Liver fibrosis

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Abstract
Liver fibrosis is a wound-healing response generated against an insult to the liver that causes liver injury. It has the potential to progress into cirrhosis, and if not prevented, it may lead to liver cancer and liver failure. The activation of hepatic stellate cells (HSCs) is the central event underlying liver fibrosis. In addition to HSCs, numerous studies have supported the potential contribution of bone marrow-derived cells and myofibroblasts to liver fibrosis. The liver is a heterogeneous organ; thus, molecular and cellular events that underlie liver fibrogenesis are complex. This review aims to focus on major events that occur during liver fibrogenesis. In addition, important antifibrotic therapeutic approaches and experimental liver fibrosis models will be discussed.
Keywords: *Fibrosis, liver, MMPs, experimental models*

INTRODUCTION

Liver fibrosis is a response generated as a result of chronic liver injury due to various factors, such as alcohol consumption, non-alcoholic steatohepatitis (NASH), viral hepatitis [hepatitis B (HBV) and hepatitis C], autoimmune hepatitis, non-alcoholic fatty liver disease (NAFLD), and cholestatic liver diseases. The common effect of all of these factors on the liver is the generation of a chronic inflammation resulting in an abnormal wound healing response. Different cell types and mediators participate to encapsulate injury. The generation of a fibrotic response in the liver gives rise to the accumulation of extracellular matrix (ECM) components, leading to fibrous scar formation (1,2). The architecture of the liver is disrupted by the presence of a fibrous scar, which causes hepatocyte loss and the deregulation of the normal functioning of the liver, ultimately resulting in liver failure (3). Liver fibrosis is a reversible process, unless it is progressive and leads to cirrhosis. The removal of the fibrotic response-causing agent aids in the regression of fibrosis as long as the liver is not at the stage of advanced cirrhosis (4,5).

Pathogenesis of liver fibrosis

Liver fibrosis is a serious health problem. If not treated, it may lead to advanced liver cirrhosis and hepatocellular carcinoma (HCC). Fibrogenesis is initiated by myofibroblast activation and proliferation because activated myofibroblasts are the major source of ECM in the injured liver (2,3). Although activated hepatic stellate cells (aHSCs) are the major source of myofibroblasts in the fibrotic liver, they are not the only precursors. Endogenous portal fibroblasts, fibrocytes, bone marrow-derived cells, and liver parenchymal cell-derived myofibroblasts that undergo epithelial-mesenchymal transition (EMT) give rise to a significant percent of myofibroblasts in the fibrotic liver. Different cell types activate myofibroblasts depending on the etiology of liver fibrosis (6). A previous study demonstrated that aHSCs are the source of myofibroblasts in a carbon tetrachloride (CCl_4)-induced liver fibrosis model, whereas portal fibroblasts give rise to myofibroblasts in the cholestatic liver (6). Bone marrow-derived cells represent a substantial fraction of the total fibrogenic population in a more chronic injury.

In the quiescent state, HSCs are known as quiescent HSCs (qHSCs), and they are responsible for the storage of vitamin A in the liver. As a result of liver injury, qHSCs are activated by inflammatory mediators, which in turn differentiate into myofibroblasts (7). In this way, tissue remodeling is initiated in the liver by the secretion of ECM proteins and matrix metalloproteinases (MMPs) by aHSCs (8,9).

Cellular and physiological events in liver fibrosis

Mechanisms of liver fibrosis

Different factors, including toxins, hepatitis, steatohepatitis, and autoimmune disorders, promote the activation of HSCs, which in turn acquire a myofibroblast-like phenotype (2). HSC activation comprises two major phases,

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namely, initiation and perpetuation, and these processes are followed by a final resolution phase in case the injury cause still exists (10). Soon after the injury, the initiation phase commences to generate a response against the factors that cause injury at the gene expression level, resulting in phenotypic changes. Early activation is driven by the initial paracrine stimulation and presence of damaged hepatocyte products, causing priming of the cells for activation. Continuous stimuli for activation result in perpetuation that is mainly based on the activity of primed cells for activation. Cell behavior, such as proliferation, fibrogenesis, contractility, matrix degradation, chemotaxis, retinoid loss, and cytokine release, changes as a result of this continuous activation. Overall, these changes contribute to the accumulation of ECM. HSCs undergo apoptosis, senescence, or become quiescent in the case of fibrosis resolution (11). Several studies have shown that different pathways and numerous cell-cell interactions control fibrogenesis and fibrosis regression (Table 1).

