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ACTIVATION OF HEPATIC STELLATE CELLS – A KEY ISSUE IN LIVER FIBROSIS

Helen L Reeves and Scott L Friedman

Division of Liver Diseases, Mount Sinai School of Medicine, New York, NY 10029-6574

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1. ABSTRACT

Hepatic fibrosis describes the presence of excess collagen due to new fiber formation, laid down as part of the tissue repair response to chronic liver injury. The causes of injury include toxins, disorders of the immune system, viral and parasitic infections, as well as rarer liver diseases such as haemochromatosis, Wilson's disease and galactosaemia. Whatever the cause of injury, the cells and soluble factors contributing to this wound healing response are similar. The principal effector of hepatic fibrogenesis is now widely recognized as the hepatic stellate cell. Stellate cells are usually quiescent cells, but in response to liver injury they undergo an activation process in which they become highly proliferative and synthesize a fibrotic matrix rich in type I collagen. Initiation of stellate cell activation is largely due to paracrine stimulation, whereas perpetuation of activation involves autocrine as well as paracrine loops, and is dependent on a number of functional changes. The principal paracrine and autocrine factors currently thought to be involved in these processes are discussed in this review, as are the roles of the extracellular matrix, the nuclear receptor superfamily, non-peptide ligands, and oxidative stress.

2. INTRODUCTION

Hepatic fibrosis is a wound healing response in which damaged regions are encapsulated by extracellular matrix (ECM), or scar. (1) The cells and soluble factors participating in this response in the liver are similar to those involved in parenchymal injury to kidney, lung or skin, and are principally the hepatic stellate cells. In normal liver, hepatic stellate cells are non-parenchymal, quiescent cells whose main functions is to store vitamin A and probably to maintain the normal basement membrane type matrix. However, numerous *in vivo* and *in vitro* studies indicate that in response to liver injury stellate cells undergo an "activation" process in which they lose vitamin A, become highly proliferative, and synthesize 'fibrotic' matrix rich in type I collagen.

It is not certain that all cells have the capacity for activation, but it is likely that an increasing percentage of cells become activated with continued insult. Activation consists of two major phases, initiation and perpetuation. Initiation refers to early changes in gene expression and phenotype, which render the cells responsive to other cytokines and stimuli, while perpetuation results from the effects of these stimuli on maintaining the activated phenotype and generating fibrogenesis. Initiation is largely due to paracrine stimulation, whereas perpetuation involves autocrine as well as paracrine loops, and is dependent on a number of functional changes. These include stellate cell proliferation and chemotaxis, leukocyte chemotaxis, matrix degradation, fibrogenesis, increased contractility, and

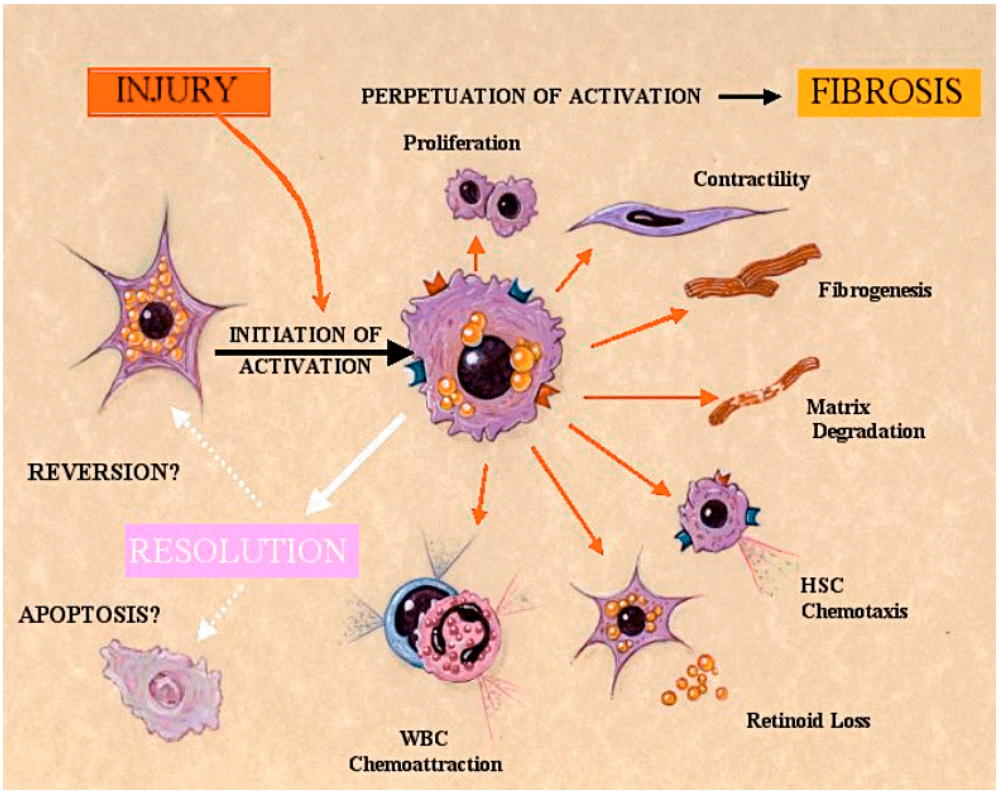

Figure 1. Hepatic stellate cell (HSC) activation and its phenotypic features following liver injury. HSC undergo activation, transforming from a quiescent vitamin A-rich cell, to a proliferative, fibrogenic, contractile myofibroblast. The major phenotypic changes that occur are shown. Resolution of liver injury, if it occurs, may include selective clearance of activated stellate cells by apoptosis, or possibly their reversion to a quiescent phenotype. (From Friedman SL. Molecular regulation of hepatic fibrosis, an integrated response to tissue injury. J Biol Chem 275:2248,2000; with permission).

retinoid loss. These major features of stellate cell activation are summarized in Figure 1 and Tables 1 and 2. The principal paracrine and autocrine factors currently thought to be involved in the processes of initiation and perpetuation are discussed below, as are the roles of the extracellular matrix, the nuclear receptor superfamily, non-peptide ligands, and oxidative stress.

3. PARACRINE SOLUBLE FACTORS

Paracrine stimuli derive from neighboring cells, namely injured hepatocytes, endothelial cells, Kupffer cells and platelets. Hepatocytes release a multitude of peptide growth factors including TGF beta and TGF alpha, and also release lipid peroxides that may be important in some forms of liver injury. Endothelial cells release endothelin 1 (ET-1) (2, 3) and cellular fibronectin (4), both of which have activating effects on stellate cells. Endothelial cells may also participate in the activation of TGF beta. (5) Kupffer cell influx coincides with the appearance of markers of stellate cell activation *in vivo*, (6) and *in vitro* stellate cell studies have demonstrated that conditioned medium from Kupffer cells accelerates stellate cell activation. (7, 8) Kupffer cells can stimulate matrix synthesis, cell proliferation and release of retinoids by stellate cells through the actions of cytokines, particularly TGF beta, and reactive oxygen species. Platelets are also recognized as a potent sources of growth factors injured liver, most notably as the source of platelet derived growth factor (PDGF). They also produce TGF beta 1 and epidermal growth factor (EGF). Other sources of paracrine peptide growth factors include lymphocytes and monocytes.

