Animal models for the study of liver fibrosis: new insights from knockout mouse models

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Hayashi H, Sakai T. Animal models for the study of liver fibrosis: new insights from knockout mouse models. Am J Physiol Gastrointest Liver Physiol 300: G729–G738, 2011. First published February 24, 2011; doi:10.1152/ajpgi.00013.2011.—Fibrosis arises as part of a wound-healing response that maintains organ structure and integrity following tissue damage but also contributes to a variety of human pathologies such as liver fibrosis. Liver fibrosis is an abnormal response of the liver to persistent injury with the excessive accumulation of collagenous extracellular matrices. Currently there is no effective treatment, and many patients end up with a progressive form of the disease, eventually requiring a liver transplant. The clarification of mechanisms underlying pathogenesis of liver fibrosis and the development of effective therapy are of clinical importance. Experimental animal models, in particular targeted gene knockouts (loss of function) in mice, have become a powerful resource to address the molecular mechanisms or significance of the targeted gene in hepatic functions and diseases. This review will focus on the recent advances in knowledge obtained from genetically engineered mice that provide novel insights into the pathophysiology of liver fibrosis.

Liver fibrosis; knockout mice; extracellular matrix

Pathogenesis of Liver Fibrosis

Liver fibrosis is defined as an abnormal response of the liver to persistent injury, characterized by the excessive accumulation of collagenous extracellular matrices (ECMs), and therefore involves both wound healing and fibrotic processes. After an injury, parenchymal cells regenerate and replace the necrotic or apoptotic cells, which is associated with an inflammatory response and deposition of ECM. This process requires both a well-orchestrated proliferation of cells and the reconstruction of ECM. If the injury persists, the damaged tissues/organs undergo substitution by overabundant ECM and suffer from extensive, pathological fibrosis. Thus, liver fibrosis is of great clinical importance, since normal liver architecture is disrupted and liver function is ultimately impaired. There are several major causes of liver fibrosis, as described below.

Viral hepatitis fibrosis. This is caused by chronic hepatitis B, C, or D. Chronic hepatitis C virus infection affects more than 170 million individuals and causes 300,000 deaths annually in the world from cirrhosis and hepatocellular carcinoma (80). Histologically, the liver shows lymphocyte infiltration, piecemeal necrosis of hepatocytes, and fibrosis in the perportal areas.

Alcoholic liver fibrosis. This type is caused by acetaldehyde, an oxidized metabolite of alcohol. The incidence is positively related to the amount of alcohol consumption in Western countries. The total cases of alcoholic fibrosis in the United States are about three times higher than the number of those arising from hepatitis C. The histological features of alcohol-induced hepatic injury vary, depending on the extent and stage: steatosis (fatty change), lobular inflammation, periportal fibrosis, Mallory bodies, nuclear vacuolation, bile duct proliferation, and fibrosis or cirrhosis (60).

Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. Nonalcoholic fatty liver disease (NAFLD) is now the most common cause of chronic liver disease among children and adults in the United States (95, 100). NAFLD is a clinicopathological entity defined as a presence of hepatic steatosis in individuals who drink little or no alcohol. NAFLD represents a spectrum of liver disease ranging from bland steatosis to nonalcoholic steatohepatitis (NASH). NASH is a progressive form of liver disease that leads to advanced fibrosis. Studies have found that 26–37% of patients with NASH demonstrate progression of fibrosis over time, for periods up to 5.6 years, with up to 9% progressing to cirrhosis (1, 28, 34, 62). Histologically, NASH is characterized by macrovesicular steatosis (the fat globules vary in size from very small to nearly filling the hepatocyte) and ballooning degeneration of hepatocytes with or without Mallory bodies, with or without fibrosis (55).

Biliary fibrosis. Medicine defines two types: primary and secondary biliary fibrosis. Primary biliary hepatic fibrosis is an autoimmune disorder in which fibrosis is caused by chronic intrahepatic bile retention. It often affects females in the age range of 40 to 60 years old. Histologically, the fibrosis appears around the microbile duct and perportal areas. Secondary biliary fibrosis occurs following the obstruction of bile ducts, which results in the progressive fibrosis in perportal areas.
**Parasitic infection-mediated fibrosis.** This type is frequent in developing countries and is often caused by schistosomiasis. The principal immune response is directed against the parasite’s eggs and leads to a fibrotic response, whereas the adult worm is weakly immunogenic.

**Genetic Studies in Rodents**

**Models of experimental liver fibrosis.** Several models of experimental liver fibrosis have been established (Table 1).

**CHEMICALLY INDUCED FIBROSIS USING HEPATOTOXIC AGENTS.** Carbon tetrachloride (CCl4) is the most commonly used liver-damaging agent to induce liver fibrosis. The trichloromethyl radical, a metabolite produced by cytochrome P-450 in hepatocytes, leads to lipid peroxidation and membrane damage, which results in a reversible acute centrilobular liver necrosis.