Among the mechanisms of liver fibrogenesis, growth factor signaling has a significant role in the activation of HSCs, mainly through platelet-derived growth factor (PDGF) signaling. Cellular proliferation is initiated when PDGF recognizes its receptors that promote the dimerization of receptor subunits and autophosphorylation. This process initiates Ras-mitogen-activated protein kinase pathway activation, increases intracellular calcium, and results in protein kinase C activation (12,13). The paramount importance of PDGF signaling has been shown after blocking with PDGF receptor inhibitors, which may be a promising potential target for drug development. In animal models, this approach has been shown to result in antifibrotic activity during fibrosis (14). HSC proliferation is also stimulated by growth factors, such as transforming growth factor (TGF)-α and epidermal growth factor (15-16). Hepatic angiogenesis is initiated by the recognition of vascular endothelial growth factor (VEGF) by its receptors. Overall, these growth factors promote the remodeling of ECM, resulting in collagen formation (17).

In the normal liver, collagens IV and VI are present in the space of Disse. However, during fibrogenesis, they are replaced by collagens I and II and fibronectin (18). Normally, TGF-β1 is found inactive, but following activation, it initiates a signaling pathway through Smad proteins, resulting in collagen production. Moreover, TGF-β1 promotes the transdifferentiation of quiescent HSCs into myofibroblasts that secrete ECM (19). In addition, leptin signaling also contributes to fibrogenesis through TGF-β1. Kupffer cells have been shown to release TGF-β1 following liver injury, which is a downstream event of leptin signaling initiation (20).

HSCs are capable of expressing different chemokine receptors; such as CXC chemokine receptor (CXCR) 3; C-C chemokine receptor (CCR) 5; CCR7; and ligands, including chemokine ligand (CCL) 2, CCL3, CCL5, CXC chemokine ligand (CXCL) 1, CXCL8, CXCL9, and CXCL10. Chemokines are a class of small chemotactic molecules that regulate inflammation. Even though the role of each specific chemokine in liver fibrogenesis is still being studied, the migration ability of fibrogenic cells to the injury site is known to be promoted by chemokines, thus increasing the number of cells and inflammation at the site of injury. Both profibrogenic and antifibrogenic effects occur on interaction between chemokine receptors and ligands. CCR5, CCR1, and CXCL4 cause fibrosis, whereas the interaction of CXCL9 and CXCR3 protects against fibrosis (21-23).

Adipokines are another major player during liver fibrogenesis (24). They are secreted from adipose tissue. One of them, leptin, is a well-known circulating adipogenic hormone that has the ability to promote fibrogenesis.

Table 1. Major signaling pathways and effectors in liver fibrosis

Pathway	Effectors
Growth factor signaling	PDGF, TGF-α, EGF, VEGF
Fibrogenic signaling pathway	TGF-β1
Chemokine pathways	CCR5, CCR1, CXCL4, CXCL9, CXCR3
Adipokine pathways	Leptin, adiponectin
Neuroendocrine pathways	Cannabinoid and opioid signaling, thyroid hormones, serotonin

CCR: C-C chemokine receptor; CXCL: CXC chemokine ligand; CXCR: CXC chemokine receptor; EGF: epidermal growth factor; PDGF: platelet-derived growth factor; TGF: transforming growth factor; VEGF: vascular endothelial growth factor

Circulating leptin in the blood is proportional to the adipose mass in the body, and increased levels of leptin in the body are associated with fibrogenesis (25,26). In contrast, adiponectin as a counter-regulatory hormone has been suggested to have antifibrogenic activity, which is partially confirmed with the finding that adiponectin expression decreases during hepatic fibrogenesis (27).

Neurochemical and neurotrophic factors also have effects on HSCs and thus during liver fibrosis. Liver injury induces the up-regulation of the neuroendocrine system, and activated HSCs start to express receptors that regulate cannabinoid (CB) signaling (28). CB1 signaling has been known to promote hepatic fibrogenesis, whereas CB2 signaling has an antifibrotic effect (29). Moreover, other neurotrophic factors also contribute to fibrosis, such as opioid signaling that promotes HSC proliferation and collagen production (30). Additionally, thyroid hormones are known to increase HSC activation, whereas serotonin is pro-fibrotic (31,32).

