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Advances in Anti-fibrotic Therapy

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Summary

Sustained progress in defining the molecular pathophysiology of hepatic fibrosis has led to a comprehensive framework for developing anti-fibrotic therapies. Indeed, the single greatest limitation in bringing new drugs to the clinical setting is lack of clarity about clinical trial and treatment endpoints, not the lack of promising agents. A range of treatments, including those developed for other indications, as well as those specifically developed for hepatic fibrosis, are nearing or in clinical trials. Most are focused on attacking features of either hepatic injury and/or activated stellate cells and myofibroblasts, which are the primary sources of extracellular matrix, or scar proteins. Thus, features of injury and stellate cell activation provides a useful template for classifying these emerging agents, and point to a new class of therapies for patients with fibrosing liver disease.

Keywords

hepatic fibrosis; liver fibrosis; cirrhosis; hepatoprotection; stellate cells; myofibroblasts; cytokines; cytokine receptors; matrix degradation

Introduction

The rapid advances in understanding the pathophysiology of liver fibrosis have generated intense interest in exploiting these insights to develop anti-fibrotic therapies for patients with chronic liver diseases. While no agents are yet approved for this indication, there is a flurry of activity in both the academic and commercial sectors to develop effective anti-fibrotic drugs. However, a remaining obstacle is the need to establish effective endpoints of anti-fibrotic drugs that are not reliant on liver biopsy, since changes in extracellular matrix content are likely to evolve more slowly than molecular markers of fibrogenic activity. Thus, considerable effort is also being invested not only in new therapies, but also in defining novel markers of liver injury, fibrogenesis, and extracellular (ie., scar) content in the liver. These advances will further accelerate the progress already generated in developing anti-fibrotic therapies.

Pathophysiology of Hepatic Fibrosis – A Framework for Therapies

The now well-established pathways of hepatic fibrosis offer a useful template for defining points of therapeutic intervention¹. Thus, a review of the cellular mechanisms of fibrosis are provided here, with emphasis on those pathways particularly amenable to therapeutic intervention.

Initiating signals of injury

Hepatic injury leads to initiation of fibrogenesis due to elaboration of key signals derived from hepatocytes, inflammatory cells and other non-parenchymal cells, in particular sinusoidal endothelial cells and Kupffer cells (liver macrophages). These fibrogenic stimuli include reactive oxygen species, hypoxia, inflammatory and immune responses, apoptosis and steatosis.

Oxidative stress—Oxidative stress through generation of reactive oxygen species (ROS) plays an important role in producing liver damage and initiating hepatic fibrogenesis. Oxidative disruption of lipids, proteins and DNA induces necrosis and apoptosis of hepatocytes, and amplifies the inflammatory response, resulting in the initiation of fibrosis. ROS also stimulate the production of profibrogenic mediators from Kupffer cells and both resident and circulating-inflammatory cells. These ROS are also directly fibrogenic and proliferative towards hepatic stellate cells (HSCs)^{2, 3}.

Hypoxia—Hypoxia has been recognized as a critical, early fibrogenic stimulus in which it up-regulates HIF-1 α expression by hepatic stellate cells (HSCs), which are a central regulator of fibrogenesis (see below). This in turn induces vascular endothelial cell growth factor (VEGF) and its receptors, and stimulates type I collagen synthesis in HSCs⁴⁻⁵. Hypoxia also potentiates transforming growth factor- β 1 (TGF- β 1) expression,⁶ contributing to both autocrine and paracrine loops that drive angiogenesis and fibrogenesis. Fibrosis and hypoxia amplify each other in the presence of persistent parenchymal injury, leading to a vicious cycle that disrupts normal tissue repair.

Inflammation and immune responses—Inflammation is an important element in the initiation and progression of hepatic fibrosis⁷. Inflammatory cells belonging to both innate immunity (e.g., NK cells and macrophages) and adaptive immunity (e.g., T and B cells) are involved in the development of liver injury and fibrogenesis. They regulate pathogen elimination, cell killing (e.g., hepatocytes damage during anti-viral immune reaction), the regulation of inflammatory cells, recruiting and activating myofibroblasts, and spontaneous recovery of fibrosis^{8, 9}.

Kupffer cells, the liver's tissue-specific macrophage population, are important effector cells in the hepatic inflammatory response. Nuclear factor kappa-B (NF- κ B) activation in Kupffer cells drives expression of a number of inflammatory genes, including chemokines and other inflammatory mediators¹⁰⁻¹².

Apoptosis—Apoptosis or programmed cell death is a common feature of chronic liver disease, in particular apoptosis of hepatocytes. Apoptosis results in the generation of apoptotic bodies, which are then cleared by phagocytosis. While apoptosis was often thought to be non-inflammatory, in fact it is a pro-inflammatory and fibrogenic stimulus. Kupffer cells secrete death ligands and tumor necrosis factor alpha (TNF-alpha) after engulfing apoptotic bodies¹³. Similarly, the engulfment of apoptotic bodies by HSCs triggers a profibrogenic response with production of oxidative radicals, and up-regulation of both TGF- β 1 and collagen I expression¹⁴⁻¹⁵.