3.1. Transforming growth factor-beta

The TGF betas consist of three vertebrate isoforms TGF beta1, TGF beta2 and TGF beta3. TGF beta1 in particular is known to be a potent modulator of cell proliferation, cell differentiation and fibrogenesis. TGF beta1 is usually secreted as a homodimer latent polypeptide (L-TGF beta), and a large pool of L-TGF beta exists in serum and bound to proteoglycans within ECM. L-TGF beta is activated into its mature form by the dissociation of its inhibitory latency-associated peptide (LAP), and this involves its binding to the insulin-like growth factor receptor-II/mannose-6-phosphate receptor (IGF-II/M6P receptor). The active/mature TGF beta1 is a 25kD homodimer, which binds to specific TGF beta receptors. TGF betas signal through ligand-dependent heterotetrameric complexes with TbetaR Type I and Type II, which have transmembrane receptor serine/threonine kinases. Ligand binding to TbetaRII recruits and phosphorylates TbetaRI, which in turn propagates the signal to downstream intracellular targets through

Table 1. Paracrine factors involved in the initiation of stellate cell activation

Cellular source	Paracrine factor
Hepatocytes	Lipid peroxides, TGFβ1, TGFα, IL-6, IGF-1, IGFBP, M-CSF, GM-CSF
Kupffer cells	Lipid peroxides, TGFβ1, TGFα, IL-6, TNFα, PDGF, gelatinase B
Endothelial cells	TGFβ1, ET-1, PDGF, cellular fibronectin, activate TGFβ1
Platelets	PDGF, TGFβ1, EGF
Lymphocyte	TGFα, Interleukins
Monocytes	TNFα,TGFβ1, PDGF

Table 2. Autocrine and paracrine loops involved in the perpetuation of stellate cell activation

Functional Change	Factors
Proliferation	PDGF, EGF, TGFα, bFGF, RANTES, IGF-1, CTGF
Stellate cell chemotaxis	PDGF, bFGF, IGF-1, M-CSF, MCP-1
Leukocyte chemotaxis	M-CSF,MCP-1
Fibrogenesis	TGFβ1, acetaldehyde, retinoids, IL1-β, IL-6 TNFα
Contractility	ET-1, PAF, Nitric Oxide, thrombin
Matrix degradation	MMP-2 + MMP-9 degrade normal ECM, ↓ MMP-1 activity (degrades scar ECM), ↑TIMP1 expression
Retinoid loss	↓ligands for RAR + RXR which maintain quiescence

phosphorylation. (9) The intracellular targets include a family of bifunctional molecules known as SMADs, whose responses differ in acute and chronic injury, favoring matrix production in the latter. (10)

TGF beta1 is present in both normal and fibrotic liver, (11) but is increased in cirrhosis (12, 13, 14) and experimental hepatic fibrosis. (15, 16,17,18) The primary source of TGF beta1 in the liver is thought to be Kupffer cells, but autocrine, stellate cell-derived TGF beta may be equally important. In rat CCl_4–induced injury, TGF beta1 mRNA increases dramatically with a similar time course to the Kupffer cell population, (15) and it localized immunohistochemically to this cell type. (19) However small amounts are also produced by endothelial cells and hepatocytes and stellate cells. (20, 21) In stellate cells TGF beta1 upregulates the expression of collagens I, II and IV, fibronectin and laminin and accelerates transformation of quiescent stellate cells to myofibroblasts. (22) Apart from accelerating activation and stimulating matrix synthesis, TGF beta1 has other profibrogenic effects. In fibroblasts it reduces collagenase and stromelysin gene expression and upregulates the expression of protease inhibitors such as TIMP-1 and plasminogen activator inhibitor, which may protect the matrix from degradation. (23, 24) In addition, Pinzani et al (25) have shown that TGF beta1 increases the mitogenic potency of the principal stellate cell mitogen PDGF-BB, and that this is as a result of a TGF beta1 stimulated increase in the expression of the PDGFbeta receptor subunit in cultured stellate cells.

A critical role of TGF beta1 fibrogenesis is suggested by studies in transgenic mice. An active form of TGF beta1 expressed in transgenic mice, resulting in chronic TGF beta1 production, causes hepatic fibrosis, with increased collagen deposition, hepatocyte apoptosis, and alpha-SMA expression. (26, 27, 28) TGF beta1-knockout mice with CCl_4 induced liver injury still develop fibrosis, albeit at reduced levels, suggesting that TGF beta1 is predominantly involved in the perpetuation of stellate cell activation and acceleration of fibrosis, rather than being a critical factor initiating activation of stellate cells.

The activation of the TGF beta downstream mediators, the Smads, is currently being studied in stellate cells. (29) It appears that their regulation is at the level of phosphorylation, as the expression level of all Smads remains largely unchanged in the activation of process of stellate cells. (30) The induction of collagen expression by TGF beta is mediated by the phosphorylation of Smad2 and Smad3, and subsequent nuclear translocation of the Smad complex. (30) In addition, an *in vivo* study using Smad 3 knockout mice has shown that there is less accumulation of collagen mRNA after acute liver injury compared to wild type animals. (31) Whether or not this translates into less actual fibrosis remains to be seen, but early studies imply that Smad manipulation may be a target for antifibrotic therapies.

3.2. Tumor necrosis factor-alpha

Tumor necrosis factor-alpha (TNF alpha) is a cytokine with proinflammatory and immunoregulatory properties. It is produced predominantly at sites of inflammation by activated monocytes and macrophages and previous studies have demonstrated that it plays a role in tissue repair following tissue injury. It is capable of regulating cell proliferation and apoptosis, controlling ECM synthesis and MMP production and inducing the expression of adhesion molecules.