Inflammatory pathways play a significant role in liver fibrogenesis. There is a positive feedback loop between inflammatory and fibrogenic cells, which in turn results in amplified fibrosis. The activation of HSCs is promoted with many other cell types, such as natural killer (NK) cells, T cells, Kupffer cells, macrophages, dendritic cells, and endothelial cells (23). Bacterial lipopolysaccharide is a well-known fibrosis-promoting agent by the activation of Toll-like receptor (TLR) 4 signaling, which is expressed on macrophages and HSCs (33,34). In contrast, Kupffer cell activity increases the activity of nuclear factor-kappa β, which in turn promotes pro-inflammatory cytokine secretion (35).

Oxidative stress and apoptotic cells are capable of inducing an immune response (36,37). Liver injury results in the apoptosis of cells, which are phagocytosed by HSCs, resulting in increased nicotinamide adenine dinucleotide phosphate oxidase and cell survival (38,39). In contrast, apoptotic hepatocyte DNA was demonstrated to interact with TLR9 on HSCs, resulting in the activation of TRL9, thus increasing collagen production and HSC migration (40). Finally, activated HSCs are killed by NK cells, demonstrating an antifibrotic effect by inducing HSC apoptosis (41).

Hepatic fibrosis is based on the wound-healing response, wherein angiogenesis has a significant role in liver regeneration. Because angiogenesis is known to promote liver carcinogenesis, the balance among angiogenic factors should be finely regulated (42). HSCs are located in the perisinusoidal space and regulate intrahepatic blood flow with the aid of their contractile abilities (43). Progressive liver injury results in the formation of vascular disorganization in certain areas, resulting in hypoxia, initiating angiogenesis. A hypoxic environment induces VEGF and PDGF cytokine activities that promote both fibrogenic and angiogenic responses (42). Activated HSCs by hypoxia initiate interactions with PDGF and VEGF signaling, which play an important role in angiogenesis (23).

Reversibility of liver fibrosis

The reversibility of liver fibrosis was a controversial issue for a long time because some of the earlier studies argued that liver fibrosis is irreversible. However, more recent studies have supported the idea that it is a reversible process if the injury-causing stimulus is withdrawn and demonstrated this argument in both experimental liver fibrosis models and clinical samples of a cirrhotic human liver (44-46). With the withdrawal of the causative agent, a cascade of events occurs to initiate the reversion of the fibrotic response. A decrease in cytokine levels, the loss of fibrous scars and myofibroblasts through senescence and apoptosis, and an increase in the collagenase activity are the initial events that occur during the reversion of liver fibrosis (47,48).

Moreover, the loss of myofibroblasts results in a decrease in tissue inhibitors of metalloproteinase (TIMP) levels and an increase in MMP activity, thus degrading ECM (49). MMPs are calcium-dependent enzymes that specifically degrade collagens and non-collagenous ECM substrates (50). Stellate cells secrete basement membrane proteases, MMP-2, MMP-9, and stromelysin (MMP-3), and interstitial collagenase, MMP-13 (50). The inactivation of proteases by binding to TIMPs is also emerging as an important locus of control because the sustained production of these proteins during liver injury could inhibit the activity of interstitial collagenases, leading to reduced degradation of the accumulating matrix (50). In addition, TIMP-1 is anti-apoptotic for stellate cells, which may result in an increased number of activated stellate cells (50).

The loss of myofibroblasts is not the only component of liver fibrosis regression. Macrophages that have a significant role in the progression and resolution of liver fibrosis by producing cytokines and chemokines to induce HSC

transition into ECM-myofibroblasts are also crucial for liver fibrosis regression (51,52). During the progression of liver fibrosis, macrophages augment fibrogenesis, whereas during resolution through the increased production of MMP-13, matrix degradation is increased (52).

Senescence in liver fibrosis

HSCs are observed at the senescence state in the cirrhotic liver, a state in which they stay non-proliferative, lack a collagen-producing capacity, and produce more inflammatory cytokines (53). A previous study demonstrated that p53 has a role in the restriction of liver fibrosis development through its association with cellular senescence via p21 induction (54). Moreover, in another study, the expression of p53 in the senescent HSCs was shown to have an association with the inhibition of HCC development (55). In contrast, another study has claimed that the senescence-associated secretory phenotype in HSCs induces the development of obesity-associated HCC (56). Thus, the role of HSC senescence in liver fibrosis remains controversial and needs more findings.