Steatosis—Hepatic steatosis most commonly arises in liver diseases from insulin resistance and mitochondrial dysfunction. The steatosis in chronic hepatitis C, alcoholic steatohepatitis (ASH) and non-alcoholic steatohepatitis (NASH) are all risk factors for fibrosis^{16, 17}.

Even in simple steatosis there is evidence of stellate cell activation as assessed by expression of alpha smooth muscle actin in a study of patients with alcoholic fatty liver¹⁸. While steatosis

may be not sufficient to perpetuate fibrosis by itself, it represents a “first hit” that renders hepatocytes susceptible to a “second hit” (e.g., oxidative stress, viral infection, or LPS), which propagates damage and provokes sustained fibrosis. A number of pathways may contribute to steatosis-related fibrogenesis in liver. These include: 1) enhanced oxidative stress; 2) increased susceptibility to apoptosis; 3) a dysregulated response to cellular injury; 4) Peroxisome proliferator-activated receptor (PPAR) signaling and activity; 5) dysregulation of leptin expression and signaling¹⁹.

Cellular Sources of Fibrogenesis – Stellate cell activation and Myofibroblasts

The identification of *stellate cell activation* as a key event in fibrogenesis has provided an important template for understanding the liver's response to injury (see Figure 1). Stellate cell ‘activation’ refers to the conversion of a resting vitamin A-rich cell to one that is proliferating, fibrogenic and contractile. While it is increasingly clear that other mesenchymal cell populations also contribute to extracellular matrix accumulation¹, stellate cell activation remains the most dominant pathway leading to hepatic fibrosis. Activation consists of two major phases, *Initiation* and *Perpetuation*, followed by *Resolution* of fibrosis if injury subsides

- **Initiation** (also called a "pre-inflammatory stage"), refers to early changes in gene expression and phenotype that render the cells responsive to other cytokines and stimuli. Initiation results mostly from paracrine stimulation, including altered surrounding extracellular matrix, as well as exposure to lipid peroxide, LPS and products of damaged hepatocytes.
- **Perpetuation** results from the effects of these stimuli on maintaining the activated phenotype and generating fibrosis. Perpetuation involves autocrine as well as paracrine loops. It is comprised of several discrete responses including proliferation, contractility, fibrogenesis, matrix degradation, retinoid loss, and inflammatory cell infiltration.
- **Resolution** of fibrosis refers to pathways that either drive the stellate cell to apoptosis, senescence, or contribute to reversion of activated stellate cells to a more quiescent phenotype.

Initiation

Early events in liver injury that initiate stellate cell activation are outlined above. Paracrine stimulation is provided by all neighboring cell types, including: sinusoidal endothelium, Kupffer cells, hepatocytes, and platelets. Endothelial cells are also likely to participate in conversion of TGFβ from the latent to active, profibrogenic form. Platelets are another important source of paracrine stimuli, including PDGF, TGFβ1, and EGF.

As noted above, Kupffer cell infiltration and activation also contribute to stellate cell activation. Kupffer cells stimulate matrix synthesis, cell proliferation, and release of retinoids by stellate cells through the actions of cytokines (especially TGFβ1) and reactive oxygen intermediates/lipid peroxides²⁰.

Hepatocytes are a potent source of fibrogenic lipid peroxides, as reviewed above. Hepatocyte apoptosis following injury also promotes stellate cell initiation through a process mediated by Fas 15²¹. This process may involve the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)^{15, 21}. Apoptotic fragments released from hepatocytes are also fibrogenic towards cultured stellate cells¹³, and activate Kupffer cells²².

Perpetuation

Perpetuation of stellate cell activation involves at least seven discrete changes in cell behavior: proliferation, chemotaxis, fibrogenesis, contractility, matrix degradation, retinoid loss, and WBC chemoattractant / cytokine release. The net effect of these changes is to increase accumulation of extracellular matrix. Cytokine release by stellate cells can amplify the inflammatory and fibrogenic tissue responses, and matrix proteases may hasten the replacement of normal matrix with one typical of the wound "scar."

Proliferation—PDGF is the most potent stellate cell mitogen identified²³. Induction of PDGF receptors early in stellate cell activation increases responsiveness to this potent mitogen²⁴. Downstream pathways of PDGF signaling have been carefully characterized in stellate cells and include PI3 kinase and Na⁺/H⁺ exchange^{25, 26}. Other mitogenic stimuli in stellate cells include vascular endothelial cell growth factor²⁷, thrombin and its receptor^{28, 29}, EGF, TGF α , keratinocyte growth factor³⁰ and bFGF³¹.

Chemotaxis—Stellate cells can migrate towards cytokine chemoattractants leading to their accumulation in zones of injury. Potent stellate cell chemoattractants include PDGF^{32, 33}, MCP-1³⁴ and CXCR3³⁵. In contrast, adenosine³⁶ blunts chemotaxis and may immobilize cells once they reach the site of injury. The mechanical features of stellate cell chemotaxis have recently been explored, revealing that PDGF-stimulated chemotaxis is associated with cell spreading at the tip, movement of the cell body towards the stimulant, and retraction of trailing protrusions associated with transient myosin phosphorylation³⁷.

