Molecular Regulation of Hepatic Fibrosis, an Integrated Cellular Response to Tissue Injury*

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Encapsulation of injury with fibrosis is a highly evolved response of adult tissues. In liver, the components of the process have been greatly clarified, leading to a coherent view of how wound healing occurs in response to injury. Hepatic fibrogenesis provides an important biological and clinical context for emerging concepts in molecular biochemistry that is relevant to many other tissues.

Background, Disease Context of Hepatic Fibrosis

Chronic injury leading to fibrosis in liver occurs in response to a variety of insults, including viral hepatitis (especially hepatitis B and C), alcohol abuse, drugs, metabolic diseases due to overload of iron or copper, autoimmune attack of hepatocytes or bile duct epithelium, or congenital abnormalities (1). Typically, injury is present for months to years before significant scar accumulates, although the time course may be accelerated in congenital liver disease. Liver fibrosis is reversible, whereas cirrhosis, the end-stage consequence of fibrosis, is generally irreversible. Thus, efforts to understand fibrosis focus primarily on events that lead to the early accumulation of scar in hopes of identifying therapeutic targets to slow its progression.

Like other parenchyma, the normal liver contains an epithelial component (hepatocytes), an endothelial lining (which in liver is distinguished by fenestrae or pores), tissue macrophages (Kupffer cells), and a perivascular mesenchymal cell called the stellate cell (previously called Ito cell, lipocyte, perisinusoidal cell, or fat-storing cell) (Fig. 1); stellate cells are the key fibrogenic cell (see next section). The cellular elements of liver are organized within the sinusoid, or microvascular unit, with the subendothelial space of Disse separating the epithelium (hepatocytes) from the sinusoidal endothelium. In normal liver this space contains a basement membrane-like matrix, although it is not electron-dense like a typical basement membrane. The normal subendothelial extracellular matrix (ECM)1 is essential for maintaining the differentiated function of all resident liver cells. Establishing the importance of the normal ECM in liver has illuminated recent attempts to develop artificial liver support by recognizing that all cellular elements and supporting structures (not just the hepatocyte compartment) must be reconstituted to preserve differentiated function of liver ex vivo (2).

As the liver becomes fibrotic, there are both quantitative and qualitative changes in composition of the hepatic ECM. The total content of collagens and noncollagenous components increases 3–5-fold, accompanied by the shift in the type of ECM in subendothelial space from the normal low density basement membrane-like matrix to interstitial type matrix containing fibril-forming collagens.

Hepatic Stellate Cells, Principal Fibrogenic Cell Type of Liver

Hepatic stellate cells comprise 15% of the total number of resident liver cells. In normal liver they are the principal storage site for retinoids (3). Stellate cells constitute a heterogeneous group of cells that are functionally and anatomically similar but different in their expression of cytoskeletal filaments, their retinoid content, and their potential for ECM production (4).

Stellate cells have an intriguing embryologic origin, with recent evidence suggesting that they are neural crest-derived because they express glial fibrillary acidic protein and nestin (Ref. 5 and references therein). A neural crest origin is further supported by studies in rat neural crest stem cells, which differentiate into myofibroblasts that express smooth muscle α-actin (6), a marker of activated stellate cells. These observations raise the possibility of using neural crest-specific promoters to drive transgene expression selectively in stellate cells in vivo and the prospect of reconstituting stellate cells from a neural crest precursor as part of efforts to repopulate liver.

The perivascular orientation and long cytoplasmic processes of stellate cells facilitate their interactions with neighboring cell types. These processes are adjacent to hepatic nerves, which can respond to α-adrenergic stimulation with an influx of cytosolic calcium and release of osmolytes (7).