Autophagy in liver fibrosis

Autophagy is a cellular event that occurs to maintain cellular homeostasis by degrading damaged organelles and protein aggregates, which is also observed in HSC activation, and the inhibition of autophagy has been demonstrated to suppress HSC proliferation and activation (57). In contrast, other studies have shown that the induction of autophagy in hepatocytes aids in the treatment of some other liver diseases, such as α1 anti-trypsin deficiency, NASH, and alcoholic liver disease (58).

Angiogenesis and liver fibrosis

Angiogenesis is the formation of new blood vessels, which is induced by hypoxia in several organs. In addition to its contribution to tumor growth progression, angiogenesis is crucial for the growth and repair of injured tissues. Moreover, angiogenesis has a role in the pathogenesis of several inflammatory diseases (59-61). Although angiogenesis occurs in various organs in the body, it is a more complex process in the liver. Angiogenesis initiated by chronic liver injury is based on different factors; hypoxia caused by inflammation and fibrosis and wound healing initiated by increased cytokine and growth factor levels. Several studies claim that anti-angiogenic therapy without blocking the wound-healing response is important for the prevention of liver fibrosis (62-64).

EMT in liver fibrosis

Initially, EMT was suggested to contribute to liver fibrosis by generating collagen-producing myofibroblasts. However, this finding has become another controversial issue in liver fibrosis owing to opposite findings regarding the role of EMT in this process (65). One of the reasons for the opposite findings on EMT and liver fibrosis is that lineage tracing is an experimental technique that has pitfalls, such as Cre-mediated recombination is not 100% efficient and the number of markers analyzed to detect the presence of EMT may not be adequate. Furthermore, similar to many other studies, experimental liver fibrosis models may not completely reflect the actual EMT that occurs during chronic human liver diseases.

Experimental liver fibrosis models

In vivo models of liver fibrosis

Chemical-based models

Various chemical agents are used to induce liver fibrosis in animal models with different methods of administration. Intraperitoneal (i.p.) injection is the fastest way to trigger liver fibrosis. In contrast, oral administration or inhalation-based administration is also used, but it takes more time than i.p. injection. In any case, chemical-based liver fibrosis models are frequently used because they are easily replicable (66, 67).

Ethanol is one of the frequently used chemicals to study liver fibrosis because alcohol consumption is among the causes of chronic liver diseases worldwide. Ethanol administration induces the activation of HSCs, the apoptosis of hepatocytes, and inflammation (68). CCl_4 is a well-known hepatotoxin that is popular in liver fibrosis and cirrhosis studies in rodents. This model effectively mimics liver fibrosis induced by toxic damage. Although the i.p. injection of CCl_4 is widespread, it can also be administered subcutaneously, orally, or via inhalation. Thioacetamide is another chemical agent that is similar to CCl_4 in the way that both need to be metabolically activated to be toxic. The outcome of thioacetamide administration has been found to be high oxidative damage associated with the activation of HSCs (69). Dimethylnitrosamine (DMN) and diethylnitrosamine (DEN) are other chemicals that are carcinogenic and frequently preferred to generate liver fibrosis models in animals. The biotransformation of DMN and DEN generates reactive oxygen species, which in turn

react with nucleic acids, lipids, and proteins, resulting in cellular malfunction and necrosis (70-73).

Diet-based models

Even though it is possible to induce NAFLD progression to NASH using certain diets in experimental animals, these models do not completely mimic the human pathology (74). A model was found to mimic hepatic stress due to fatty acid transition from the adipose tissue to the liver and increased triglyceride production (75). A methionine- and choline-deficient (MCD) diet is preferred for NASH studies, but this model ignores obesity and insulin-resistance problems (76). MCD diet problems are overcome by a high-fat (HF) diet because it induces insulin resistance and an increase in body weight. A HF diet model is very similar to human NASH but requires a very long time to develop in animals. An alternative high-cholesterol diet was proposed, but this also has the disadvantage of a lack of obesity and insulin resistance (77). A choline-deficient L-amino acid-defined diet is similar to the MCD diet, but in addition, it induces obesity and insulin resistance. In the long term, this model is important for NAFLD, NASH, and HCC studies because it promotes liver tumor formation associated with liver fibrosis (78).