Fibrogenesis—Stellate cells generate fibrosis not only by increased cell numbers, but also by increasing matrix production per cell. The best-studied component of hepatic scar is collagen type I, the expression of which is regulated both transcriptionally and post-transcriptionally in hepatic stellate cells by a growing number of stimuli and pathways.

The most potent stimulus for production collagen I and other matrix constituents by stellate cells is TGF β 1, which is derived from both paracrine and autocrine sources (see 38, 39 for reviews). Signals downstream of TGF β 1 include a family of bifunctional molecules known as Smads, upon which many extracellular and intracellular signals converge to fine-tune and enhance TGF β 's effects during fibrogenesis 38. TGF β 1 also stimulates the production of other matrix components including cellular fibronectin and proteoglycans 40.

Connective tissue growth factor (CTGF/CCN2) is also a potent fibrogenic signal towards stellate cells^{41, 42} and may be upregulated by hyperglycemia and hyperinsulinemia 43. While stimulation of CTGF production has traditionally been considered TGF β -dependent 44, there is also TGF β -independent regulation⁴⁵.

Contractility—The collagenous bands typical of end-stage cirrhosis contain large numbers of activated stellate cells that contribute to contractility of cells and the entire organ 46. These impede portal blood flow by constricting individual sinusoids and by contracting the cirrhotic liver. Endothelin-1 and nitric oxide are major counter-regulators controlling stellate cell contractility, in addition to a growing list of additional mediators including angiotensinogen II, eicosanoids, atrial natriuretic peptide, somatostatin and carbon monoxide, among others (see 46, 47 for reviews). As stellate cells activate, the expression of the cytoskeletal protein α -SMA is increased which confers increased contractile potential.

Matrix degradation—Fibrosis reflects a balance between matrix production and degradation. The degradation of extracellular matrix is a key event in hepatic fibrosis. Early

disruption of the normal hepatic matrix by matrix-degrading proteases hastens its replacement by scar matrix, which has deleterious effects on cell function.

A major determinant of progressive fibrosis is failure to degrade the increased interstitial, or scar matrix. Matrix metalloproteinase-1 (MMP-1) is the main protease that can degrade type I collagen, the principal collagen in fibrotic liver. Sources of this enzyme are not as clearly established as for the type IV collagenases. Stellate cells express MMP-1 mRNA but little enzyme can be detected ⁴⁸.

Regulation of matrix metalloproteinase activity occurs at many levels, among which is their inactivation by binding to tissue inhibitors of metalloproteinases (TIMPs) ⁴⁹. Stellate cells also produce functional TIMP-1 and TIMP-250, and sustained production of these proteins during liver injury could inhibit the activity of interstitial collagenases, leading to reduced degradation of the accumulating matrix during liver injury. TIMP-1 also is anti-apoptotic towards stellate cells⁵¹, and thus its sustained expression in liver injury will enlarge the population of activated stellate cells by preventing their clearance. In support of TIMP's role *in vivo*, transgenic overexpression of TIMP-1 in liver, delays regression of liver fibrosis in experimental animals ⁵². Thus, as described below, TIMP antagonism represents a very appealing antifibrotic target.

Stellate cells express uroplasminogen activator receptor (uPA-R) and its inhibitor (PAI-1), as well as other components of the plasmin system ⁵³, ⁵⁴. These findings suggest that stellate cells contain most, if not all of the molecules necessary to either activate or inhibit metalloproteinases.

Retinoid loss

Activation of stellate cells is accompanied by the loss of the characteristic perinuclear retinoid (vitamin A) droplets. In culture, retinoid is stored as retinyl esters whereas the form of retinoid released outside the cell during activation is retinol, suggesting that there is intracellular hydrolysis of esters prior to export ⁵⁵. Interesting, mice genetically deficient in lecithin:retinol acyltransferase (LRAT) lack lipid droplets in their stellate cells, but it is unknown what impact this has on the fibrogenic potential ⁵⁶. Thus, whether retinoid loss is required for stellate cells to activate, and which retinoids might accelerate or prevent activation are not clarified.

Resolution

As attention has turned to the treatment of liver fibrosis, the issue of how stellate cell activation resolves has become quite critical ⁵⁷, ⁵⁸. Two potential pathways account for reduction in activated stellate cells, either reversion to a quiescent phenotype or clearance through apoptosis or senescence.

A large amount of evidence supports the importance of stellate cell apoptosis during regression of liver fibrosis ⁴⁹, ⁵⁹. In culture, stellate cells are sensitive to CD95-L and TRAIL-mediated apoptosis, and NK cells can induce apoptosis of stellate cells by a TRAIL mediated mechanism ⁶⁰. Nerve growth factor (NGF) derived from hepatocytes is also apoptotic towards stellate cells ⁶¹, and is antagonized by serotonin receptor signaling ⁶².

Natural killer cells may be an important determinant of stellate cell apoptosis and fibrosis regression. The anti-fibrotic role of NK cells is also consistent with the clinical data of increased liver fibrosis in the setting of therapeutic immunosuppression. The effect of single immunosuppressive agents on NK cell function is minimal, but the combination of cyclosporine and corticosteroids results in significant loss of NK cell cytotoxicity ⁶³.