Hepatic Stellate Cell Activation, a Highly Orchestrated Response to Tissue Injury

Following liver injury of any etiology, hepatic stellate cells undergo a response known as "activation," which is the transition of quiescent cells into proliferative, fibrogenic, and contractile myofibroblasts. Stellate cell activation is a remarkably pleiotropic yet tightly programmed response occurring in a reproducible sequence (Fig. 2). The organization of stellate cell activation into a defined temporal sequence provides a framework in which cellular events can be placed into a discrete biologic context. Early events have been termed initiation (also referred to as the "prvmtflammatory" stage). Initiation encompasses rapid changes in gene expression and phenotype that render the cells responsive to cytokines and other local stimuli. Initiation is associated with transcriptional events and induction of immediate early genes. It results from paracrine stimulation due to rapid, disruptive effects of liver injury on the homeostasis of neighboring cells and from early changes in ECM composition. Perpetuation incorporates those cellular events that amplify the activated phenotype through enhanced cytokine expression and responsiveness; this component of activation results from autocrine and paracrine stimulation, as well as from accelerated ECM remodeling.

Initiation: Paracrine Stimulation and Transcriptional Events

Stimuli initiating stellate cell activation derive from injured hepatocytes and neighboring endothelial and Kupffer cells in addition to rapid, subtle changes in ECM composition. Hepatocytes and Kupffer cells are a potent source of reactive oxygen intermediates (ROI) (8). These compounds exert paracrine stimulation of stellate cells. Moreover, their activity is amplified in vivo by depletion of antioxidants as typically occurs in diseased liver. In cultured stellate cells, conditioned medium from hepatocytes undergoing oxidative stress increases proliferation and collagen synthesis (9). Overexpression in stellate cells of the enzyme cytchrome P4502E1, which generates ROI, stimulates collagen I gene expression; this effect is attenuated by antioxidants (10).

Endothelial cells play a dual role in early stellate cell activation. Injury to sinusoidal endothelial cells stimulates production of a splice variant of cellular fibronectin (EIIIA isoform), which has an activating effect on stellate cells (11). Additionally, endothelial cells

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1 The abbreviations used are: ECM, extracellular matrix; ROI, reactive oxygen intermediates; TGF-β, transforming growth factor-β; KLF, Kruppel-like factor; RTK, receptor tyrosine kinase; DDR, discoidin domain receptor; PDGF, platelet-derived growth factor; ERK, extracellular signal-regulated kinase; ET-1, endothelin-1; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; MCP-1, monocyte chemotactic protein-1; RA, retinoid acid; IL-10, interleukin-10.
convert latent transforming growth factor-β1 (TGF-β1) to the active, fibrogenic form through the activation of plasmin (12).

Molecular approaches to explore stellate cell gene regulation during early activation have identified differentially up-regulated genes (13–15). An advantage to the stellate cell system in these studies has been the opportunity to analyze "in vivo" gene expression in freshly purified, homogenous cell isolates. These efforts have yielded a transcription factor (13), an adhesion molecule (ICAM-1) (14), and interestingly, the prion protein (15), among others.

One representative effort to identify regulatory genes during early stellate cell activation has resulted in the cloning of a Kruppel-like factor (KLF) zinc finger gene, Zf9/COPEB/GBF (recently renamed "KLF6"). KLF6 mRNA is rapidly induced in liver injury in vivo and in culture (13) and can transactivate genes regulating ECM accumulation (16). At least two other KLF proteins also regulate stellate cell activation. Sp1, the first member of the KLF family, binds more actively to its consensus motif in activated versus quiescent stellate cells (17, 18). Basic transcription element binding protein 1 (BTEB1) mediates the increase in collagen gene expression, which occurs in response to UV radiation or expression of the transcription factor Jun (19).

Perpetuation: Paracrine and Autocrine Cytokine Activity and ECM Remodeling Sustain the Activated Phenotype

Perpetuation of stellate cell activation involves key phenotypic responses mediated by increased cytokine effects and remodeling of ECM (12). Enhanced cytokine responses occurs through multiple mechanisms (12); among these, increased expression of cell membrane receptors and enhanced signaling are especially important (see Ref. 20 for review). In particular, receptor tyrosine kinases (RTKs), which mediate many of the stellate cell’s responses to cytokines, are broadly up-regulated during liver injury (21).