Acute and chronic liver diseases in humans and animal models are accompanied by elevated levels of TNF alpha and TNF alpha receptors. (32, 33, 34, 35 36, 37) Kupffer cells are thought to represent the major source of TNF alpha in the liver, and TNF alpha can be upregulated by exposure of Kupffer cells to lipopolysaccarhide, viruses or alcohol. (38,39) TNF alpha accelerates stellate cell activation *in vitro* as determined by morphological criteria, the loss of retinyl palmitate, and enhanced expression of

alpha-SMA and TGF beta receptor type I. (22, 40) Interestingly, TNF alpha is not a classical 'fibrogenic' mediator like TGF beta1. In fact, while in stellate cells TNF alpha stimulates synthesis of fibronectin, (22) and tenascin, it reduces the synthesis of type I (41, 42, 43) and type III (40) collagen. TNF alpha also leads to increased expression of the MMP stromelysin/transin by hepatic stellate cells, (44) suggesting a role in matrix degradation rather than synthesis. Thus, the main role of TNF alpha in hepatic fibrosis may be the initiation of stellate cell activation rather than the stimulation of ECM produced by activated cells. In support of this hypothesis, it is produced by inflammatory cells early in liver injury and increases the synthesis of monocyte chemotactic protein-1 (45) and neutrophil chemoattractants, (46) by transforming stellate cells.

3.3. Endothelins

The endothelins are a group of three related isopeptides, ET-1, ET-2 and ET-3 (47, 48) that arise by proteolytic cleavage of prepropeptides to proendothelins, which are transformed to mature peptides by endothelin-converting enzymes. The isopeptides are expressed in a number of tissues including the liver. (49, 50) There are three types of G protein-coupled endothelin receptors; ET-A, ET-B (51, 52) and ET-C, (53) which have different binding affinities for the three peptides. Endothelins have a variety of metabolic effects, but are regarded primarily as potent vasoconstrictors. It is thought that contractility of stellate cells may be a major determinant of early and late increases in portal resistance during liver fibrosis, and that the major contractile stimulus towards stellate cells is ET-1. (54)

In normal human liver ET-1 is expressed at low levels, while in cirrhotic liver there is a marked increase in ET-1 synthesis particularly by sinusoidal endothelial cells, bile duct cells and stellate cells. (50) An increase in ET-1 mRNA levels has also been detected in the livers of cirrhotic rats. (49) In advanced fibrosis ET-1 may take part in the contraction of collagen bands resulting in distortion of the liver lobule. (55) *In vitro* studies support a role for ET-1 in stellate cell activation and fibrogenesis. ET-1 promotes stellate cell activation as assessed by alpha-SMA expression, (56) and stellate cell synthesis of ET-1 by both stellate cells and endothelial cells can be increased by the fibrogenic mediators, PDGF and TGF beta. (50) ET-1 induced stellate cell activation has been shown to be mediated by ET-B receptors, as blocking these receptors in an animal model of liver injury reduces fibronectin and collagen synthesis. ET-1 also regulates stellate cell proliferation, and it is the relative prevalence of ET-A and ET-B receptors that determines the net effect. Quiescent cells express both ET-A and ET-B receptors, and in early culture ET-1 promotes proliferation. This is attributed to an ET-A effect, as the proliferative effect in quiescent cells is blocked by an ET-A antagonist. (50) As stellate cells transform, there is a shift to predominantly ET-B receptors. (50) ET-1 inhibits proliferation of fully activated cells, mediated by the ET-B receptor. (57)

3.4. Platelet derived growth factor

PDGF is a dimeric protein consisting of two related polypeptide chains that can form three isoforms: PDGF-AA, PDGF-BB, and PDGF-AB. It is the single most potent stellate cell mitogen currently identified. Two PDGF receptor subunits, alpha and beta have been identified. (58) A pathogenic role for platelet-derived growth factor (PDGF) has been demonstrated in several fibrogenic disorders, including the liver. The genes encoding PDGF and its receptor subunits are markedly overexpressed in cirrhotic human liver, (59) and PDGF gene expression is markedly up-regulated in rat liver 48 hours after a single oral administration of CCl_4. (60) PDGF acts as a powerful chemotactic and mitogenic factor for resident mesenchymal cells, and in liver injury plays a major role in the perpetuation of stellate cell activation and the subsequent development of fibrosis.

The mitogenic potential of PDGF in stellate cells requires the activation-dependent expression of its receptor. (8) Quiescent stellate cells express the alpha receptor but not the beta receptor subunit. In response to activation there is an increase in the synthesis of the beta type receptor while expression of the alpha type receptor remains unchanged, resulting in the predominant expression of the beta type receptor. (61) These results are in agreement with the observation that PDGF-AB and PDGF-BB increase proliferation markedly whilst PDGF-AA is a weaker mitogen. (62) TGF beta is thought to be involved in the upregulation of the beta receptor and increases the mitogenic effect of PDGF-BB but not that of PDGF-AA or PDGF-AB. Cultured human stellate cells express mRNA for both the A and B genes and secrete active PDGF into their medium, suggesting the presence of an autocrine loop that maintains cells in their proliferative state. (63) The signaling pathways downstream of the PDGF receptor have now also been characterized in stellate cells. (64)

3.5. Transforming growth factor-alpha

Transforming growth factor-alpha (TGF alpha) is a polypeptide that belongs to the family of epidermal growth factor-like ligands and is synthesized in many normal tissues (65) including the liver. Immunohistochemical studies have confirmed its presence in adult and fetal liver, (66) particularly in perivenular hepatocytes. (67) It is thought to play an important role in hepatocyte regeneration following hepatocyte injury, (67, 68) and has also been implicated in hepatocarcinogenesis. (69,70)

TGF alpha is not found in sinusoidal cells in normal liver. However, it is synthesized by activated macrophages, (71,72) including Kupffer cells, (73, 74) and has also been shown to be synthesized by activated rat stellate cells. (75,20) Mature TGF alpha is a 6KDa polypeptide that is released from a larger membrane-bound propeptide after protease cleavage. It binds EGF receptors on stellate cells and stimulates proliferation in primary culture in a dose-dependent manner, (76) with less potency than PDGF. (77) It has been suggested that stimulated responses of stellate cells to TGF alpha are phenotype dependent. For example, in quiescent stellate cells TGF alpha stimulates growth with little effect on ECM synthesis, whereas in myofibroblast-like cells, it stimulates

proteoglycan synthesis and may be anti-proliferative. (78) In addition to its effects on proliferation, TGF alpha has been reported to accelerate stellate cell activation, as determined by alpha-SMA expression, and it has been suggested that this may be mediated via oxidative stress and c-myb expression. (79)

3.6. Insulin like growth factor

Insulin-like growth factor (IGF) 1 and 2 secreted by hepatocytes may contribute to the paracrine regulation of stellate cell proliferation. (80) The IGFs and their binding proteins (IGFBPs) and receptors play an essential role in normal physiology and disease states, and are currently under study in a number of different fields of research including liver fibrosis. The IGFs are potent mitogens whose actions are determined by the availability of free IGFs to interact with their receptors. There are two known IGF receptors, which are both integral membrane proteins. The IGF-I receptor signals multiple cascades via its inherent tyrosine kinase activity, whereas the IGF-II/M6P receptor is primarily involved in targeting enzymes to various subcellular compartments. (81) The IGFBPs are secreted by cells and accumulate in the ECM or on the external surface of the cell. In addition to modulation of IGF/IGF receptor interactions, they may have some IGF independent effects, possibly via the interaction with integrins or other cell membrane proteins.