A recent study has documented that stellate cells also undergo senescence during fibrosis regression, rendering them more susceptible to attack by NK cells⁶⁴. This observation prevents new opportunities to exploit known pathways of cellular senescence to define new therapies and to understand the role of senescence in non-malignant diseases⁶⁵. Presently it is uncertain, however, whether the same stellate cells that express senescence markers are also undergoing apoptosis, as the two pathways typically represent distinct molecular programs.

Therapies for Hepatic Fibrosis – Current and Future

Points of attack against hepatic fibrosis are derived from our current understanding of liver injury, inflammation and stellate cell activation. The ideal therapies will be those that are orally available, well tolerated during chronic usage, and do not simply prevent progression of fibrosis, but rather regress scar, leading to stabilization or improvement in liver function. While specific therapies developed solely for hepatic fibrosis are attractive, in reality there may be many existing therapies developed for other indications, with well-established safety profiles whose mechanism of action may also be anti-fibrotic. Examples of this group include angiotensin converting enzyme 1 inhibitors and angiotensin receptor blockers, antioxidants, and receptor tyrosine kinase antagonists, among others.

In addition to broadly active oral therapies, monoclonal antibodies and other parenteral therapies may also prove valuable, as these reagents have amassed a solid safety and efficacy profile in a number of chronic diseases when used in weekly or monthly infusions. Moreover, efforts to target therapies specifically to activated stellate cells have begun to succeed. In particular, a study using a vitamin A-containing liposome to deliver an siRNA to a collagen heat shock protein (Hsp47) has shown remarkable specificity in three different animal models of liver fibrosis^{1, 66}. This impressive stellate cell targeting could be exploited for a range of therapies to limit collateral injury to other cell types, or to deliver diagnostic imaging agents to liver.

The broad targets of anti-fibrotic therapy can be divided among several categories: a) Cure the primary disease to prevent injury. b) Reduce inflammation or the host response in order to avoid stimulating stellate cell activation. c) ‘hepatoprotection’ to reduce hepatocyte injury, thereby attenuating downstream signals of activation to stellate cells d) Directly downregulate stellate cell activation. e) Neutralize proliferative, fibrogenic, contractile and/or pro-inflammatory responses of stellate cells. f) Stimulate apoptosis of stellate cells. g) Increase the degradation of scar matrix, either by stimulating cells that produce matrix proteases, down-regulating their inhibitors, or by direct administration of matrix proteases.

a. Cure the Primary Disease

Clearing the primary cause of liver disease remains to date the most effective ‘anti-fibrotic’, and increasing evidence in viral liver disease suggests that viral clearance or suppression not only improves histology but also may reduce portal pressure⁶⁷. Other conditions where controlling the primary diseases has salutary effects include abstinence in alcoholic liver disease, removal of excess iron or copper in precirrhotic genetic hemochromatosis or Wilson’s, clearance of HBV or HCV in chronic viral hepatitis, eradication of organisms in schistosomiasis, or decompression in mechanical bile duct obstruction. Most recently weight loss in patients with NASH has led to reduced fibrogenesis^{68, 69}.

b. Reduce inflammation and immune response

Reduced fibrosis has been reported in HCV patients successfully treated with pegylated α -interferon and ribavirin⁷⁰, presumably through its effect on viral replication and liver injury. Sustained viral clearance has been associated with marked regression of fibrosis, so that long

term follow-up of patients successfully cleared of HCV may show more dramatic reversal of disease than at early time points. Importantly, some anti-fibrotic effect is observed even in the absence of viral clearance⁷¹. In experimental biliary fibrosis α -interferon also reduces fibrosis⁷², raising the possibility of a direct anti-fibrotic mechanism in addition to its anti-viral effect. However, to date long term studies of 'interferon maintenance' in the absence of antiviral clearance have not yet proven beneficial.

A number of agents have anti-inflammatory activity in vitro and in vivo which may reduce the stimuli to stellate cell activation. Corticosteroids have been used for decades to treat several types of liver disease, in particular autoimmune hepatitis⁷³. Antagonists to TNF α , or NF κ B modulators have some rationale, as do a growing number of biologically active agents currently used in other chronic inflammatory diseases, in particular inflammatory liver disease. Pentoxifylline may exert its anti-fibrotic activity by downregulating TNF α signaling⁷⁴.

The renin-angiotensin system may also amplify inflammation through generation of oxidant stress, and therefore either angiotensin converting enzyme antagonists and/or angiotensinogen II type I receptor antagonists may have an anti-inflammatory as well as antifibrogenic activity^{75, 76}.

Ursodeoxycholic acid has a beneficial effect on fibrosis in primary biliary cirrhosis^{77, 78}, possibly in part due to its anti-inflammatory activity. Similarly, a nitric-oxide releasing derivative of ursodeoxycholic acid reduces inflammation, fibrosis and portal pressure in an animal model⁷⁹. More, recently ligands of the FXR receptor have been developed which enhance cholestasis and are anti-fibrotic in animal models⁸⁰; clinical trials of these agents are expected in the coming years.

c. Hepatoprotection

A new class of drugs, broadly referred to as 'hepatoprotectants', are showing considerable promise in pre-clinical and clinical studies, including hepatocyte growth factor (HGF), HGF deletion variants, and HGF synthetic mimetics^{81–83}, as well as insulin-like growth factor 84, and a small molecule caspase inhibitor that improves AST levels in patients with chronic HCV and is currently in clinical trials⁸⁵.