Continued ECM remodeling during this phase underlies virtually all cellular responses characterizing progressive liver injury. The low density subendothelial matrix is progressively replaced by one rich in fibril-forming collagen. This fundamental shift in ECM composition affects the behavior of hepatocytes, sinusoidal endothelium, and stellate cells (Fig. 1).

**FIG. 1. Sinusoidal events during fibrosing liver injury.** Changes in the subendothelial space of Disse and sinusoid as fibrosis develops in response to liver injury include alterations in both cellular responses and extracellular matrix composition. Stellate cell activation leads to accumulation of scar (fibril-forming) matrix. This in turn contributes to the loss of hepatocyte microvilli and sinusoidal endothelial fenestrae, which result in deterioration of hepatic function. Kupffer cell (macrophage) activation accompanies liver injury and contributes to paracrine activation of stellate cells.

**FIG. 2. Phenotypic features of hepatic stellate cell activation during liver injury and resolution.** Following liver injury, hepatic stellate cells (HSC) undergo "activation," which connotes a transition from quiescent vitamin A-rich cells into proliferative, fibrogenic, and contractile myofibroblasts. The major phenotypic changes after activation include proliferation, contractility, fibrogenesis, matrix degradation, chemotaxis, retinoid loss, and white blood cell (WBC) chemoattraction. Key mediators underlying these effects are shown. The fate of activated stellate cells during resolution of liver injury is uncertain but may include reversion to a quiescent phenotype and/or selective clearance by apoptosis.
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Fibril-forming ECM also accelerates stellate cell activation. These effects are mediated not only through interactions with integrins, the classic ECM receptors, but also through binding to at least one RTK. Several integrins and their downstream effectors have been identified in stellate cells, including α1β1, α5β1, αvβ3, and αvβ5 (22). Recently, a subfamily of receptor tyrosine kinases, discoidin domain receptors (DDRs), has been characterized, which unlike other RTKs signal in response to fibrillar collagen rather than growth factors (22, 23). With the identification of discoidin domain receptor-2 (DDR2) mRNA in stellate cells (21), a mode of matrix-cell interaction in liver has emerged that may explain why fibril-forming matrix (especially collagen type I) provokes activation of stellate cells during sinusoidal fibrosis. Thus, as the subendothelial basement membrane is replaced by fibrillar collagen stellate cell activation may be perpetuated via binding of collagen to the DDR2 receptor (21, 22).

Phenotypic Responses of Activated Stellate Cells

Discrete phenotype responses of stellate cells can be identified as their activation in response to liver injury is perpetuated. These include: (a) proliferation; (b) contractility; (c) fibrogenesis; (d) matrix degradation; (e) chemotaxis; (f) retinoid loss; and (g) cytokine release and white blood cell chemotraction.

Proliferation—Increased numbers of stellate cells in injured liver arise in part from local proliferation in response to polypeptide growth factors, most of which signal through receptor tyrosine kinases. Platelet-derived growth factor (PDGF) is the best characterized and most potent among these proliferative factors in hepatic fibrosis. Injury is associated with both increased autocrine PDGF and up-regulation of PDGF receptor (20). Activated PDGF receptor recruits the signaling molecule Ras, followed by activation of the ERK/mitogen-activated protein kinase pathway. Additionally, activation of phosphoinositol 3-kinase is necessary for both mitogenesis and chemotaxis by pathways largely independent of ERK activation (24). The proliferative response to PDGF also requires sustained influx of extracellular Ca2+ and increased intracellular pH (20). The activities of a PDGF-regulated Na+/H+ exchanger (25) and a Na+/Ca2+ exchanger (26) both increase in rat stellate cells during activation in culture and in CCl4 liver injury, respectively. This up-regulation is mediated by calcium/calcmodulin- and protein kinase C-dependent pathways (25).

Contractility—Contractility by activated stellate cells represents an important mechanism underlying increased portal resistance during liver injury. The key contractile stimulus toward stellate cells is endothelin-1 (ET-1), which is autocrine in hepatic stellate cells (27). Up-regulation of ET-1 production is accompanied by increased endothelin-converting enzyme-1, which activates the latent ET-1 (29). ET-1, in addition to its potent contractile effect, also regulates stellate cell proliferation (28, 30).