Activated stellate cells express both IGF-1 and IGFBPs, (82, 83, 84) and IGF-1 has been confirmed as a stellate cell mitogen. (82) However, the IGF-I receptor is expressed in early stellate cell culture, and subsequently reduces as cells transform. (83, 84) This in conjunction with the increase in IGFBPs as stellate cells transform has lead to the suggestion that IGF-I mediated effects may be important in the initiation rather than perpetuation of stellate cell activation. IGF-1 is in fact equipotent to PDGF in the stimulation of the Ras/ERK mitogenic cascade, but only stimulates a fraction of the proliferation. (85) It may be that its principal role in HSC activation is as a survival factor, via its stimulation of the PI3K/PKB cascade. (86) Conversely, stellate cell expression of the IGF-II receptor increases as cells activate in response to either CCl_4 liver injury (87, 88) or to PDGF in culture. (89) The time-course of its expression and its ability to activate latent TGF beta suggest that this receptor plays a role in the perpetuation rather than initiation of stellate cell activation.

3.7. Other profibrogenic paracrine peptides

Additional less well characterized peptides involved in stellate cell activation are included in the summary in Tables 1 and 2, and some of these are mentioned here. Prostanoids such as thromboxane, prostaglandins and prostacyclin are secreted from endothelial cells, (90) and have important effects on stellate cell contractility. Another peptide with vasoactive properties is thrombin, which is rapidly generated after liver damage, and may be involved in tissue remodeling and/or scarring during liver damage. (91) Established growth factors such as bFGF and VEGF also exert biological effects on hepatic stellate cells. These are known to play roles in angiogenesis and chronic wound healing. bFGF is a stellate cell mitogen and chemoattractant. Its mRNA and protein levels are dramatically increased in an animal model of fibrosis, supporting a role for this peptide in hepatic fibrogenesis. (92) VEGF binds two receptor tyrosine kinases, VEGFR1 (Flt-1) and VEGFR2 (FLK-1). VEGF stimulates activated HSC growth and HSC activation is associated with an increase in both VEGF and VEGR expression. (93, 94, 95) At later stages of activation, VEGFR1 progressively increases, whereas VEGFR2 decreases, but the relevance of these changes remains to be determined. At these later stages, VEGF attenuates the contractile properties of HSC and expression. (96)

Expression of connective tissue growth factor (CTGF) is also increased in human and experimental cirrhosis. (97, 98, 99) In situ hybridization studies indicate that the source of CTGF is the HSC *in vivo*, and expression of CTGF in stellate cells in culture increases as cells become activated. Thus, this cytokine may also modulate hepatic fibrosis.

3.8. Paracrine peptides with an inhibitory role in fibrogenesis

It is becoming clear that a number of paracrine peptides may inhibit the initiation or perpetuation of stellate cell activation. The most established of these are the interferons (IFNs), but this group may also include some of the interleukins, such as IL-10.

3.8.1. Interferons

IFNs are cytokines or soluble extracellular signaling proteins that were initially described as agents interfering with virus replication, (100) however, it has become clear that they have diverse effects on cell growth and differentiation, with a particularly prominent effect on the pattern and magnitude of the immune response. The interferons are divided into two groups designated type I (IFN-alpha, IFN-beta, and IFN-omega) and type II (IFN-gamma). The type I IFNs share a common receptor complex, whereas type II IFN binds to a distinct receptor. (101) In general, leukocytes produce IFN-alpha and IFN-beta species in response to viral exposure, whereas IFN-gamma is produced by T-lymphocytes when stimulated with a variety of different antigens, including mitogens such as staphylococcal enterotoxin A.

Inhibition of collagen synthesis by interferons was first reported in cultured fibroblasts in 1984, (102) and subsequently evidence that interferons are antifibrogenic in the liver has come from both human (103, 104, 105, 106, 107, 108) and experimental studies. (109, 110, 111, 112) In addition, some *in vivo* experimental studies have shown that IFNs reduce stellate cell activation. (113, 114,115) *In vitro* studies in human liver myofibroblast cells have shown a reduction in cell proliferation and/or the synthesis of ECM components such as type I collagen in the presence of IFN-alpha or IFN-gamma. (116, 117) Similarly, in rat stellate cells, IFN-gamma inhibits the proliferation and activation of quiescent cells, (118) as well as the synthesis of ECM proteins, including interstitial matrix proteins (fibronectin, tenascin, collagen type III), and basement

membrane proteins (collagen type IV, entactin, laminin). (119, 120)

3.8.2. IL-10

Stellate cells also produce the classical anti-inflammatory cytokine interleukin-10 (IL-10), and its expression increases during activation. (121, 122) In progressive human fibrosis due to hepatitis C virus (HCV) the levels of IL-10 are reduced, (123) suggesting that IL-10 may have an antifibrogenic role. This is supported by recent studies showing increased fibrosis in IL-10 knockout mice exposed to liver injury. (124, 125) The inhibitory effect of IL-10 on fibrogenesis may be secondary to its anti-inflammatory properties, but recent evidence also suggests a direct autocrine effect on stellate cells. Wang et al (122) recently showed a decrease in mRNA levels of interstitial collagenase and an increase in procollagen type I gene and protein expression in stellate cells treated with neutralizing anti-IL-10 antibodies *in vitro*. Clinical trials looking at the therapeutic role of this cytokine are in progress, and an uncontrolled pilot study has suggested that in patients with hepatitis secondary to HCV, subcutaneous treatment with IL-10 for 90 days significantly reduces both inflammation and fibrosis on liver biopsy. (126)

3.8.3. IL-1

In fibroblasts, interleukin-1 (IL-1) inhibits the synthesis of collagen (127) and promotes collagen degradation by stimulating collagenase production. (128) In stellate cells, IL-1alpha stimulates proliferation in a concentration dependent manner, whilst inhibiting collagen production. (42) It is synthesized in Kupffer cells (129) and endothelial cells. (130)

4. AUTOCRINE STIMULATION OF HEPATIC STELLATE CELLS

'Autocrine stimulation' implies that a growth factor is synthesized by a cell, which binds and responds to this same faction. Accordingly, activated stellate cells are able to produce many soluble mediators that regulate their proliferation, activation and synthesis of ECM components. In fact, autocrine signaling may be most important in HSC activation. There is evidence for autocrine signaling of TGF beta and EGF/TGF alpha, fibronectin (22, 131) and type I and III collagens. (131) Notably, activated stellate cells also express IGF-II/M6P receptors (87) and produce urokinase, (132) both of which are known to play a role in the activation of L-TGF beta. (133, 87) TGF beta is also involved in the upregulation of the PDGF beta receptor, thereby increasing the mitogenic effect of PDGF-BB. (25) As cultured human stellate cells secrete active PDGF into their medium, the presence of a PDGF autocrine loop that sustains cells in their proliferative state has also been suggested. (63) As discussed previously, stellate cells synthesize ET-1 and express ET-A and ET-B receptors, as well as synthesizing IGF-1, IGFBPs and expressing IGF-Rs, constituting two other potential autocrine regulatory loops. Similarly, interleukin-6 (IL-6), an acute phase protein, is secreted by stellate cells in culture and stimulates the expression of collagen type I in these cells. (130, 134).