**CHOLESTATIC FIBROSIS BY BILE DUCT LIGATION.** Bile duct ligation is a convenient and well-studied experimental disease model that induces biliary fibrosis and cirrhosis such as extra-hepatic biliary atresia and primary sclerosing cholangitis.

**IMMUNOLOGICALLY MEDIATED FIBROSIS.** Concanavalin A is commonly used to induce immune-mediated liver fibrosis. Histologically, the induced liver fibrosis resembles human chronic hepatitis (54, 63). *Schistosoma mansoni* infection is another immunologically mediated fibrosis model (19).

**EXPERIMENTAL LIVER FIBROSIS USING TRANSGENIC MICE.** Over the past decade, targeted gene knockouts (loss of function) in mice have become a powerful strategy to address the basis of mono- and polygenic disorders. A great advance is the targeting of stable or inducible gene disruption exclusively to the liver using liver-specific or cell type-specific gene promoters such as albumin to target hepatocytes. The use of these liver-specific promoters in the Cre-loxP system not only permits the conditional expression and silencing of genes in the liver but also makes it possible to control the temporal expression/silencing of genes by fusing Cre with a mutant estrogen receptor (Cre-ERT2) in which Cre recombinase is induced by the injection of the estrogen analog tamoxifen (44). Considerable data on candidate genes for hepatic fibrogenesis have been accumulated through targeted gene disruption in mice (Table 2).

**Target signaling cascades/molecules for liver fibrosis.** Hepatocyte apoptosis/necrosis. Hepatocytes are damaged by hepatotoxic reagents, including alcohol, bile acids, and viral infection. Upon tissue damage, injured hepatocytes release reactive oxygen species (ROS) and fibrogenic mediators, which induce recruitment of white blood cells. Damaged hepatocytes that undergo apoptosis can be phagocytosed by macrophages and Kupffer cells, which stimulates the fibrogenic actions of liver myofibroblasts (15). An interesting observation is that DNA from apoptotic hepatocytes can act as a mediator of hepatic stellate cell (HSC) differentiation and inhibits platelet-derived growth factor-mediated chemotaxis via Toll-like receptor 9 in vitro, suggesting that such an apoptotic hepatocyte DNA provides a stop signal to retain HSCs at sites of cellular apoptosis (114). Activation of two alternative mechanisms can result in hepatocyte apoptosis: a death receptor-mediated pathway or an intrinsic organelle-dependent pathway (27). The hepatocyte damage mediated by a death receptor is a common mechanism for apoptosis, and it requires activation of Fas/CD95 or tumor necrosis factor (TNF) receptor-1. Fas-deficient mice show decreased fibrogenesis after bile duct ligation (17). In contrast, hepatocyte-specific Bcl-xL (antiapoptotic member of the Bcl-2 family)-deficient mice show spontaneous and continuous apoptosis in hepatocytes and develop liver fibrosis at an advanced age (103). The intrinsic organelle-dependent pathway is caused by lysosomal permeabilization and the release of cathepsin B into the cytoplasm, which causes mitochondrial damage and subsequent hepatocyte death. Indeed, in cathepsin B-null mice, hepatocyte apoptosis, liver damage, and liver fibrosis are attenuated after bile duct ligation (16). CCAAT/enhancer-binding protein homologous protein (CHOP), also known as growth arrest- and DNA damage-inducible gene 153, is a transcriptional regulator induced by endoplasmic reticulum (ER) stress and is a key factor in the ER stress-mediated apoptotic pathway. CHOP-deficient mice have been shown to be resistant to apoptosis in various disease models. In the cholestasis-induced liver fibrosis model, CHOP-null mice show reduced hepatocyte cell death and subsequent liver fibrogenesis (104). Thus, apoptotic signals in the injured liver play an important role in the progression of liver fibrosis (Fig. 1).

**PROFIBROGENIC GROWTH FACTORS.** Transforming growth factor-β (TGF-β) regulates a wide variety of cellular processes, including apoptosis of hepatocytes, activation and recruitment of inflammatory cells into injured liver, and transdifferentiation of some liver-resident cells, such as quiescent HSCs, into