HGF—There is strong support for the anti-fibrotic effect of HGF in animal models of fibrosis^{86, 87}. Mechanisms of its anti-fibrotic activity include suppressing TGF β expression⁸² and inducing collagenase expression⁸⁸. Further studies have shown growth inhibition and apoptosis of HSCs, as well as blocking biliary epithelial cells from undergoing epithelial-to-mesenchymal transition⁸¹. More recently, a mechanism was uncovered by which HGF inhibits TGF β from activating its downstream targets, thereby suppressing TGF β mediated transcription of collagen type I in activated HSCs. This effect is mediated by increased interaction between galectin-7 and phosphorylated Smad3, effectively sequestering the p-Smad3 to the cytoplasm and preventing collagen promoter activation⁸⁹.

Caspase inhibitors—Apoptosis of hepatocytes is recognized as an inflammatory stimulus that is pro-fibrogenic^{15, 90–92}. As a result, small molecules have been developed that specifically block caspases, key intracellular effectors of apoptotic signaling⁹³. These agents are currently in clinical trials. The main theoretical concern is that by blocking this pathway that removes cells that may have acquired DNA damage, there will be an enhanced risk of malignancy.

FXR ligands—Farnesoid X Receptor, FXR, is a member of the nuclear receptor transcription factor family, which can be activated by binding of bile acids, of which chenodeoxycholic acid (CDCA) is the most active endogenous ligand. FXR has been implicated in regulating genes

controlling bile flow and secretion^{94, 95}. An additional, novel role for FXR was uncovered with its identification in stellate cells, where it downregulates cellular activation⁸⁰. In these studies, the endogenous FXR ligand, CDCA showed a significant anti-fibrotic effect in animal models. In vivo and in vitro evidence suggested that FXR ligands upregulate SHP in HSCs, and markedly reduce collagen I levels⁸⁰. Furthermore, the FXR-SHP cascade effectively inhibits TIMP-1 expression in stellate cells, mediated by interaction of SHP with JunD, resulting in the inability of JunD to bind the TIMP-1 promoter. MMP-2 activity levels are also increased by 100%⁹⁶. The inhibition of TIMP-1 is pivotal for two reasons: first, metalloprotease activity will no longer be inhibited, and second stellate cells lose the crucial survival signals they receive from TIMP-1 (see above). Interestingly, FXR ligands can reverse the down regulation of PPAR γ in stellate cells in models of rodent liver fibrosis (porcine serum, CCl₄ and bile duct ligation), and submaximal effective doses of PPAR γ agonists and FXR ligands synergistically reduce levels of collagen type I⁹⁷. Combination targeting of these two pathways could reduce the likelihood of side effects resulting from higher doses of a PPAR γ agonist.

d. Inhibit Stellate Cell Activation

Reducing the transformation of quiescent stellate cells to activated myofibroblasts is a particularly attractive target given its central role in the fibrotic response. The most practical approach is to reduce oxidant stress, which is an important stimulus to activation. Anti-oxidants, including alpha-tocopherol (vitamin E) suppress fibrogenesis in some⁹⁸, but not all⁹⁹ studies of experimental fibrogenesis. Other anti-oxidants also can reduce stellate cell activation in culture¹⁰⁰, which provides a rationale for anti-oxidant trials in humans, although as noted above, more potent formulations than those currently available may be required.

Cannabinoids have emerged as a very attractive pathway for antagonizing hepatic fibrosis. There are two identified cannabinoid G-protein-coupled receptors (CB1 and CB2), and studies in human stellate cells demonstrate that activation of CB2 is anti fibrogenic¹⁰¹. Stimulation of cultured stellate cells with an endogenous cannabinoid, anandamide, provokes stellate cell death, albeit through a CB2 ligand-independent pathway¹⁰². Moreover, CB1 receptor is induced primarily in hepatic stellate cells as they activate into myofibroblasts during liver injury. Antagonism of this receptor in an acute model of injury due to CCl₄ or in isolated cells leads to decreased expression of TGF β 1, the most potent fibrogenic cytokine, reduced cellular proliferation and increased myofibroblast apoptosis, all of which would effectively reduce fibrosis¹⁰³. Currently, the drug rimonabant, a CB1 antagonist¹⁰⁴, is undergoing clinical trials in non-alcoholic fatty liver, and a potential anti-fibrotic effect is being monitored in these trials. In contrast to CB1 signaling, the CB2 pathway is anti-fibrotic¹⁰¹, and thus agonists rather than antagonists to this pathway can reduce collagen accumulation in animal models¹⁰⁵.

Interestingly, endogenous opioids, like cannabinoids have profibrogenic activity¹⁰⁶. Thus, opioid antagonists have been examined in animal models, where they demonstrate anti-fibrotic activity¹⁰⁷.