5.0 OTHER SECRETORY PRODUCTS OF HEPATIC STELLATE CELLS

Hepatic stellate cells produce additional soluble factors, some of which, such as hepatocyte growth factor (HGF) (135), have little direct effect on HSC. Other secretory products, such as chemokines, platelet activating factor, and leptin, may play a significant role in stellate cell activation, and these are reviewed in this section.

5.1. Chemokines

Chemokines are an expanding family of chemotactic cytokines that attract and activate leukocytes. Stellate cells release neutrophil and monocyte chemoattractants that amplify inflammation during liver injury. Among these are macrophage colony stimulating factor (M-CSF), monocyte chemotactic protein-1 (MCP-1), and RANTES (Regulated upon Activation, Normal T cell Expressed and Secreted). (45, 136, 137, 64)

M-CSF is a potent hemopoetic growth factor that modulates macrophage proliferation and differentiation. mRNA for M-CSF is constitutively expressed in a variety of parenchymal organs including the liver, (138) and expression of the M-CSF gene is increased in both human liver disease and experimental models of fibrosis. (139, 140) Cultured stellate cells express M-CSF mRNA and secrete the active protein into their media. (141).

MCP-1 is a potent chemoattractant for monocytes, lymphocytes, and mesenchymal cells, including stellate cells themselves. Its mRNA is not expressed in normal liver, but its expression is up-regulated in liver tissue from patients with chronic active hepatitis. (136) Activated human stellate cells in culture express MCP-1 mRNA and secrete the active protein into their medium. The secretion of MCP-1 by stellate cells is regulated through beta1 integrin stimulation, (137) and is increased by proinflammatory cytokines, such as IL-1alpha, IFN-gamma, TNF alpha (45) and macrophage inflammatory protein 2 (MIP-2) (46). A high level of expression of MCP-1 by HSC in chronic liver injury in vivo has been confirmed by in situ hybridization and Immunohistochemical studies, and contributes to the maintenance of the inflammatory infiltrate during chronic liver injury. (136) Interestingly a group from Fukuoka, Japan, have reported preliminary success in suppressing experimentally induced fibrosis in rats using gene therapy to deliver a deletion mutant of MCP-1, which blocks the MCP-1 receptor, inhibiting leukocyte chemotaxis. (142).

The chemokine RANTES plays a particular role in eosinophil attraction, and has received much of its liver related attention in the post transplantation literature as a potential mediator of acute rejection. A recent report, however, suggest that this chemokine may also have a role to play in liver fibrogenesis. Stellate cells in culture stimulated with TNF alpha secrete high levels of RANTES, which in turn, induces the generation of ROS, the phosphorylation of the ERK mitogen activated kinase cascade, and stellate cell proliferation. (143).

5.2. Platelet activating factor

Platelet activating factor (PAF) is a phospholipid with potent, diverse physiological actions, including regulatory roles in inflammation and vasoconstriction.

(144) Its primary role is as a mediator of intercellular interactions. It is synthesized by a variety of cell types, and subsequently binds to receptors on the plasma membranes of adjacent cells, resulting in their activation and a change in phenotype. The best characterized of these juxtacrine interactions is that between endothelial cells and leukocytes. When inflamed, endothelial cells express both PAF and the adhesion molecule P-selectin. P-selectin tethers the leukocyte to the endothelial cell, allowing PAF to bind a PAF receptor (PAFR) on the leukocyte. The subsequent adhesion and activation of the leukocyte is dependent upon its expression of the $beta_2$ integrin receptor.

In view of the role of PAF in mediating intercellular interactions and altered cell phenotypes, it is not hard to envisage a role in stellate cell activation, particularly in view of the many cell types involved in liver injury. PAF increases the expression of extracellular matrix proteins (145) and is believed to play a role in the development of both renal interstitial fibrosis and pulmonary fibrosis. In the liver it is synthesized by Kupffer cells (146) very early after acute injury (147) and also by stellate cells, (148) where stimulation with the calcium ionophore A23187, thrombin and lipopolysaccharide all induce significant increases in PAF secretion. A recent study suggests that PAF is the predominant inflammatory lipid mediator produced by hepatic cells after CCl_4 / free radical- initiated liver damage. (149) Furthermore, it appears that cells expressing the PAF receptor are protected from TNF alpha induced apoptosis (NF-κB dependent mechanism) (150). The role of PAF in the liver has not been well characterized as yet, but this may well change, particularly in view of the efforts being invested in the development of antagonists to PAFR.

5.3. Leptin

Leptin released by activated stellate cells within the space of Disse acts as a paracrine modulator of hepatic fibrogenesis. It is a hormone product of the obese gene expressed primarily by adipocytes. Plasma leptin levels correlate with percent body fat, and it is likely that leptin signaling is important for weight regulation (151). It binds and activates specific receptors on hypothalamic neurons that govern energy homeostasis, and also regulates both insulin secretion and tissue responsiveness to insulin. Leptin release is stimulated by cytokines, and it also enhances the secretion of TNF alpha, IL-6 and IL-12 from isolated macrophages in response to LPS. It is possible, therefore, that leptin may amplify some proinflammatory responses, and may well influence the progression from hepatic steatosis to steatohepatitis (152).

Recent reports suggest that leptin may also play a role in liver fibrogenesis. Circulating leptin expressed per kilogram of fat mass is elevated in patients with alcohol induced cirrhosis, and activated hepatic stellate cells grown in culture express leptin (153). Ikejima et al (154) have demonstrated that recombinant leptin augments the profibrogenic responses induced by hepatotoxic chemicals, possibly by the up-regulation of TGF-beta1.