Surgery-based models

Bile duct ligation (BDL) is the most common surgery-based model that induces biliary fibrosis and cholestatic injury, which is based on the double ligation of the bile duct (79). In this way, increased biliary pressure is generated together with inflammation and cytokine secretion, resulting in cholestasis and liver damage. The applicability and replicability of BDL are not high, and this model has been suggested for use in short-term liver fibrosis studies associated with cholestasis (80).

Genetically modified models

The use of genetically modified animals in liver fibrosis studies has both advantages and disadvantages. It allows researchers to investigate specific proteins and signaling pathways underlying liver fibrosis, but on the other hand, it is hard to develop liver fibrosis in these animals without a second stimulus (81-83). In multidrug resistance-associated protein 2-deficient mice, a high level of hepatocyte necrosis and portal inflammation, a strong human cholangitis-like phenotype, and periductal fibrosis have been observed (84). Moreover, these mice were found to be capable of developing biliary fibrosis at 4-8 weeks and HCC at 4-6 months (84). *Alms1Fat ausi* mutant mice form another group of genetically modified animals for liver fibrosis studies because of the effect of both diet and genetics on liver fibrosis progression in these mice. They are ideal for studies investigating NAFLD progression to NASH (85).

Infection-based models

In humans, hepatitis virus induces liver fibrosis, but it is not the case in rodents. Thus, genetically engineered animals that are capable of expressing the HBV envelope-coding region under the control of the albumin promoter are frequently used for hepatitis studies (86). The use of immunodeficient mice transfected with the HBV plasmid is an alternative for these animals (87). In addition to these viral infection-based models, different parasite infection-based models are used to study chronic liver diseases(66). Overall, all of these infection-based models aim to increase the cytokine levels, resulting in the activation of HSCs and liver fibrosis.

In vitro models of Liver Fibrosis

Although in vivo models are more effective in reflecting the actual hepatic environment, in vitro models are also frequently used in liver studies. Primary HSCs isolated from the liver are good models for this, but a low viability of these cells after isolation is a common problem. Moreover, HSCs are activated just after they are embedded on a culture dish, which does not reflect the real mechanism underlying liver fibrogenesis. Obtaining pure HSCs is another problem for liver fibrosis studies because HSC cultures may be easily contaminated with other liver cell types. Cell lines are used as an alternative for primary cells, but like in many other studies, they do not completely reflect the in vivo scenario in the liver, even though they are easily available and unlimited (66).

ANTIFIBROTIC THERAPIES

Thanks to continued experimental advances in the past years, new promising and exciting therapeutic approaches can be developed. One of the active research areas to develop new therapy is toward targeting fibrogenic events in the liver.

TGF-β1 is a well-known molecule that occurs in fibrotic events in all organs. However, its systemic inhibition may increase overall inflammation. Thus, targeting certain steps in the activation of TGF-β1 may be helpful to decrease the fibrotic response in the liver. Integrins and connective tissue growth factor are good candidates for

targeting the TGF-β1 pathway because they play significant roles in TGF-β1 release and activation, respectively (88, 89). A study has demonstrated that cannabinoid receptor 1 (CB1) deactivation attenuates experimental liver fibrosis, but an antagonist of CB1 was shown to have side effects in another study. Reducing redox injury is another alternative for antifibrotic therapy, such as the use of antioxidants. Unfortunately, owing to differences between animals and humans, testing the effect of antioxidants on liver fibrosis is more complex than predicted, and more clinical trials need to be conducted (90).

Another possible area to develop new therapies is in the targeting of fibrosis reversal. In this context, targeting macrophage recruitment may be a useful approach in rodents because it is central in fibrogenesis and its regression. However, because macrophage subpopulations in humans have not been clearly characterized yet, macrophage-targeting studies would not be helpful, until human macrophage biology is completely understood (90).

Liver fibrosis is a dynamic process; thus, targeting one pathway in this process may not be enough to induce its reversal. Combination therapies that target the central components that underlie liver fibrosis are important, such as ECM and certain cell types that play roles in this process. Overall, combination approaches for antifibrotic therapies are very encouraging. However, toxic and off-target effects of these combination therapies should not be ignored in future studies, like in many other therapeutic approaches for different diseases.

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