**Table 1. Hepatotoxic agents for in vivo models of liver fibrosis**

<table>
<thead>
<tr>
<th>Agents</th>
<th>Effects</th>
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<tbody>
<tr>
<td>Chemically induced fibrosis</td>
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<tr>
<td>Carbon tetrachloride (1 ml/kg body wt injected ip 2 times weekly from 4 to 12 wk) (21)</td>
<td>Centrilobular hepatocyte death</td>
</tr>
<tr>
<td>Thioacetamide (300 mg/l of drinking water or 200 mg/kg of body wt injected ip 2 or 3 times/wk for up to 12 wk) (61)</td>
<td>Centrilobular hepatocyte death</td>
</tr>
<tr>
<td>Dimethyl nitrosamine</td>
<td>Hemorrhagic centrilobular necrosis, destruction of sinusoidal endothelial cells leads to coagulation</td>
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<tr>
<td>Cholestatic fibrosis</td>
<td></td>
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<tr>
<td>Bile duct ligation</td>
<td>Periportal hepatocyte death</td>
</tr>
<tr>
<td>Immunologically mediated fibrosis</td>
<td></td>
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<tr>
<td>Concavanalin A (up to 20 mg/kg of body wt injected iv 1 time/wk for up to 20 wk) (106)</td>
<td>Centrilobular and perisinusoidal fibrosis</td>
</tr>
<tr>
<td><em>Schistosoma mansoni</em> (exposure percutaneously to cercariae of <em>S. mansoni</em>) (97)</td>
<td>Granuloma formation and periportal fibrosis</td>
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i.p., intraperitoneally; i.v., intravenously.

AJPGI-Gastrointest Liver Physiol • VOL 300 • MAY 2011 • www.ajpgi.org
collagen-producing myofibroblasts (6, 88). Three TGF-β isoforms (β1, β2, and β3) have been identified, but only TGF-β1 is linked to liver fibrogenesis (35). Investigators studying conditional TGF-β1 transgenic mice using the tetracycline regulatory system (5, 31) have achieved elegant observations: although the overexpression of TGF-β1 spontaneously induces HSC activation and liver fibrosis without any treatments, the switching off of TGF-β production in the advanced stages of liver fibrosis results in the reversal of fibrosis (108).

TGF-β is secreted in a biologically inactive (latent) form in a complex with TGF-β latency-associated protein and latent TGF-β-binding proteins. The tissue concentration of these latent complexes is maintained at a constant level (84). In response to injury, local latent TGF-β complexes are converted into active TGF-β. There are several mechanisms for activation, such as via chaotropic agents, proteases, integrins (αvβ6, αvβ8), and thrombospondin-1, all of which are likely to be tissue specific (3, 42). The expression level of integrin αvβ6 is specifically increased on cholangiopithelial cells after bile duct ligation, and, in integrin β6-null mice, periductal fibrogenesis is attenuated with decreased TGF-β signaling (112).

