutional review board approval because no patient information was disclosed.

Pathology

In all experiments, the left liver lobe was excised and fixed in 10% neutral-buffered formalin and processed into paraffin sections for H&E or immunohistochemistry (IHC) and scoring by the Brunt classification. Portal and lobular inflammation were noted to be lymphocytes present in the lobules or portal areas and were scored as follows: 1 = rare foci; 2 = up to 5 foci; 3 = >5 foci. Centrilobular necrosis and parenchymal necrosis were each separately scored. The scores for centrilobular necrosis were as follows: 1 = hepatocyte necrosis affecting only zone 3; 2 = in addition to zone 3 necrosis, occasional bridging necrosis was seen; 3 = pronounced bridging and confluent necrosis. Parenchymal necrosis was noted to be spotty necrosis or apoptosis in zones 2 and 1. The scores for parenchymal necrosis were as follows: 1 = ≤1 focus; 2 = 5–10 foci; 3 = ≥10 foci at 100×. Ductular reaction was noted to be proliferation of bile ductules at the margins of the portal tracts, and the score was as follows: 1 = rare bile ductules present; 2 = irregular buds of bile ductules affecting some portal tracts; 3 = when bile ductules are more prominent and affect the majority of portal areas and/or strings of

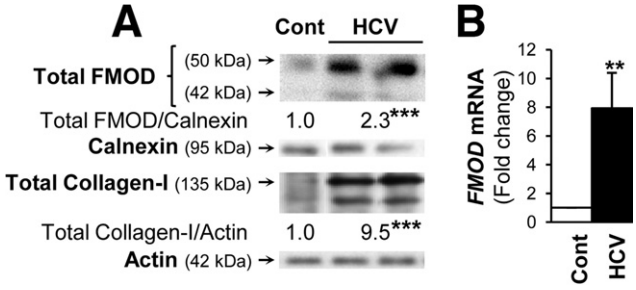

Figure 1. FMOD expression increases in patients with HCV-induced cirrhosis. (*A*) Patients with biopsy-proven stage 3 HCV-induced cirrhosis showed an increase in FMOD and collagen I protein levels compared with healthy individuals. Likewise, there was a significant elevation in *FMOD* mRNA levels in patients with stage 3 HCV-induced cirrhosis compared with healthy liver explants. (*B*) The *bar graph* represents the fold change in *FMOD* mRNA normalized by that of *GAPDH*. n = 5; $^{**}P < .01$ and $^{***}P < .001$ for HCV-induced cirrhosis vs healthy explants.