At least two G-protein-coupled receptors mediate the effects of ET-1. Unlike receptor tyrosine kinases, which are generally induced during activation, ET receptor types A and B are expressed on both quiescent and activated stellate cells (27). However, the relative prevalence of ETA and ETB receptors changes with the cellular activation, and each mediates divergent responses (29). The proliferative effect of ET-1 in quiescent cells correlates with increased Ras/ERK activity, which is blocked with ETA agonists (30). In contrast, the growth inhibitory effect of ET-1 in activated cells is mediated by the ETB receptor (31) via a prostaglandin/CAMP pathway that leads to down-regulation of ERK and c-Jun kinase (JNK) (31).

Fibrogenesis—TGF-β1 is the dominant stimulus to ECM production by stellate cells (see Ref. 12 for references therein). A role for TGF-β1 in promoting rather than initiating stellate cell activation has been established by examining the behavior of stellate cells in TGF-β1 knockout mice with acute liver injury. These animals have markedly reduced collagen accumulation in response to liver injury as expected but still have increased smooth muscle α-actin, indicative of stellate cell activation (32).

TGF-β1 is increased in experimental and human hepatic fibrosis. There are many sources of this cytokine; however, autocrine expression is most important (see Ref. 33 for review). Transcriptional up-regulation of the TGF-β1 gene has been demonstrated in culture-activated stellate cells (16). TGF-β1 activity is also enhanced in activated stellate cells through proteolysis of latent TGF-β1 into the active cytokine by a urokinase-type plasminogen activator (12). Release and activity of TGF-β1 are controlled by a number of intracellular binding proteins (34). A splice variant of latent TGF-β1 binding protein, which lacks a proteinase-sensitive hinge, has been identified in stellate cells (35); this could alter the biologic availability of TGF-β1 during fibrosis. Enhanced TGF-β1 signaling also underlies the response to injury in stellate cells. Increased binding of TGF-β1 to its signaling (types I and II) receptors occurs, yet type II receptor mRNA is decreased during stellate cell activation (12, 36).

Up-regulation of collagen synthesis during activation is among the most striking molecular responses of stellate cells to injury and is mediated by both transcriptional and post-transcriptional mechanisms, not all of which can be ascribed to TGF-β1. Transcriptional activation of the type I collagen has been extensively characterized (13, 37, 38). In addition, the half-life of collagen α1(I) mRNA increases 20-fold in activated compared with quiescent stellate cells (39). A conserved stem-loop structure at the 5′-end of the collagen α1(I) mRNA mediates this enhanced mRNA stability through an interaction with the 3′-untranslated region (40).

Matrix Degradation—Changes in matrix protease activity lead to remodeling of the hepatic ECM during liver injury, which both directly and indirectly accelerates stellate cell activation. Stellate cells express virtually all the key components required for matrix degradation (see Refs. 4, 41, and 42 for reviews). In particular they are a key source of matrix metalloproteinase-2 (MMP-2) as well as stromelysin/MMP-3 (41), each of which degrades the normal subendothelial ECM. This degradation of the normal subendothelial ECM facilitates its replacement by fibril-forming collagen, which further activates stellate cell growth and MMP-2 production in a positive feedback loop (43, 44). Initial evidence suggests that both of these effects of fibrillar collagen on stellate cells can be mediated by receptor tyrosine kinase DDR2 (45).

Through the up-regulation of tissue inhibitor of metalloproteinase-1 and -2 (TIMP-1 and -2), activated stellate cells can also inhibit the activity of interstitial collagenases, which additionally favors the accumulation of scar (41). Up-regulation of TIMP-1 gene expression requires a high mobility activator protein-1 binding activity, which is absent from quiescent stellate cells; this mode of regulating TIMP-1 gene expression has not previously been described (46).