### Table 2. Genetic factors associated with liver fibrogenesis in knockout mouse models

<table>
<thead>
<tr>
<th>Disrupted Gene and Effects</th>
<th>Agents to Induce Fibrosis</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Increasing liver fibrogenesis</strong></td>
<td></td>
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<tr>
<td>IL-10</td>
<td>Carbon tetrachloride</td>
<td>Streek et al. (101)</td>
</tr>
<tr>
<td>gp130</td>
<td>Carbon tetrachloride</td>
<td>Streek et al. (101)</td>
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<tr>
<td>SOCS3</td>
<td>Concanaualin A</td>
<td>Ogata et al. (76)</td>
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<tr>
<td>SOCS1 (±)</td>
<td>Dimethylhydrazine</td>
<td>Yoshida et al. (120)</td>
</tr>
<tr>
<td>Stat5</td>
<td>Carbon tetrachloride</td>
<td>Hosui et al. (40)</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>Carbon tetrachloride</td>
<td>Takehara et al. (103)</td>
</tr>
<tr>
<td>Fibroblast growth factor receptor 4</td>
<td>Carbon tetrachloride</td>
<td>Louis et al. (64)</td>
</tr>
<tr>
<td>Tissue-type plasminogen activator</td>
<td>Carbon tetrachloride</td>
<td>Yu et al. (123)</td>
</tr>
<tr>
<td>Adipocytokine</td>
<td>Carbon tetrachloride</td>
<td>Hsiao et al. (41)</td>
</tr>
<tr>
<td>Telomerase RNA</td>
<td>Carbon tetrachloride</td>
<td>Kamada et al. (51)</td>
</tr>
<tr>
<td>β5</td>
<td>Carbon tetrachloride</td>
<td>Rudolph et al. (85)</td>
</tr>
<tr>
<td>Cannabinoid receptor type 2</td>
<td>Carbon tetrachloride</td>
<td>Krizhanovsky et al. (56)</td>
</tr>
<tr>
<td>Angiotensin II type 2 receptor</td>
<td>Carbon tetrachloride</td>
<td>Julien et al. (50)</td>
</tr>
<tr>
<td>Early growth response-1</td>
<td>Carbon tetrachloride</td>
<td>Nabeshima et al. (74)</td>
</tr>
<tr>
<td><strong>Decreasing liver fibrogenesis</strong></td>
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<tr>
<td>TNF-α</td>
<td>Bile duct ligation</td>
<td>Gabele et al. (29)</td>
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<tr>
<td>TNF receptor type 1</td>
<td>Carbon tetrachloride</td>
<td>Sudo et al. (102)</td>
</tr>
<tr>
<td>TNF receptor 1 and 2</td>
<td>Bile duct ligation</td>
<td>Simeonova et al. (96)</td>
</tr>
<tr>
<td>Fas</td>
<td>Bile duct ligation</td>
<td>Canbay et al. (17)</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>Bile duct ligation</td>
<td>Canbay et al. (16)</td>
</tr>
<tr>
<td>CHOP</td>
<td>Bile duct ligation</td>
<td>Tamaki et al. (104)</td>
</tr>
<tr>
<td>p21</td>
<td>Bile duct ligation</td>
<td>Lunz III et al. (65)</td>
</tr>
<tr>
<td>IL-1 receptor</td>
<td>Thioacetamide</td>
<td>Gieling et al. (30)</td>
</tr>
<tr>
<td>IL-13</td>
<td><em>Schistosoma mansoni</em> infection</td>
<td>Kaviratne et al. (53)</td>
</tr>
<tr>
<td>Toll-like receptor 4</td>
<td>Carbon tetrachloride/bile duct ligation/thioacetamide</td>
<td>Seki et al. (93)</td>
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<tr>
<td>CD14</td>
<td>Bile duct ligation</td>
<td>Isayama et al. (47)</td>
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<tr>
<td>LPS-binding protein</td>
<td>Bile duct ligation</td>
<td>Isayama et al. (47)</td>
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<tr>
<td>Integrin αvβ6</td>
<td>Bile duct ligation</td>
<td>Wang et al. (112)</td>
</tr>
<tr>
<td>Smad3</td>
<td>Dimethylhydrazine</td>
<td>Latella et al. (57)</td>
</tr>
<tr>
<td>NADPH oxisdase (p73iso)</td>
<td>Bile duct ligation</td>
<td>Bataller et al. (10)</td>
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<tr>
<td>Angiotensin II type 1A receptor</td>
<td>Carbon tetrachloride</td>
<td>Kanno et al. (52)</td>
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<tr>
<td>Galactin 3</td>
<td>Carbon tetrachloride</td>
<td>Yang et al. (119)</td>
</tr>
<tr>
<td>Cannabinoid receptor type 1</td>
<td>Carbon tetrachloride/bile duct ligation/thioacetamide</td>
<td>Henderson et al. (36)</td>
</tr>
<tr>
<td>Fibroblast growth factor 1 and 2</td>
<td>Carbon tetrachloride</td>
<td>Gabele et al. (29)</td>
</tr>
<tr>
<td>Leptin</td>
<td>Carbon tetrachloride</td>
<td>Leclercq et al. (59)</td>
</tr>
<tr>
<td>Complement 5</td>
<td>Thioacetamide</td>
<td>Honda et al. (39)</td>
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<tr>
<td>Tenascin C</td>
<td>Carbon tetrachloride</td>
<td>Hillebrand et al. (37)</td>
</tr>
<tr>
<td>CC chemokine receptor 1, 5</td>
<td>Carbon tetrachloride/bile duct ligation</td>
<td>Seki et al. (91)</td>
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<tr>
<td>CC chemokine receptor 2</td>
<td>Thioacetamide</td>
<td>Seki et al. (92)</td>
</tr>
<tr>
<td>Matrix metalloproteinase 12</td>
<td><em>Schistosoma mansoni</em> infection</td>
<td>Bonacchi et al. (13)</td>
</tr>
<tr>
<td>Matrix metalloproteinase 13</td>
<td>Bile duct ligation</td>
<td>Madala et al. (66)</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor</td>
<td>Bile duct ligation</td>
<td>Uchimami et al. (107)</td>
</tr>
<tr>
<td>No effects</td>
<td>Carbon tetrachloride</td>
<td>Wang et al. (113)</td>
</tr>
<tr>
<td>TNF receptor type 2</td>
<td>Carbon tetrachloride</td>
<td>Sudo et al. (102)</td>
</tr>
<tr>
<td>TIMP2</td>
<td><em>Schistosoma mansoni</em> infection</td>
<td>Vaillant et al. (109)</td>
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</table>

IL-1, interleukin; gp, glycoprotein; DDC, 3,5-diethoxy carbonyl-1,4-dihydrocollidine; STAT, signal transducer and activator of transcription; TNF, tumor necrosis factor; CHOP, CCAAT/enhancer-binding protein (C/EBP) homologous protein; LPS, lipopolysaccharide; NADPH, nicotinamide adenine dinucleotide-phosphate; TIMP, tissue inhibitor of matrix metalloproteinase.
**Mouse Models for Liver Fibrosis**

In TGF-β superfamily mediated signaling, all TGF-β ligands bind to cell surface TGF-β receptors. Upon ligand binding, the activated TGF-β receptor phosphorylates the transcription factor Smads, which then translocate to the nucleus to regulate gene expression (69, 72). Smad3-null mice exhibit reduced liver fibrosis with reduced myofibroblast activation and ECM production in response to liver injury. Moreover, the disruption of Smad7, an inhibitory Smad that downregulates TGF-β signaling, results in an enhancement of liver damage and fibrogenesis after chronic injury by CCl₄ (33). Furthermore, the hepatocyte-specific overexpression of Smad7 attenuates TGF-β-mediated Smad signaling, which thereby reduces TGF-β-dependent epithelial-mesenchymal transition of hepatocytes and fibrogenesis after chronic injury induced by CCl₄ (24).