6. THE EFFECT OF RETINOIDS AND THE ROLE OF THE NUCLEAR RECEPTOR SUPERFAMILY

Both chronic liver disease (155, 156) and activated stellate cells in culture (157, 158, 159) are associated with loss of cellular vitamin A, but whether this is a coincidental 'bystander effect', or whether it is an integral part of stellate cell activation remains uncertain. Over a decade ago, it was reported that vitamin A could protect against fibrosis in the CCl_4 induced animal model of liver injury. (160, 161) The observation, however, that hypervitaminosis A is associated with increased hepatic fibrosis in humans (162) and alcohol fed rats, (163, 164) was inconsistent, and it has taken a number of years of *in vitro* study before our general understanding of vitamin A metabolism has shed light on this complex field.

Dietary vitamin A is esterified with long chain fatty acids by enterocytes. These retinyl esters are taken up by hepatocytes and hydrolyzed to retinol. Retinol has a number of potential fates within the hepatocyte (165), one of which is being exported to HSC by cellular retinol binding protein 1 (CRBP-1). The addition of exogenous retinol to cultured stellate cells *in vitro* can maintain a quiescent phenotype, and when added to activated stellate cells, can cause reversion of phenotypic changes, inhibition of proliferation, (166, 167) and a reduction in collagen synthesis. (167) Retinol can also inhibit the production of TGF beta mRNA by stellate cells *in vitro*, (167) as well as PDGF-mediated stellate cell proliferation. (168) However, retinol is not the only form of vitamin A within the HSC. In fact, retinol can be re-esterified for storage in HSC, bind to retinol binding protein for export back to the hepatocyte, or, importantly, be oxidized to retinoic acid. (165)

Isomers of retinoic acid are important ligands for the nuclear retinoic acid (RAR) and retinoid X receptors (RXR). RXR and RAR receptors are ligand dependent transcriptional regulators that belong to the nuclear hormone receptor superfamily. They are further subdivided into alpha, beta and gamma receptors. Maintenance of the quiescent phenotype of HSC may be dependent on adequate levels of all-trans and 9-cis retinoic acid, and on sufficient expression of the receptors, RAR beta and RXR alpha. During activation, stellate cells express less RAR beta, (169, 170) which in other cell types is required for retinol mediated inhibition of cell proliferation. Thus, the expression of RAR beta by quiescent stellate cells may contribute to their poor proliferative capacity, representing an anti-proliferative mechanism that is lost as cells activate. This loss of RAR beta would be exacerbated further by the loss of cellular retinoids during activation. Paradoxically, exposure of stellate cells to supraphysiological concentrations of retinoic acid is profibrogenic, possibly because it induces plasmin mediated activation of latent TGF beta (171; 172). This may partly explain the increased level of fibrosis associated with hypervitaminosis A.

The nuclear hormone receptor superfamily also includes peroxisome proliferator activated receptors (PPARs), the vitamin D receptor, thyroid receptor, several steroid receptors and orphan receptors (ligands as yet

unknown). These receptors may be present in cells as monomers in equilibrium, but in order to affect transcription they form homo- or heterodimers with other family members, and bind serum response elements within the promoter regions of specific genes, awaiting ligand induced activation. The unliganded heterodimers tend to act as transcriptional silencers because they recruit corepressors and histone deacetylators (HDACs). (173; 174)

The PPARs were so named in 1990 when they were discovered to be the receptors responsible for the induction of proliferation in rat liver peroxisomes in response to a diverse set of compounds. (175) Peroxisomes are organelles that are involved in the oxidation of fatty acids, producing hydrogen peroxide in the process. It is now recognized that PPARs are involved in hepatic lipid metabolism and adipocyte differentiation. They function as heterodimers with RXRs, and like the latter, are also subdivided into subtypes of alpha, beta and gamma receptors. PPAR alpha is the most abundant form in the liver, but HSC are reported to express the gamma isoform, and recent evidence suggests that loss or inactivation of PPAR gamma receptors may contribute to stellate cell activation and predispose to hepatic fibrosis. (176) PPAR gamma mRNA is expressed in HSCs of normal rat liver and its expression is greatly reduced in HSC from cholestatic liver fibrosis induced by bile duct ligation. A decrease in PPARgamma protein is detected in culture activated HSC, supporting the idea that its expression is diminished with activation. Furthermore, the treatment of culture activated HSC with PPAR gamma ligands restores the level of PPAR gamma mRNA to amounts representative of quiescent HSC, and inhibits HSC proliferation, alpha-SMA, type 1 collagen, and MCP-1 expression.

In summary, RXRs, RARs and PPARs are emerging as important regulatory molecules in hepatic stellate cells, and manipulation of these receptors could potentially be used to control fibrogenesis in chronic lever disease.

7. MODULATION BY THE EXTRACELLULAR MATRIX

For many years the extracellular matrix (ECM) was regarded as a passive framework binding tissues together, but this view has now changed. In fact, the ECM represents an important regulator of HSC activation. It is made up of collagens, glycoproteins and glycosaminoglycans, the relative quantities of which change in a chronically injured liver. The normal liver ECM is rich in type IV collagen, a network-forming collagen, but as fibrosis progresses this is replaced by more rigid fibrillar collagens such as types I and III collagen. These, together with fibronectin, form an electron dense matrix within the space of Disse. This change, termed ‘capillarization’, (177) is associated with a decrease in the number of endothelial fenestrae (178) and a loss of differentiated hepatocyte function. (179)

The ECM not only provides the mechanical framework for cellular adhesion, migration, and cellular interactions, but it can also be regarded as a reservoir of agents which play important roles in the processes of stellate cell activation and matrix remodeling. It contains a host of tethered molecules waiting to be released or activated. These include growth factors such as PDGF, bFGF, TGF beta and TNF alpha, as well as enzymes responsible for matrix processing, such as matrix metalloproteinases and procollagen peptidases.