The cytokine γ interferon has inhibitory effects on stellate cell activation in animal models of fibrosis¹⁰⁸. A clinical trial of γ interferon did not show the expected anti-fibrotic benefit in patients with HCV, possibly because only patients with advanced fibrosis were enrolled and the treatment period (one year) may have been too short. In contrast, a beneficial effect of γ interferon was reported in patients with hepatitis B infection¹⁰⁹.

PPAR γ nuclear receptors are expressed in stellate cells, and synthetic PPAR γ ligands (thiazolidinediones) down-regulate stellate cell activation^{110, 111}. Given their widespread use in diabetes, clinical trials of 2nd and 3rd generation thiazolidinediones (ie., lacking the

hepatotoxicity seen with 1st generation agents such as troglitazone) are now being tested in clinical trials both in NASH and other fibrotic liver diseases.

Leptin is produced by activated stellate cells ¹¹², which not only affects lipid metabolism, but also can directly influence wound healing. In fact, animals deficient in leptin have reduced hepatic injury and fibrosis ^{113, 114}. Based on this finding, the discovery of adiponectin, a natural counter-regulator to leptin, may lead to use of this agent in fibrosis, particularly associated with NASH ^{115, 116}.

e. Neutralize proliferative, fibrogenic, contractile and/or pro-inflammatory responses of stellate cells

Many proliferative cytokines including PDGF, FGF and TGF α signal through tyrosine kinase receptors, inhibitors of which are already undergoing clinical trials in other tissues ¹¹⁷. Because the intracellular signaling pathways for these receptors are well understood, inhibitors to signaling molecules are being explored in vivo or in cultured stellate cells. Antagonists to a range of pathways are under evaluation that block PDGF ^{118, 119} or VEGF receptors ¹²⁰, and compounds that modulate intracellular cyclic AMP ¹²¹ or block ion transporters ¹²².

The recent success in developing Gleevec a safe, effective small molecule tyrosine kinase antagonist in human leukemia and mesenchymal cell tumors ^{123, 124} augurs well for the potential of this approach in other indications, including liver fibrosis. In fact, Gleevec is anti-fibrotic in experimental liver fibrosis ¹²⁵, albeit only in ongoing rather than established fibrosis ¹²⁶. Moreover, combinations of Gleevec with other anti-fibrotics may be possible ¹¹⁸. Other orally available, low molecular weight small molecules are under development to block cytokine receptor or intracellular signaling. One such compound is a selective inhibitor of Rho-mediated focal adhesions, which can reduce experimental liver fibrosis ¹²⁷. Antisense to PDGF B chain also blocks experimental hepatic fibrosis ¹²⁸. Since siRNA technology is increasingly become clinically applicable ⁶⁶, this approach merits further evaluation.

Inhibition of matrix production has been the primary target of most anti-fibrotic therapies to date. This has been attempted directly by blocking matrix synthesis and processing, or indirectly by inhibiting the activity of TGF β 1, the major fibrogenic cytokine. The emerging importance of translational regulation of collagen gene expression ^{129–132} could lead to specific translational inhibitors with therapeutic value. Colchicine generated excitement at one time because of its apparent efficacy in a small group of patients ¹³³; however, a more recent study in alcoholic cirrhosis showed no benefit ¹³⁴.

TGF β antagonists are being extensively tested because neutralizing this potent cytokine would have the dual effect of inhibiting matrix production and accelerating its degradation. Animal and culture studies using soluble TGF β receptors or other means of neutralizing the cytokine including monoclonal antibodies and protease inhibitors to block TGF β activation, have established proof-of-principle for these approaches ^{135–137}. Moreover, the natural compound curcumin may also block TGF β signaling ^{138, 139}. Concerns that inhibiting TGF β may alter hepatocellular growth or apoptosis will need to be addressed as these antagonists reach clinical trials, but in other tissues there is great promise for this approach. A number of even newer TGF β antagonists are also being developed and may undergo testing soon. These could include recombinant Smad7, which antagonizes TGF β activity in stellate cells ¹⁴⁰.

As described above, a recent study elegantly employed liposomes containing both vitamin A and an siRNA against the collagen chaperone HSP47 to attack collagen production by stellate cells ⁶⁶. Incorporation of vitamin A in the liposomal complex greatly enhanced their specificity for stellate cells, which serve as the primary site of storage for dietary vitamin A (retinoid). Levels of Hsp47 in the endoplasmic reticulum have previously been shown to correlate closely

with collagen production, and its expression in the liver is localized to activated collagen producing stellate cells ^{141, 142}.

Rapamycin, an immunosuppressive drug used following liver transplantation has the added benefit of inhibiting stellate cell proliferation ¹⁴³, which could attenuate the accelerated fibrosis progression in patients with recurrent HCV; however, enthusiasm for using rapamycin has been tempered by a reported increase in hepatic artery thrombosis ¹⁴⁴.

Relaxin, a natural peptide hormone that mediates parturition, has been developed as an agent to decrease collagen synthesis by stellate cells and increase matrix degradation *in vitro* and *in vivo* ¹⁴⁵. Stellate cells also express relaxin receptors ¹⁴⁶, which might represent an attractive target.