It has been demonstrated that the persistent activation of epidermal growth factor receptor (EGFR) causes the pathogenesis of tissue fibrosis in several organs, including lung and kidney (58, 67). Activation of the EGFR conveys survival signals to hepatocytes. Amphiregulin, one of the EGFR ligands, is specifically induced upon liver injury and stimulates cell proliferation and inhibits apoptosis of hepatic myofibroblasts and HSCs in vitro. Amphiregulin-null mice exhibit a remarkably reduced fibrosis compared with wild-type controls after chronic liver injury induced by CCl₄, which was associated with reduced expression of tissue inhibitor of matrix metalloproteinase (TIMP)-1 and connective tissue growth factor (78). These results indicate a novel role of the EGFR system in hepatic fibrogenesis and suggest that the amphiregulin/EGFR signaling system could be a new therapeutic target for liver fibrosis.

**Renin-Angiotensin System and NADPH.** The renin-angiotensin system is an important factor in regulating the blood pressure and body fluid homeostasis, is involved in liver fibrogenesis. Among vasoactive cytokines, angiotensin II is likely to play a major role in liver fibrogenesis. Angiotensin II is an effector peptide in the renin-angiotensin system and a major regulator for arterial pressure homeostasis in humans. The key components of this system, angiotensinogen, renin, and angiotensin-converting enzyme, are expressed in response to chronic liver damage, and angiotensin II is produced by activated HSCs (9, 77). Angiotensin II induces hepatic secretion of proinflammatory cytokines and stimulates cellular proliferation, migration, and an array of fibrogenic actions such as collagen synthesis in activated HSCs (21, 77). Angiotensin II induces hepatic secretion of proinflammatory cytokines and stimulates cellular proliferation, migration, and an array of fibrogenic actions such as collagen synthesis in activated HSCs (21, 77).

**Immune Response and Inflammatory Cytokines.** Inflammation is an important element in the initiation and progression of liver fibrosis. TNF is a pleiotropic inflammatory cytokine and is involved in cell death/apoptosis (4). Increased levels of TNF are observed in the liver after acute injury induced by CCl₄ treatment (117). TNF-α can activate HSCs in vitro through stress-activated protein kinase pathways (via p38 mitogen-activated protein kinase (83). The disruption of TNF-α attenuates liver fibrogenesis after bile duct ligation (29). Furthermore, disruption of the TNF type 1 receptor also attenuates liver fibrogenesis after CCl₄ treatments (102). Thus, TNF-α acts as a profibrogenic cytokine during the progression of liver fibrosis.

After partial hepatectomy, IL-6/glycoprotein 130 (gp130) signaling promotes hepatocyte proliferation like TNF-α does. In contrast, IL-6/gp130 and TNF-α show opposite effects on liver fibrogenesis. The disruption of IL-6 induces more advanced biliary fibrosis than control livers after bile duct ligation with less phosphorylation of the signal transducer and...
activator of transcription 3 (STAT3) and hepatocyte proliferation (26). There is an interesting observation using a conditional knockout model for gp130: while the knockout of gp130 from hepatocytes does not affect liver fibrogenesis during chronic injury induced by CCl_4, its knockout in all cell types in the liver results in more advanced fibrosis. These findings indicate that the IL-6/gp130-dependent pathway in nonparenchymal cells of the liver plays a protective role in the pathogenesis of liver fibrosis after liver injury by CCl_4 treatments (101). In a sclerosing cholangitis model using 3,5-diethoxycarbonyl-1,4-dihydrocollidine, the livers of hepatic gp130-knockout mice (gp130<sup>−/−</sup>) and hepatic gp130-knockin mice expressing a truncated gp130-knockin allele that lacks the STAT1 and -3 activation regions (gp130<sup>ΔhepaSTAT</sup>) show a high level of TNF-α expression, more hepatocyte apoptosis, enhanced HSC activation, and advanced liver fibrosis than control livers. Interestingly, mice lacking hepatic gp130 and STAT signaling show increased and earlier mortality (79). Thus, IL-6/gp130/STAT3 signaling is likely to play a protective role in liver fibrogenesis.