There is now accumulating evidence indicating that the matrix itself is of great importance in stellate cell activation. Stellate cells cultured on plastic or type I collagen become activated and express type I collagen. In contrast, stellate cells cultured on laminin rich basement membrane-like matrix derived from Englebroth-Holm-Swarm (EHS) murine tumor, retain their retinoid rich quiescent phenotype, with low levels of proliferation and secrete lower levels of collagen, which is predominantly type III. (180) Disruption of the normal basement membrane may be an early key event in the initiation of stellate cell activation, as well as its perpetuation. This damage to normal basement membrane matrix may be mediated by MMPs, in particular type IV collagenase/gelatinase B released from invading inflammatory cells or resident Kupffer cells, (181) or by type IV collagenase/gelatinase A and transin/stromelysin produced by activated stellate cells themselves. (182, 183, 184) Stellate cell activation may also be regulated by matrix production from other liver cells. Endothelial cells produce the EIIIA isoform of fibronectin following liver injury, and this has been shown to stimulate stellate cell activation in vitro. (4)

ECM-cell interactions are largely mediated via specific receptors called integrins. These are heterodimeric molecules composed of alpha and beta chains whose ligands are matrix molecules rather than cytokines. Classical integrin ligands contain an Arg-Gly-Asp (RGD) peptide sequence, and binding leads to conformational changes in the cytoplasmic domains which modify the organization of the cytoskeleton and activate signaling cascades within the cell. There are a number of alpha and beta chains, giving rise to numerous alphabeta subunit combinations, which confer specificity for different matrix components. The integrin expression varies between different cell types in the liver, and this pattern of expression altars in diseased livers. (185) Stellate cells express several integrins, including $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_V\beta_1$ and $\alpha_6\beta_4$. (4, 186, 187, 188, 189,190), and the pattern of integrins expressed changes in diseased compared to normal liver (189). $\alpha_1\beta_1$ appears to be the major subtype present in quiescent stellate cells, whereas $\alpha_2\beta_1$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$ are expressed in activated or diseased stellate cells. This change in the repertoire of receptor expression is appropriate for fibrotic matrix components (type I collagen, laminin, fibronectin), and ligand binding in response to the deposition of these matrix components

during liver injury may well play a role in the perpetuation of stellate cell activation.

In addition to integrins, a growing number of other adhesion proteins and cell matrix receptors have been characterized, including cadherins and selectins, which mediate interactions between inflammatory cells and the endothelial wall. (191; 192; 193) Furthermore, upregulation of a tyrosine kinase receptor, discoidin domain receptor 2 (DDR2) has been identified during stellate cell activation, which signals in response to fibrillar collagens, leading to enhanced matrix metalloproteinase expression and cell growth (194).

8. NON-PEPTIDE FACTORS

When chronic liver injury is not clearly associated with an abundant inflammatory cell infiltrate, other soluble substances may sustain the activation of stellate cell through pathways that are specific for a particular kind of insult. In alcoholic injury for example, acetaldehyde, the main metabolite of ethanol, has profibrogenic effects. Not only does it increase the expression of type IV collagenase by stellate cells in culture, (195) suggesting an alternative means of disrupting the normal basement membrane matrix and initiating stellate cell activation, it is also able to increase gene transcription and synthesis of different ECM components in activated stellate cells, including type I collagen (196, 197). This effect, which is associated with TGF beta gene expression, is possibly due to formation of acetaldehyde-protein adducts (198) and protein kinase C activation. (199) Acetaldehyde induces activation of additional kinase cascades in cultured HSC ($pp70^{S6K}$ and ERK 1/2), but these are blocked by a protein kinase C inhibitor, (200) supporting a major role for protein kinase C in mediating the fibrogenic effects of acetaldehyde. The stress activated Jun N-terminal kinases (JNKs) 1 and 2 are also activated by acetaldehyde in stellate cells, and JNK inhibition reduces acetaldehyde stimulated alpha (I) collagen mRNA, suggesting that this pathway too is involved. (201)

It remains unclear exactly how these effects of acetaldehyde on the signaling cascades achieve an increase in collagen gene transcription, but it is likely that acetaldehyde affects either the levels of transcription factors, such as basic transcription element binding protein (BTEB) (201), or the promoter affinity of transcription factors such as the C/EBP family, which are known to regulate collagen gene transcription (202, 203; 204; 205).

The other significant non-peptide factor thought to play a role in liver fibrosis and the activation of hepatic stellate cells is iron. Iron overload states result in sideronecrosis of hepatocytes, accumulation of iron in Kupffer cells, and lipid peroxidation (206). Damage to lipid membranes as a result of lipid peroxidation in a setting of increased reactive oxygen species (ROS) introduces the contribution of oxidative stress in liver fibrosis. Many of effects of alcohol, acetaldehyde and iron in the liver, as well as some those induced by viruses and cholestasis, are thought to mediated at least in part by oxidative stress, (207, 208, 209) and this is discussed briefly below.

9. OXIDATIVE STRESS

Oxidative stress is thought to play an important role in a number of pathological disease processes, including liver fibrosis. The term 'Oxidative stress' refers to an imbalance between free radical formation as a result of aerobic metabolism and antioxidant defenses, when the latter are not sufficient. A 'free radical' describes any atom or molecule that contains unpaired electrons. (210) The unpaired electrons alter the chemical reactivity of the atom or molecule, usually making it more reactive. The simplest free radical is the hydrogen radical, which is often cleaved from other molecules during lipid peroxidation, for example. Lipid peroxidation describes a situation where a reactive radical removes an atom of hydrogen from a polyunsaturated fatty-acid side chain in a membrane phospholipid or lipoprotein. This leaves an unpaired electron on carbon within the unsaturated fatty acid, which reacts with oxygen, resulting in a peroxyl radical. The peroxyl radical attacks an adjacent fatty acid side chain, setting up a chain reaction resulting in membrane disruption. Free radicals can also damage DNA, leading to potential carcinogenic mutations, and proteins impairing their function.

In the last decade, attention has focused on lipid peroxidation as a fibrogenic mediator in a number of different liver diseases, particularly alcoholic liver disease, hepatitis C, and iron overload. (211, 212, 213, 214, 215, 216) Type I collagen transcripts and aldehyde (4-hydroxynonenal [4-HNE], malondialdehyde [MDA]) adducts, by-products of lipid peroxidation, have been co-localized in alcohol and iron-fed rats using in situ hybridization and immunohistochemistry. (217) Similar in situ studies have shown a correlation between the presence of these aldehyde adducts and collagen gene expression by stellate cells in humans with chronic liver diseases, including Hepatitis C, haemochromatosis and alcoholic liver disease. (218, 219, 216, 220) Antioxidant levels are typically depleted in cirrhotic liver (221) which will amplify any oxidative stress and favor lipid peroxidation. Ethanol metabolism both induces oxidative stress, by increasing the production of free radicals capable of initiating lipid peroxidation, (222) and depletes antioxidant defenses including glutathione (GSH). (223, 224, 225) Furthermore, *in vivo* studies suggest that treatment with an antioxidant may reduce stellate cell activation in humans with hepatitis C, (226) and inhibit fibrogenesis in experimental iron overload. (227, 228)

Culture studies support the role of lipid peroxides in stellate cell activation. The generation of free radicals or the direct addition of the end-products of lipid peroxidation, MDA, 4-HNE and F2-isoprostanes, enhance stellate cell proliferation and/or collagen mRNA synthesis. (229, 230, 231) Induction of cytochrome P450 2E1 (CYP2E1) by ethanol is one of the central pathways by which ethanol generates oxidative stress, and induction of

this enzyme has been used as an *in vitro* tool. Transfection of CYP2E1 into the T6 stellate cell line both elevates ROS and increases mRNA for type I collagen. (232) Notably, co-culture with primary rat hepatic stellate cells and a cell line overexpressing CYP2E1 also demonstrates an increase in collagen levels, indicating that diffusable oxidants contribute to stellate cell activation. (233)

The disappointing aspect in a number of *in vitro* studies has been in the failure of induced ROS or lipid peroxides to induce collagen protein synthesis in stellate cells, and the failure of an effect of depletion of the critical antioxidant, GSH, to alter collagen synthesis. (234) Thus, oxidative stress possibly contributes indirectly to fibrosis, by affecting the activity of other cell types, and their release of profibrogenic agents. Furthermore, it is possible that the antifibrotic effects of antioxidants are a result of effects on cytokine mediated signaling, as outlined below.