Because endothelin-1 is an important regulator of wound contraction and blood flow regulation mediated by stellate cells, antagonists have been tested as both Anti-fibrotic and portal hypotensive agents. Bosentan, a mixed endothelin A & B receptor antagonist, has Anti-fibrotic activity and reduces stellate cell activation in experimental hepatic fibrosis¹⁴⁷. This and other endothelin antagonists remain attractive drug development targets ¹⁴⁸. Alternatively, delivery of nitric oxide to injured liver may have the same therapeutic effect as inhibiting endothelin-1^{79, 149}.

f. Stimulate stellate cell apoptosis

Apoptosis is the main mechanism accounting for reduced stellate cell numbers during spontaneous recovery from liver injury ^{59, 150}, with recent data (noted above) implicating senescence as well. A combination of signaling pathways may be involved in inducing this apoptosis, thereby clearing fibrogenic cells from the injury milieu. Pro-apoptotic activity towards stellate cells will need to be restricted to this cell type to avoid collateral loss of hepatocytes or other non-parenchymal cells. Below are several examples of how this could be achieved:

TIMP antagonism (see above)—TIMP-1 plays an important role in stellate cell survival by directly inhibiting apoptosis of these cells. The pro-survival effect of TIMP-1 is dependent on MMP inhibition ⁵¹. Transgenic over expression of TIMP-1 in mouse models of fibrosis leads to delayed regression, and is accompanied by decreased numbers of apoptotic stellate cells ⁵². On the contrary, antibodies to TIMP can attenuate fibrosis¹⁵¹. Similarly, use of MMP-9 mutants proteins to sequester TIMP-1 molecules reduces fibrosis accumulation by enhancing matrix resorption ¹⁵².

NK cell stimulation/immunomodulation—NK cells are members of the innate immune system and account for 50% of the lymphoid pool in the liver. In addition to aiding in defense against viral infections, NK cells can ameliorate hepatic fibrosis by killing activated stellate cells ^{60, 153}, associated with the release of two anti-fibrotic cytokines IFN α and γ interferon ^{108, 154}. Interestingly, alcohol has a dampening effect on these anti-fibrotic qualities of NK cells, which could account for the accelerated fibrosis in the setting of alcoholic liver disease ¹⁵⁵. Thus, new approaches to anti-fibrotic therapies could exploit strategies that foster and promote NK cell surveillance, or activate downstream signaling pathways that NK cells use to abolish activated stellate cells.

Pro-apoptotic therapeutics targeted to stellate cells—Attention is increasingly focused on how liver fibrosis regresses, and in particular the fate of activated stellate cells as fibrosis recedes. Mounting evidence indicates that both reversal of the activated stellate cell phenotype and apoptosis are possible. In particular, as liver fibrosis is decreased there is selective cell death of activated stellate cells ¹⁵⁰. This exciting observation has led to animal

studies using gliotoxin, which provokes selective apoptosis of stellate cells in culture and in vivo, leading to reduced fibrosis^{156, 157}. Similarly, inhibition of I κ k, whose net effect is to increase NF κ B signaling in stellate cells, may accelerate apoptosis¹⁵⁸. Apoptosis can also be provoked by disruption of integrin-mediated adhesion¹⁵⁹ or through use of TRAIL ligands¹⁶⁰. Stellate cells contain several families of apoptotic mediators, including Fas/FasL, TNF receptors, nerve growth factor receptors⁶¹, and Bcl/Bax, so that additional targets to promote apoptosis will likely be uncovered in the future¹⁶¹.

f. Increase the degradation of scar matrix

This component of treatment is very important, because anti-fibrotic therapy in human liver disease will need to provoke resorption of existing matrix in addition to preventing deposition of new scar. Direct expression of metalloproteinases in animal models of hepatic fibrosis has begun to confirm that matrix can be resorbed by expression of exogenous enzymes^{162, 163}. In addition, an experimental study has affirmed the importance of matrix degradation in the regression of hepatic fibrosis by demonstrating that a genetically altered mouse expressing mutant collagen resistant to degradation displays delayed regression of fibrosis following liver injury¹⁶⁴.

Expert Commentary

A new field of liver-directed therapies for chronic fibrosing liver disease is about to emerge. With continued elucidation of pathways of both matrix production and degradation in liver injury, new targets are steadily accruing. Combined with enthusiasm for this new 'market' by biotechnology and pharmaceutical companies, the future is extremely bright for this nascent field. Nonetheless, more robust biomarkers and clinical trial endpoints must be validated to allow these agents to establish their efficacy. Combined with emerging genetic markers of disease risk, clinical trials are likely to be progressively shorter and more accurate in establishing therapeutic benefit by focusing on patients at high risk of progression, using endpoints that change rapidly and reproducibly in response to an effective drug. Once efficacy is established in liver, many new agents are likely to be tested in other chronic fibrotic diseases, including those in lung, kidney, pancreas, heart and bone marrow, among others.