Toll-like receptors (TLR), a component of the innate immune system, comprise a highly conserved family of receptors that recognize pathogen-associated molecular patterns and thus allow the host to detect microbial infection. TLR4 acts as a receptor for lipopolysaccharide (LPS), which is a cell wall component of Gram-negative bacteria and is the strongest known inducer of inflammation. Several reports suggest that gut-derived LPS is an important mediator of hepatic fibrogenesis (47, 93). The levels of plasma LPS increase in response to liver injury induced by CCl_4, bile duct ligation, or thioacetamide, and pretreatment with a cocktail of nonabsorbable broad-spectrum antibodies inhibits such an increase (82, 93). Kupffer cells are among the first cells in the liver to be hit by gut-derived toxins and are involved in the uptake of LPS (90, 94). CD14 is a LPS receptor and is a 55-kDa glycosylphosphatidylinositol-anchored glycoprotein constitutively expressed on the surface of mature monocytes, macrophages, and neutrophils. The disruption of CD14 attenuates liver fibrogenesis after bile duct ligation with decreased macrophage/monocyte infiltration and α-smooth muscle actin expression, as a marker for activated HSCs (47). There is an important observation showing the association between innate immune responses mediated by TLR4 and TGF-β-driven fibrogenesis via activated HSCs. Quiescent HSCs express high levels of TLR4 and are highly sensitive to LPS. Activation of TLR4 signaling by LPS downregulates the TGF-β-β-α receptor, bone morphogenic protein, and the activin membrane-bound inhibitor (Bambi) via the MyD88-NF-κB pathway on quiescent HSCs, which thereby augments TGF-β-mediated HSC activation and collagen production and enhances hepatic fibrogenesis (93).

Chemokines are potential leukocyte chemoattractants that cooperate with profibrotic cytokines such as IL-13 and TGF-β in the development of fibrosis by recruiting macrophages and other effector cells. Chemokines and their receptors are upregulated in response to liver injury (2, 13, 68). The CC chemokine family has been shown to play an important role in hepatic fibrogenesis. In vitro evidence suggests that three CC chemokine members [regulated upon activation, normal T-cell expressed and secreted, monocyte chemoattractant protein (MCP)-1, and CC chemokine ligand 21] promote proliferation and migration of HSCs (89). The expression levels of several chemokines such as MCP-1, macrophage inflammatory protein-2, and IL-8/CINC and their receptors, including CXC chemokine receptor 3, CC chemokine receptor (CCR) 5, and CCR7, are upregulated in HSCs during their activating process (13–14, 89). The disruption of CCR1 and/or CCR5 reduces liver fibrogenesis and macrophage infiltration after chronic liver injury induced by CCl_4 or bile duct ligation (91). Interestingly, while CCR1 mediates profibrogenic effects in bone marrow-derived cells (primarily Kupffer cells), CCR5 mediates profibrogenic effects in resident liver cells (mainly HSCs). Another chemokine, CCR2, functions to recruit macrophages/Kupffer cells to the site of inflammation and is highly expressed both in HSCs and Kupffer cells in fibrotic liver. The disruption of CCR2 attenuates liver fibrogenesis with the decreased number of activated HSCs after chronic injury induced by CCl_4 or bile duct ligation (92). Thus CC-chemokines play an important role in pathogenesis of liver fibrosis.

Chronic hepatitis C virus infection is characterized by relentless activation of the complement system (111). In response to injury, hepatic complement 5 (C5) levels increase and C5 receptor 1 expression occurs in various hepatic cell types, including HSCs (37, 87). Recently, the gene Hc (encoding complement factor C5) has been identified as a quantitative trait gene that modifies liver fibrogenesis by experimental intercrosses between fibrosis-susceptible BALB/cJ and fibrosis-resistant A/L inbred strains (37). Hc<sup>−/−</sup> strains show less liver fibrosis than Hc<sup>+/+</sup> strains after treatment with CCl_4. The intravenous administration of the C5 receptor 1 antagonist reduces liver fibrogenesis in Hc<sup>−/−</sup> BALB/cJ mice but not Hc-null A/L mice (37).