Stellate Cell Chemotaxis—The directed migration of activated stellate cells enhances their accumulation in areas of injury. PDGF and monocyte chemotactic protein-1 (MCP-1) have been identified as chemoattractants toward activated but not quiescent stellate cells (24, 47, 48); chemotaxis requires plasminogen activator, presumably to promote matrix degradation as cells advance through ECM (42). Signaling in response to MCP-1 requires phosphoinositide 3-kinase and calcium influx, but unlike in other MCP-1-responsive cell types, its activity in stellate cells is not mediated by the chemokine receptor CCR2, raising the possibility of a novel receptor (47, 49).

Retinoid Loss—Loss of intracellular vitamin A is a notable feature of stellate cell activation, yet it remains unknown whether retinoid loss is required for stellate cells to activate and which retinoids might accelerate or prevent activation in vivo. The generation of minor metabolites of retinoic acid (RA), 9-cis RA and 9,13-di-cis RA, has been reported in an experimental model of liver fibrosis induced by porcine serum administration (50). These compounds may have a direct link to fibrogenesis because they stimulate the activation of latent TGF-β1, thereby increasing its fibrogenic activity.

Cytokine Release and Leukocyte Chemotraction—Increased production and/or activity of cytokines are critical for perpetuation of stellate cell activation. Almost all features of stellate cell activation can be attributed to autocrine cytokines (12). ECM in liver is an important reservoir of bound growth factors (12).

Stellate cells can also amplify inflammation through the release of neutrophil and monocyte chemoattractants. Key inflammatory chemokines are colony-stimulating factor and MCP-1 (49, 51). The secretion of MCP-1 is regulated through β2 integrin stimulation (20). Up-regulation of adhesion molecules accompanying stellate cell activation further amplifies inflammation during liver injury (52).
Resolution of Liver Fibrosis and the Fate of Activated Stellate Cells

During recovery from acute human and experimental liver injury the number of activated stellate cells decreases as tissue integrity is restored. This raises an intriguing question. What happens to activated stellate cells during resolution? Do they revert to quiescent cells or are they cleared? Recently answers have begun to emerge.

**Reversion?**—A key unresolved issue is whether an activated stellate cell can revert to a quiescent state. One stimulus that may control this response is interleukin-10 (IL-10). IL-10 down-regulates inflammation and increases interstitial collagenase activity (53, 54). IL-10 is induced during stellate cell activation (53) providing an autocrine negative feedback signal to limit scar accumulation.

In addition to effects of soluble cytokines, regression of stellate cell activation may be possible by reconstitution of the normal subendothelial ECM. When stellate cells are grown on a basement membrane substratum (Matrigel) they remain quiescent (45).

**Apoptosis?**—One potential fate of activated stellate cells is apoptosis (see Ref. 55 for review). Stellate cell apoptosis associated with reduced TIP-1 expression has been documented during the recovery phase of experimentally induced liver injury (56). Stellate cells also undergo apoptosis during spontaneous activation, in parallel with increased expression of CD95 L (Fas ligand), Bcl-2, and p53 (55).

**Concluding Remarks**

Reductionist models of tissue repair have evolved to clarify relationships between individual molecules, yet more complex, physiologic systems reveal tremendous redundancy both in whole animals and culture models. In 3T3 fibroblasts, for example, distinct signaling pathways mediated by different phosphotyrosines of the p56**K** receptor converge on the same set of immediate early genes, albeit to different extents (57). How then does one identify those signaling pathways truly relevant to a specific disease? Use of a system exemplified by hepatic stellate cells, in which cellular behavior is thoroughly tracked both in *vivo* and in *vitro*, provides an important advantage. In stellate cells, gene expression and protein secretion are regulated not only in a cell-specific manner but also depend upon the state of cellular activation. Thus, effects of mediators may diverge at different states of cellular activation, possibly reflecting recruitment of different pathways in early versus late liver injury. The stellate cell system, therefore, provides an ideal platform for large-scale gene analysis using cDNA or cDNA microarrays or transcriptional profiling, where relevance to behavior in *vivo* is almost certain. What is likely to emerge is an even more coherent paradigm for how complex cellular events are integrated to achieve a distinct biologic end point, the encapsulation of tissue injury by scar.

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