Oxidative stress may also play a part in growth-factor or ECM-related activation of stellate cells, as the addition of antioxidants, such as vitamin E, resveratrol or quercetin, appears to delay the culture activation of stellate cells or that induced by TGF alpha or TNF alpha (79, 235, 236) There is an important link between cytokines and ROS. Not only do ROS trigger the release of proinflammatory cytokines such as TNF alpha, but they may also be involved in mediating the intracellular signaling pathways of these cytokines. Recent evidence suggests that ROS such as superoxide anions and hydrogen peroxide function as intracellular second messengers and regulate a number of cellular processes. (237) Studies over the last decade have demonstrated that ligand stimulation of non-phagocytic cells results in an increase in intracellular ROS. Ligands include cytokines with established roles in HSC activation, such as TNF alpha (238) and TGF beta, (239) as well as peptide growth factors such as PDGF, which can induce ROS via both tyrosine kinase receptors (240, 241) and G-protein coupled receptors. (242)

Thus, while ROS are undoubtedly important in fibrogenesis, this may be via different routes, the relative contributions of which or not yet established. The principal role of ROS may be via stimulating the release of cytokines from other cells, or possibly the ECM. The reported antifibrotic effects of antioxidants may be via the reduction of oxidative stress and reduced cytokine release, or in fact by inhibiting the magnitude of signaling cascades initiated by growth factors, rather than by inhibiting the direct effects of ROS on stellate cells.

10. SUMMARY

Hepatic fibrosis is the result of a wound healing response in the presence of chronic liver injury or continued insult. The factors contributing to activation of hepatic stellate cells may vary somewhat depending on the type of liver injury, such as the contribution of acetaldehyde adducts in the case of alcohol induced damage, but on the whole, the healing response follows a similar pattern. Hepatocyte injury results in the release of proinflammatory cytokines, chemokines, and generates ROS and lipid peroxides. Damaged endothelial cells release cellular fibronectin as well as cytokines. Inflammatory cells are recruited to the region of injury, and these, as well as resident Kupffer cells and platelets, release additional growth factors, as well as metalloproteinases which degrade the normal basement membrane. Local metalloproteinase activity releases membrane bound growth factors. Initiation of activation and subsequent proliferation of resident stellate cells follows.

Additional activated stellate cells accumulate following chemotaxis. These activated cells themselves produce proinflammatory cytokines, regulatory factors, and receptors that enable them to respond to their changing environment. In an attempt to seal off the area of injury, this army of cells lay down a new, thickening, scar matrix, and produce inhibitors of the metalloproteases, which are present and ready to degrade this scar when the damage has resolved. Unfortunately, with continued liver injury, this wound healing process continues without resolution, resulting in progressive liver fibrosis, disruption of normal liver architecture, and finally, cirrhosis.

The hepatic stellate cell is central to this whole process, and future treatments will target this cell, either by inhibiting its activators, inhibiting its fibrogenic function, promoting its capacity to aid resolution of fibrosis, or even promoting its capacity to undergo spontaneous apoptosis. (243)

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Abbreviations

alpha-SMA	alpha-smooth muscle actin
BTEB	Basic transcription element binding protein
CCl_4	Carbon tetrachloride
C/EBP	CCAAT enhancer binding protein
CRBP-1	Cellular retinol binding protein 1
CTGF	Connective tissue growth factor
CYP2E1	Cytochrome P450 2E1
DDR2	Discoidin domain receptor 2
ECM	Extracellular matrix
EGF	Epidermal growth factor
ERK	Extracellular regulated kinase
ET-1	Endothelin-1
ET-A	Endothelin A receptor
ET-B	Endothelin B receptor
FAK	Focal adhesion kinase
GSH	Glutathione
HDAC	Histone deacetylator
HSC	Hepatic stellate cell
4HNE	4 Hydroxynonenal
IFN	Interferon
IGF	Insulin like growth factor
IGFBP	Insulin like growth factor binding protein
IGF-II/M6P	Insulin like growth factor II/mannose 6 phosphate
IL	Interleukin
JNK	c-jun N terminal kinase
L-TGF beta	Latent TGF beta
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemotactic protein 1
MCSF	Macrophage colony stimulating factor
MDA	Malondialdehyde
MIP-2	Macrophage inflammatory protein - 2
MMP	Matrix metalloproteinase
mRNA	messenger ribonucleic acid
NFkB	Nuclear factor kappa B
NO	Nitric oxide
PAF	Platelet activating factor
PAFR	Platelet activating factor receptor
PBC	Primary biliary cirrhosis
PDGF	Platelet derived growth factor
PI3K	Phosphoinositol 3 kinase
PKC	Protein kinase C
PPAR	Peroxisome proliferator activated receptor
RAR	Retinoic acid receptor
RANTES	Regulated upon activation normal T cell expressed and secreted
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
RXR	Retinoid X receptor
STAT	Signal transducer and activator of transcription
TGF	Transforming growth factor
TIMP	Tissue inhibitor of matrix metalloproteinase
TNF alpha	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor

Key Words: Liver; fibrosis; hepatic stellate cell, platelet derived growth factor, extracellular matrix; Kupffer cell, leptin, transforming growth factor beta, tumor necrosis factor alpha, Review

Send correspondence to: Helen L. Reeves, M.D. PhD, Postdoctoral Scientist, Division of Liver Diseases, Box 1123, Mount Sinai School of Medicine, 1425 Madison Ave., Room 11-70F, New York, NY 10029-6574, Tel 212-659-9523, Fax: 212-849-2574,E-mail: Hal1@doctors.org.uk and Scott L. Friedman, M.D., Fishberg Professor of Medicine, Chief, Division of Liver Diseases, Box 1123, Mount Sinai School of Medicine, 1425 Madison Ave., Room 11-70F, New York, NY 10029-6574, Tel 212-659-9501, Fax 212-849- 2574, E-mail: frieds02@doc.mssm.edu