**ENDOGENOUS OPIOIDS.** Endogenous opioids such as cannabinoids have a profibrogenic activity (23, 105). The increased expression of opioid receptors and met-enkephalin, which are an endogenous opioid neurotransmitter/neuromodulator with agonist activity to µ and δ opioid receptors, is observed in the injured liver (23, 73). The activation of opioid receptors results in the increased proliferation and collagen production of activated HSCs in vitro. Indeed, the opioid antagonist naltrexone reduces the expression of the activated HSC marker α-smooth muscle cell actin and collagen deposition of HSCs in dimethylsulfoxide-treated rats (23). Cannabinoids are the active component of marijuana and act via two G protein-coupled receptors, cannabinoid receptor type 1 (CB1) and type 2 (CB2). The CB1 receptor is highly induced in myofibroblastic cells of the cirrhotic liver, whereas the receptor is only faintly expressed in quiescent HSCs. The liver lacking CB1 receptor or treated with CB1 receptor antagonist SR141716A inhibits the progression of fibrosis in chronic liver injury with decreased hepatic content of profibrogenic cytokine TGF-β1 (105). The CB2 receptor is also highly upregulated in myofibroblasts of human cirrhotic liver, as well as in cultured HSCs and hepatic myofibroblasts. Activation of the CB2 pathway by Δ9-tetrahydrocannabinoid induces growth inhibition and apoptosis of myofibroblasts and HSCs in vitro, and, indeed, CB2 receptor-null mice show enhanced liver fibrogenesis in chronic liver injury induced by CCl_4 (50). Therefore, in contrast to CB1 signaling, the CB2 pathway has an anti-fibrotic role in chronic liver injury. Thus, it would be attractive to pursue combined anti-fibrotic approaches such as the use of CB1 receptor antagonists with CB2 receptor agonists.
CELLULAR SENESCENCE. Cellular senescence, an important mechanism of tumor suppression, functions by blocking the proliferation of damaged cells at risk of malignant transformation. A recent study has documented that cellular senescence acts to prevent the accumulation of ECM in chronic liver fibrosis (56): the senescent cells in fibrogenic liver are derived primarily from activated HSCs, and they reduce secretion of ECMs and enhance secretion of ECM-degrading enzymes. Furthermore, natural killer cells preferentially kill senescent activated HSCs and facilitate the resolution of fibrosis. However, in mice lacking key senescence regulators (p53 and/or INK4a/ARF), activated HSCs continue to proliferate, leading to excessive liver fibrosis. Thus, cellular senescence plays a part in the pathogenesis of liver fibrosis as a self-defense mechanism. Another study shows that telomere shortening contributes to the pathogenesis of liver cirrhosis (85): telomerase-deficient mice, null for the essential telomerase RNA (mTR) gene, exhibit an accelerating development of liver cirrhosis after chronic injury by CCl4 treatments. Furthermore, adenoviral delivery of the mTR gene into cirrhotic mTR-null mice alleviates cirrhotic pathology and improves liver function. Interestingly, significant telomere shortening is observed in hepatocytes but not HSCs in human cirrhotic liver, and this condition is correlated with cellular senescence in hepatocytes (116). These findings indicate that telomerase therapy could be beneficial for liver fibrosis.

Roles of ECM in liver fibrosis. FIBRONECTIN AND PROVISIONAL MATRIX. An essential response to wound healing is the reorganization of ECMs, which provide strength and temporary structure to damaged tissue. A paradigm of adult liver ECM remodeling during wound healing is that, like other tissues, the initial "provisional matrix" formation between plasma type fibronectin and fibrinogen stabilizes wounded areas, which acts as a nidus for subsequent collagen fibrillogenesis (20). Fibronectin is a large dimeric glycoprotein that exists in blood plasma in its soluble form and in its insoluble form as a part of the ECM of almost every tissue in an organism. Plasma fibronectin is produced solely by hepatocytes in the liver (43, 71). A prominent expression of fibronectin is observed during tissue repair. Because experimental evidence has documented that skin wounds heal normally in mice lacking plasma-type fibronectin (86), an absolute requirement for fibronectin and the role of fibronectin in response to adult tissue damage has been speculative. To define the functional identity of fibronectin in the initial stage of adult tissue remodeling, we recently established a null condition for both fibronectin isoforms (plasma and cellular types) from adult mouse liver. We have uncovered that, although it has been postulated that ECM organization and assembly depends on the fibronectin matrix in culture (98–99, 110), the lack of both fibronectin isoforms does not actually interfere with reconstruction and resolution of collagen fibril organization after liver injury induced by CCl4. Furthermore, TGF-β-signaling and type V collagen were identified as essential elements for collagen fibrillogenesis during remodeling of adult liver tissue (70). Thus, our results have wiped out the long-standing concept that collagen fibril organization requires the prior assembly of fibronectin matrix (98–99, 110) and the further interpretation that fibronectin matrix is probably serving as a scaffold for collagen fibril organization. Fibronectin scaffold is not always essential for tissue remodeling, and a certain cell type can assemble collagen fibril networks in the complete absence of fibronectin in vivo.