Five-year view

A major turning point in the establishment of this new field of fibrosis therapies will be the completion of a successful proof-of-principle trial within five years, showing clear-cut efficacy in patients with ongoing chronic liver injury. Once this occurs, a flood of new therapies are likely to enter clinical trials. While these are unlikely to be available to the practitioner within 5 years, in the subsequent 5 years, they will be transformative in managing patients with chronic liver disease, sparing many from the need for liver transplantation and possibly reducing the risk of hepatocellular carcinoma, the third leading cause of cancer mortality worldwide.

Key Issues

- Fibrogenic stimuli in liver include reactive oxygen species, hypoxia, inflammatory and immune responses, apoptosis and steatosis.
- Most therapies are focused on targeting hepatic injury and activated stellate cells, which are the primary source of ECM deposition.
- Pathways regulating different features of stellate cell activation including proliferation, contractility, and ECM production provide a useful template for identifying therapeutic targets.

- A variety of approaches have been successful in attenuating fibrosis in animal models using the above-mentioned pathways, but these need to be tested in human trials. Directly targeting accumulated fibrotic matrix with protease activity merits further development.
- There is a great need to identify effective and unequivocal end-points for anti-fibrotic drugs in humans that are dynamic and specific in order to translate the exciting data from animal models to patients.

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Figure 1. Pathways of stellate cell activation and fibrogenesis

Pathways of hepatic stellate cell activation. Features of stellate cell activation can be distinguished between those that stimulate initiation, and those that contribute to perpetuation. Initiation is provoked by soluble stimuli that include oxidant stress signals (reactive oxygen intermediates), apoptotic bodies, lipopolysaccharide (LPS) and paracrine stimuli from neighboring cell types including hepatic macrophages (Kupffer cells), sinusoidal endothelium and hepatocytes. Perpetuation follows, characterized by a number of specific phenotypic changes including proliferation, contractility, fibrogenesis, altered matrix degradation, chemotaxis, and inflammatory signaling. (Abbreviations: PDGF – platelet derived growth factor, VEGF – vascular endothelial growth factor; FGF- fibroblast growth factor; ET-1 – endothelin-1; NO – nitric oxide; TGF β 1 – transforming growth factor beta 1; CTGF – connective tissue growth factor; MMP- matrix metalloproteinase; MT-MMP – membrane type

matrix metalloproteinase; TIMP – tissue inhibitor of metalloproteinase; TLR- toll like receptor). Reprinted from Ref ¹, with permission.

TABLE 10.2**Therapeutic Strategies For Hepatic Fibrosis****Reducing injury and inflammation**

Antiviral therapy for viral hepatitis

Anthelmintic therapy for schistosomiasis

Chelation/venesection, treatment of metabolic disease

Angiotensin II type I receptor antagonists, ACE inhibitors

Ursodeoxycholic acid (anti-inflammatory)

Hepatoprotectants:

- Caspase inhibitors
- HGF/HGF mimetics
- IGF-I

Attenuating stellate cell activation

Alpha interferon

Antioxidants:

- Vitamin E, PDTC
- Angiotensin II type I receptor antagonists

Cytokine-directed therapy:

- TGF β antagonists
- Endothelin receptor antagonists
- HGF
- Pentoxifylline (TNF α antagonist)
- IFN- γ

PPAR- γ agonists

FXR agonists

Targeting Cannabinoid Receptors

- CB1 antagonists
- CB2 agonists

Aldosterone antagonists

Opioid antagonists

Inhibiting properties of activated stellate cells

Anti-proliferative:

- PDGF receptor antagonists (i.e. gleevec)
- Antisense to PDGF B chain
- Sodium exchange inhibitors
- HMG CoA reductase inhibitors
- Plasmin/thrombin receptor antagonists

Anti-contractile:

- Endothelin/endothelin receptor antagonists
- Nitric oxide donors

- Inhibition of Rho-mediated focal adhesions

Anti-fibrogenic:

- Collagen synthesis inhibitors
- Collagen folding inhibition (siRNA to Hsp47)
- TGF β inhibitors (soluble receptors, neutralizing antibodies)
- HGF/HGF mimetics
- AT receptor antagonists
- ACE inhibitors
- Integrin
- CTGF/CCN antagonists
- SMAD 7 agonists
- Relaxin

Promoting specific apoptosis of hepatic stellate cells

Gliotoxin

NGF agonists

TIMP antagonists

TRAIL ligands

Degrading scar matrix

Direct collagenase administration

Inhibitors of transglutaminase or collagen crosslinking

TIMP antagonists

TGF- β inhibitors

ACE, angiotensinogen converting enzyme; AT, angiotensin; CCN, Cysteine rich 61/Connective tissue growth factor, Neuroblastoma overexpressed, FXR, farnesyl X receptor; HGF, hepatocyte growth factor; HMG CoA, 3-Hydroxy-3-Methyl-Glutaryl Coenzyme A; NGF, nerve growth factor; NOV, nephroblastoma overexpressed; PDGF, platelet derived growth factor; PDTC, pyrrolidine dithiocarbamate; PPAR, peroxisome proliferators activated receptor; SMAD; mothers against DPP homolog 7; TIMP, tissue inhibitor of metalloproteinase; TGF- β , transforming growth factor beta.