COLLAGENS AND MATRIX METALLOPROTEINASES. Fibrillar collagens (especially type I and III) and elastin are the most abundant ECM components. The amount of type I collagen in scar tissues is significantly increased compared with normal tissues (32); the quantity of ECMs in fibrotic liver can be up to eightfold higher than that of normal liver. Fibrosis reflects a balance between production and degradation of ECM. Matrix metalloproteinases (MMPs) and their specific inhibitors (TIMPs) play a pivotal role in both fibrogenesis and fibrolysis. MMPs are a family of zinc-dependent enzymes and comprise collagenases, gelatinases, stromelysins, and membrane-type MMPs based on their substrates (11, 75). MMPs are secreted from cells into the extracellular space as proenzymes, which are then activated by a number of specific, usually cell surface-associated, cleavage mechanisms. ECM degradation is mediated by interstitial collagenase, and MMP-1 and -13 (in humans) or MMP-13 (in rodents) are the main proteases that can degrade type I collagen. In chronic injury, the hepatic expression of MMP-13 mRNA is increased during the development of fibrosis but is decreased once the fibrosis becomes prominent. On the other hand, its expression level is increased in the recovery phase of liver fibrosis (115, 118). After bile duct ligation, MMP-13 mRNA is upregulated in isolated HSCs (107). Unexpectedly, MMP-13-null mouse liver attenuates cholestasis-induced liver fibrosis after bile duct ligation, accompanied by significantly decreased expression of the inflammatory mediator TNF-α and the fibrogenic cytokine TGF-β1. Thus, despite the known function of MMPs in degrading the ECM in liver fibrosis, MMP-13 can contribute to the cholestatic liver fibrogenesis by modulating the initial inflammation (107).

MMP activities are inhibited by a family of TIMP (TIMP-1 to -4). The expression of TIMP-1 in the liver is markedly upregulated in both the murine fibrosis model as CCl4 treatments or bile duct ligation and humans fibrotic liver (12, 45). TIMP-1 has an antiapoptotic effect in activated HSCs in culture, and the overexpression of TIMP-1 in the mouse liver shows significantly attenuated spontaneous resolution of chronic liver fibrosis induced by CCl4 (121). MMP-1 and -8 and stromelysin (MMP-3) are activated by the serine protease plasmin, which is generated from circulating plasminogen by urokinase plasminogen activator (uPA) or tissue plasminogen activator (tPA) (46). The conversion from plasminogen to plasmin is inhibited by plasminogen activator inhibitor-1 (PAI-1). The disruption of uPA attenuates liver fibrogenesis after chronic injury induced by CCl4 with increased TIMP1 expression (41). The disruption of PAI-1 also attenuates liver fibrosis after bile duct ligation accompanying increased tPA but not uPA activity (113). Therefore, a balance of ECM production and degradation by MMPs and TIMPs plays a pivotal role in liver fibrogenesis.

ECM stiffness and liver fibrosis. Accumulating evidence shows that ECM stiffness is an important indicator for determining HSC behaviors (38). Continuous ECM stiffness induces transdifferentiation and maturation of myofibroblastic phenotypes in activated HSCs. If such a stiffer ECM persists, it leads to higher myofibroblast contraction and ECM secretion, which leads to further ECM stiffening. Consequently, these stiff ECMs dramatically impede organ architecture and

AJP-Gastrointest Liver Physiol • VOL 300 • MAY 2011 • www.ajpgi.org
function. Furthermore, these stiff ECMs have a correlation to the reversion of activated HSCs. In mouse liver expressing mutated type I collagen, which confers resistance to collage-
nase digestion, apoptosis of activated HSCs decreased during the recovery period following liver injury (49). As predicted from this study, the degradation of ECM promotes apoptosis in activated HSCs (48). Clinically, new diagnostic modalities such as transient elastography suggest a positive correlation between the stiffness of liver and the fibrosis stage and could be useful tools to monitor fibrosis progression and regression as a noninvasive evaluation of liver fibrosis (18).

Future Directions

Gene knockout technology has led to many breakthroughs in liver fibrogenesis research. In contrast, if and how each phenotype in these experimental animal models reflects aspects of human liver fibrogenesis is still poorly understood. The functional links of each target molecule during hepatic fibrogenesis need to be clarified. To date, many studies using gene knockout mice have focused on the progression of liver fibrogenesis. Indeed, most antifibrotic approaches have focused on myofi-
broblasts, including HSCs, since the key pathogenic event in liver fibrosis is the activation of these cell types. Relatively few studies have focused on the resolution of liver fibrosis. The pertinent questions in this regard are whether liver fibrosis can completely regress to normal liver and whether all types of liver fibrosis achieve this regression to normal by the same molecular mechanisms. Indeed, in contrast to the traditional view that liver cirrhosis is an irreversible disease, accumulating evidence indicates that even advanced fibrosis is reversible. In animal models of liver injury, regression of fibrosis has been well documented after the cessation of liver damage. Although emerging antifibrotic interventions have a therapeutic effect in experimental animal models of liver fibrosis, their efficacy and safety in humans remains to be elucidated in the clinical setting. Nevertheless, the translation of basic antifibrotic research into improved therapeutic approaches is an essential step in the man-
agement of patients with chronic liver diseases, having a signifi-
cant beneficial public health impact.

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